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Biochemical profile and clinical significance of MAPK/KINASE pathway genes in the diagnosis of thyroid neoplasms

Perfil bioquímico e significado clínico de genes da via MAPK/KINASE no diagnóstico de neoplasias de tireoide

Perfil bioquímico y significado clínico de los genes de la vía MAPK /CINASA en el diagnóstico de neoplasias tiroides

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Abstract
Thyroid neoplasms are the main types of endocrine malignancy, their incidence increasing in recent years. Routine diagnosis may present inconclusive results, leading to the need for using additional techniques that are more precise, such as molecular analysis. Therefore, it is essential to search for molecular markers for the diagnosis of these malignancies and their different histological types. The objective of this study was to identify diagnostic molecular markers for papillary thyroid carcinomas (PTC) and goiter lesions. For this, expression of genes belonging to the MAPK/KINASE pathway was assessed by the RT-qPCR technique. Additionally, complete biochemical profiles of the samples were obtained using Fourier transform infrared spectroscopy (FTIR). Results of RT-qPCR suggest that FOS, JUN, MAP2K6, CCNA1, SFN genes have the potential to be tumor markers of thyroid lesions, and the MAP2K6, CCNA1, SFN genes further have the potential to distinguish PTC samples from other thyroid lesions. FTIR results showed that PTC lesions can be distinguished from normal and benign tissues with 95.83% efficiency; changes in nucleic acids being the major classifying factor. Overall, results suggest potential of molecular and FTIR analysis in diagnosis of thyroid cancer.
Keywords: Thyroid; Gene expression; Molecular marker; MAPK/KINASE; Spectroscopy; FTIR.

Resumo
As neoplasias da tireoide são os principais tipos de malignidade endócrina, apresentando incidência aumentada nos últimos anos. O diagnóstico de rotina pode apresentar resultados inconclusivos, levando à necessidade de utilização de técnicas adicionais mais precisas, tais como a análise molecular. Portanto, é essencial a busca de marcadores

moleculares para o diagnóstico dessas neoplasias e seus diferentes tipos histológicos. O objetivo deste estudo foi identificar marcadores moleculares diagnósticos para carcinoma papilífero de tireoide (CPT) e lesões de bócio. Para isso, a expressão de genes pertencentes à via MAPK/KNASE foi avaliada pela técnica de RT-qPCR. Adicionalmente, perfis bioquímicos completos das amostras foram obtidos por meio da espectroscopia de infravermelho com transformada de Fourier (FTIR). Os resultados do RT-qPCR sugerem que os genes *FOS, JUN, MAP2K6, CCNA1, SFN* têm o potencial de serem marcadores tumorais de lesões da tireoide, e os genes *MAP2K6, CCNA1, SFN* têm ainda o potencial de distinguir amostras de CPT de outras lesões de tireoide. Os resultados do FTIR mostraram que as lesões de CPT podem ser diferenciadas de tecidos normais e benignos com 95,83% de eficiência; sendo as alterações nos ácidos nucleicos o principal fator de classificação. No geral, os resultados sugerem o potencial da análise molecular e do FTIR no diagnóstico do câncer de tireoide.
Palavras-chave: Tireoide; Expressão gênica; Marcador molecular; *MAPK/KINASE;* Espectroscopia; FTIR.

Resumen
Las neoplasias tiroideas son los principales tipos de malignidad endocrina, incrementándose su incidencia en los últimos años. El diagnóstico de rutina puede presentar resultados no concluyentes, lo que obliga a utilizar técnicas adicionales más precisas, como el análisis molecular. Por lo tanto, es fundamental la búsqueda de marcadores moleculares para el diagnóstico de estas neoplasias malignas y sus diferentes tipos histológicos. El objetivo de este estudio fue identificar marcadores moleculares de diagnóstico para carcinomas papilares de tiroides (CPT) y lesiones de bocio. Para ello, se evaluó la expresión de genes pertenecientes a la vía MAPK/KINASE mediante la técnica RT-qPCR. Además, se obtuvieron perfiles bioquímicos completos de las muestras mediante espectroscopia infrarroja transformada de Fourier (FTIR). Los resultados de RT-qPCR sugieren que los genes FOS, JUN, MAP2K6, CCNA1, SFN tienen el potencial de ser marcadores tumorales de lesiones tiroideas, y los genes MAP2K6, CCNA1, SFN tienen además el potencial de distinguir muestras de CPT de otras lesiones tiroideas. Los resultados de FTIR mostraron que las lesiones de CPT se pueden distinguir de los tejidos normales y benignos con una eficiencia del 95,83 %; siendo los cambios en los ácidos nucleicos el principal factor de clasificación. En general, los resultados sugieren el potencial del análisis molecular y FTIR en el diagnóstico del cáncer de tiroides.
Palabras clave: Tiroides; La expresion genica; Marcador molecular; MAPK/KINASE; Espectroscopia; FTIR.

1. Introduction

Papillary thyroid carcinoma (PTC) is the main histological type and represents more than 85% of all well-differentiated thyroid cancers of follicular origin, occurring more frequently in women (Mansour et al., 2018). Risk parameters include family history, radiation exposure history, clinical findings, and patient age. Although this type of tumor has a slow asymptomatic evolution and is an indolent cancer with a good prognosis and low mortality rate, some clinicopathological characteristics are known to be correlated with fatal outcomes. For example, large primary tumors and distant metastases are risk factor for recurrence and low patient survival (Coca-Pelaz et al., 2020; Yu et al., 2020).

Fine Needle Aspiration (FNA) biopsy is currently the gold standard for the diagnosis of the thyroid nodules. However, the sensitivity of this procedure is variable, with high rate of indeterminate or inconclusive findings, causing difficulties in the clinical management of patients (Zhang et al, 2017; Mayson et al., 2019). Genomic and gene expression alterations that result in tumor development can alternatively be used for more precise diagnosis (Lan et al., 2018). Since molecular alterations that can subsequently transform a normal tissue malignant can be identified using molecular biology techniques, they can be ideal tools for the early diagnosis of lesions (Hu et al., 2017; Mansour et al., 2018).

The Mitogen-Activated Protein Kinase (MAPK/KINASE) signaling pathway is the main pathway implicated in the development of thyroid cancer. This is a conserved signal transduction pathway that uses a series of protein kinases to transmit signals from the cell membrane to the nucleus, controlling a wide variety of essential cellular processes, including proliferation, differentiation, motility and apoptosis. Mutations in specific genes of the pathway have been associated with specific tumor types, increase the risk of tumor recurrence, lymph node metastasis and advanced thyroid cancer (Silva et al. 2020; Tang et al., 2020; Subbiah, Baik and Kirkwood et al., 2020; Lu et al., 2020). Hyperactivation of the MAPK/KINASE pathway is also related to several pathologies, including neurodegenerative diseases and the development of diabetes and cancer (Katz, Arrit, Yarden, 2007; Krishna et al., 2008; Zaballos and Santisteban, 2017; Silva et al. 2020; Khan et al., 2021; Thiel et al., 2021).

Although precise in pinpointing the change, gene expression studies can be time-consuming and cumbersome. Fourier transform infrared spectroscopy (FTIR) can rapidly and easily identify biochemical changes in tissues, and may be more applicable in clinical settings (Depciuch et al., 2018). For example, studies on colon (Li et al., 2005), prostate (Siqueira and Lima, 2016), breast (Elmi et al., 2017), cervical and gastric cancers (Liu et al., 2017) have shown FTIR to be a promising technique for differentiating the biochemical characteristics of neoplastic and normal tissues. Further, FTIR is non-invasive, rapid, and combined with multivariate analysis, is objective.

Therefore, in this study, we have analyzed MAPK/KINASE pathway gene expression levels of normal thyroid, goiter, and PTC tissues using RT-qPCR. We have also obtained FTR spectra of these samples and explored the possibility of classifying them using multivariate statistical analysis.

2. Methodology

2.1 Samples

It is an experimental study that was approved by the Research Ethics Committee/UNIVAP (no. 2.741.302/CEP/2018). Seventy samples, obtained of 46 patients with thyroid lesions previously collected by surgical procedure with excisional biopsies were provided by Biobank of Academic network of Research on Cancer of University of São Paulo of Instituto do Câncer do Estado de São Paulo (ICESP). Thyroid tissue samples were microdissected to ensure at least 70% of tumor cells were included and analyzed histopathologically by the Department of Pathology at ICESP. The samples were analyzed by histopathology, and the medical reports were signed by the Pathology Department of ICESP, following the Brazilian Society of Pathology (BSP) diagnostic criteria for thyroid cancer. The samples were analyzed by RT-qPCR and FTIR espectroscopy in Laboratório de Biologia Molecular do Câncer, Instituto de Pesquisa e Desenvolvimento, Universidade do Vale do Paraíba, Brazil.

For the expression gene analysis by RT-qPCR, 50 samples were used, distributed in the following groups: goiter tissue (n= 10), normal tissue adjacent to goiter (n= 10), PTC samples (n=15), adjacent normal tissue to the PTC (n=15). For the FTIR spectral analysis, 20 samples were used and distributed into the following groups: normal tissue adjacent to the goiter (n=5), goiter (n=7) and PTC (n=8). Six non-tumor tissue samples were used to normalize the RT-qPCR technique. The number of samples used in each technique was different because of the size of samples, which were at times insufficient for both FTIR and RT-qPCR analysis.

2.2 MAPK/KINASE pathway genes analysis

The CCNA1, SFN, JUN, KSR1, MAPK2K6, HSPA5, CDKN1C, MAPK81P2, FOS genes belonging MAPK/KINASE pathway were selected for the RT-qPCR analysis based on results of previous study by our research group (Silva et al., 2020) that classified thyroid tissues through MAP Kinase Signaling Pathway platform (PAHS-061ZA-24) (SA Biosciences, Qiagen).

The MRPL19 gene was defined as the endogenous control, providing an increase in the accuracy and resolution of the gene expression data, even facilitating the detection of small alterations in expression.

RNA extraction was performed using three protocols: AllPrep DNA RNA Mini Kit (Qiagen), Rneasy Mini Kit (Qiagen) and Trizol® Reagent (Life Technologies), followed by the RNeasy® purification protocol MinEluteTM Cleanup Kit (Qiagen, Germany). After RNA purification, the DNA was digested by the DNase I Amplification Grade Kit (1U/µL). Quantitative characterization of the extracted RNA samples was performed by the ultraviolet absorption spectroscopy method in the NanoDrop (ND-1000 Spectrophotometer v.3.0.1, Labtrade). RNA integrity was assessed in the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA) and agarose gel electrophoresis in 1.5% Tris-Borate-EDTA 1X (TBE) stained

with ethidium bromide. cDNA synthesis reactions were performed using a thermocycler (Biocycler, MJ96G, USA) through the Superscript™ IV preamp system, which includes SuperScript® IV / RNaseOUT™ Enzyme Mix, 2X First-Strand Reaction Mix, and Annealing Buffer (Invitrogen, Life Technologies, Carlsbad, CA).

RT-qPCR reactions for nine target genes (CCNA1, SFN, JUN, KSR1, MAPK2K6, HSPA5, CDKN1C, MAPK81P2, FOS and) and the endogenous gene MRPL19 were performed using Platinum® SYBR® Green RT- qPCRSuper Mix UDG (Applied Biosystems, Life Technologies, Carlsbad, CA) on the ABI Prism 7500 Sequence Detection System (Life Technologies, Foster City, CA, USA). Primers for amplification of the genes were designed in Primer Express software (version 3.0) (PE Applied Biosystems, Foster City, CA, USA). To avoid the amplification of contaminating genomic DNA, primers were placed at the junction exon-exon. The concentration of the used primers was 10 µm. RT-qPCR conditions consisted of an initial denaturation at 95 °C for 2 minutes followed by 40 cycles of three steps: denaturation at 95 °C for 15 seconds, annealing at 50 °C for 30 seconds, and extension at 60 °C for 1 minute; and finally a final step at 4°C. For all primers, standard curves were constructed with serial dilutions of cDNA from normal tissue and thyroid carcinoma (100, 20, 4, 0.8 and 1.6 ng/ul) and allowed the definition of the dilution 1:5 in sample preparation. The dissociation curve for target and endogenous gene demonstrated the high specificity of the primers used in amplification, in addition to the absence of contamination, non-specific amplification or formation of primer dimers.

Delta Delta Ct (ΔΔCt) method was used for calculating the relative expression for each sample and obtaining the QR (relative quantification or fold-change) values (Pfaffl, 2001). Fold-change values greater than 2 (QR> 2.0) indicate increase in expression for the target gene, and fold-change less than 0.5 (QR <0.5) demonstrates decrease in gene expression, with a degree of significance of p-value≤ 0.05. Statistical analyzes were performed using the GraphPad In Stat software (version 5.0) (GraphPad Software, San Diego, CA, USA, www.graphpad.com) using the Mann Whitney Test and the T Test.

2.3 FTIR spectroscopy analysis

This methodology follows the study of Silva et al. 2020. The samples stored in a Freezer at -80°C were thawed at room temperature, then tissue kept in a room with low humidity for 100 minutes (1h 40 min) to remove excess water from the sample. The equipment for obtaining the spectra was a FT-IR spectrophotometer (Spotlight 400 FT-IR Imaging System, Perkin Elmer) coupled to an ATR unit (diamond surface) (Attenuated Total Reflection). The samples were positioned on the diamond crystal face of the ATR unit and the tip of the micrometer clamp was pressed into the surface to allow proper contact to obtain a characteristic spectrum. The 64 scans background was performed before each analysis. Three absorbance spectra were acquired by 32 scans in the mid-infrared range (wavelength 1000-4000 cm^{-1}) with a resolution of 8 cm^{-1} per sample.

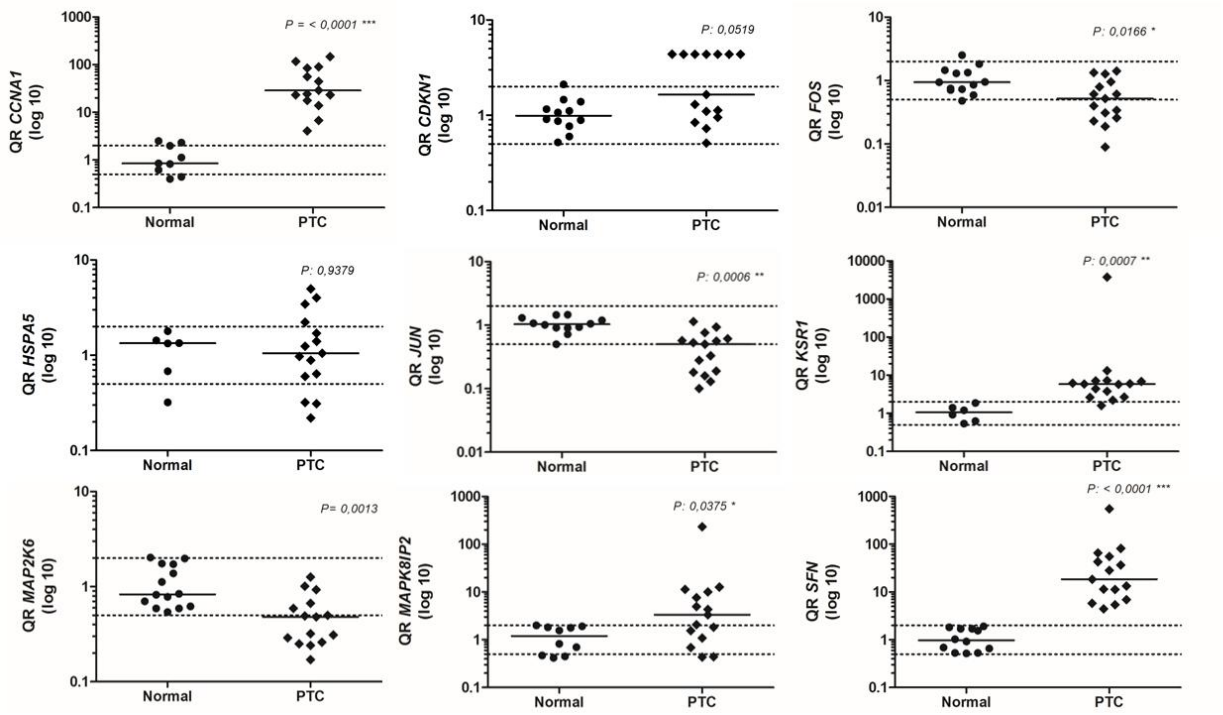
The spectra of each group were baseline corrected by subtracting a polynomial of order 8 for mean spectrum calculation, and the vector normalized and smoothed using OPUS 8 software (OPUS 8.0, Bruker Optik GmbH, Germany). The evaluation of average spectra, in cases in which intensity variations and displacement with respect to the appearance of the peaks between spectra groups, were performed using the Origin software (Origin 7.5 SR6, Origin Lab Corp, USA). The Principal Components Analysis (PCA) was performed using the MATLAB® 2015 software (MATLAB, MathWorks Inc., USA) and for this multivariate statistical analysis all spectra were first derived to correct baseline effects, and vector normalized. The first 10 PCs were used to perform Linear Discriminant Analysis (LDA) and then validated using a single-output cross-validation (LOOCV) using MATLAB.

3. Results and Discussion

The study explores two approaches to diagnostic approaches – RT-qPCR and FTIR, for their capability to distinguish normal, goiter and malignant (PTC) thyroid tissues.

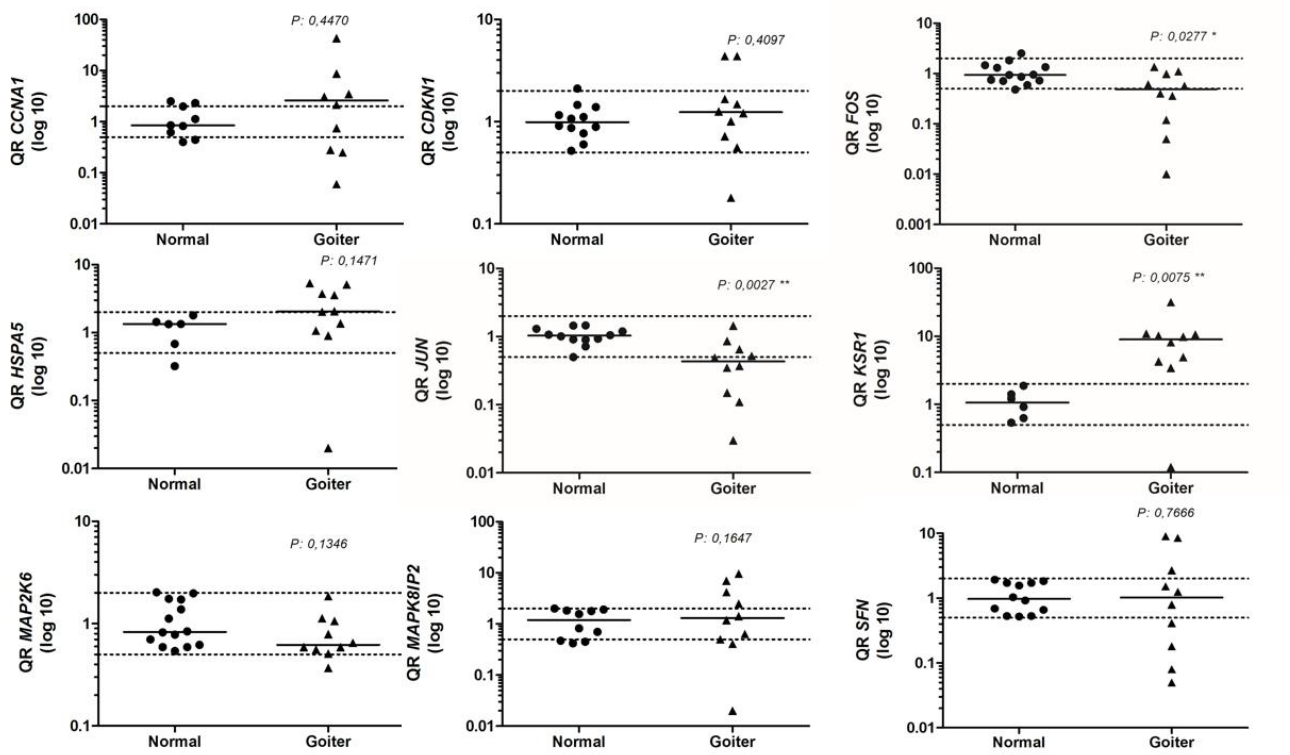
Using RT-qPCR, expression levels of CCNA1, SFN, JUN, KSR1, MAPK2K6, HSPA5, CDKN1C, MAPK81P2, FOS genes were studied. When comparing PTC tissues with normal thyroid tissues (all 25 samples) (control group), the FOS (0.0166), JUN (P = 0.0006) and MAP2K6 (P = 0.0013) genes showed a significant decrease in expression in tumor samples when compared to the control group and the genes CCNA1 (P = <0.0001), KSR1 (P = 0.0007), SFN (P = <0.0001) and MAPK8IP2 (P = 0.0375) showed increased expression in PTC samples when compared with the control group (Figure 1). When comparing goiter tissue with all samples of normal thyroid tissue (all 25 samples) (control group), the FOS (P = 0.0277) and JUN (P = 0.0027) genes showed reduced expression in goiter samples when compared to the control group and the KSR1 gene (P = 0.0075) showed increased expression in goiter samples when compared to the control group (Figure 2).

Figure 1. Comparison between medians of gene expression levels by qRT-PCR from CCNA1, FOS, JUN, KSR1, MAP2K6, MAPK8IP2, HSPA5, SFN and CDKN1C genes in PTC and normal samples (control group).



FOS, JUN and MAP2K6 genes showed a significant decrease in expression in tumor samples when compared to the control group. QR: relative quantification of the gene. PTC: papillary thyroid carcinoma. Normal: thyroid tissue without histopathologically identified lesion. * P value significant. Source: Authors.

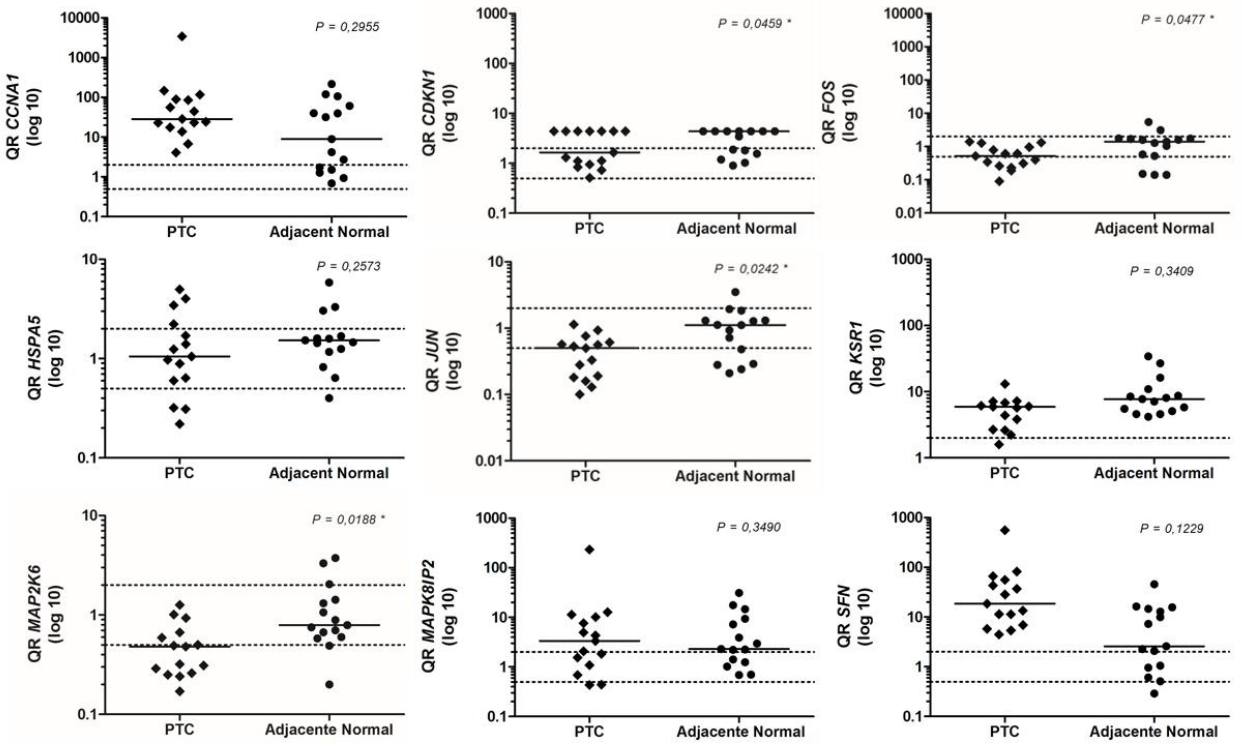
Figure 2. Comparison between medians of gene expression levels by the RT-qPCR from CCNA1, FOS, JUN, KSR1, MAP2K6, MAPK8IP2, HSPA5, SFN and CDKN1C genes in goiter and normal group (control group).



FOS and JUN genes showed reduced expression in goiter samples when compared to the control group. QR: relative quantification of the gene. Goiter: goiter samples. Normal: thyroid tissue without histopathologically identified lesion. * P value significant. Source: Authors.

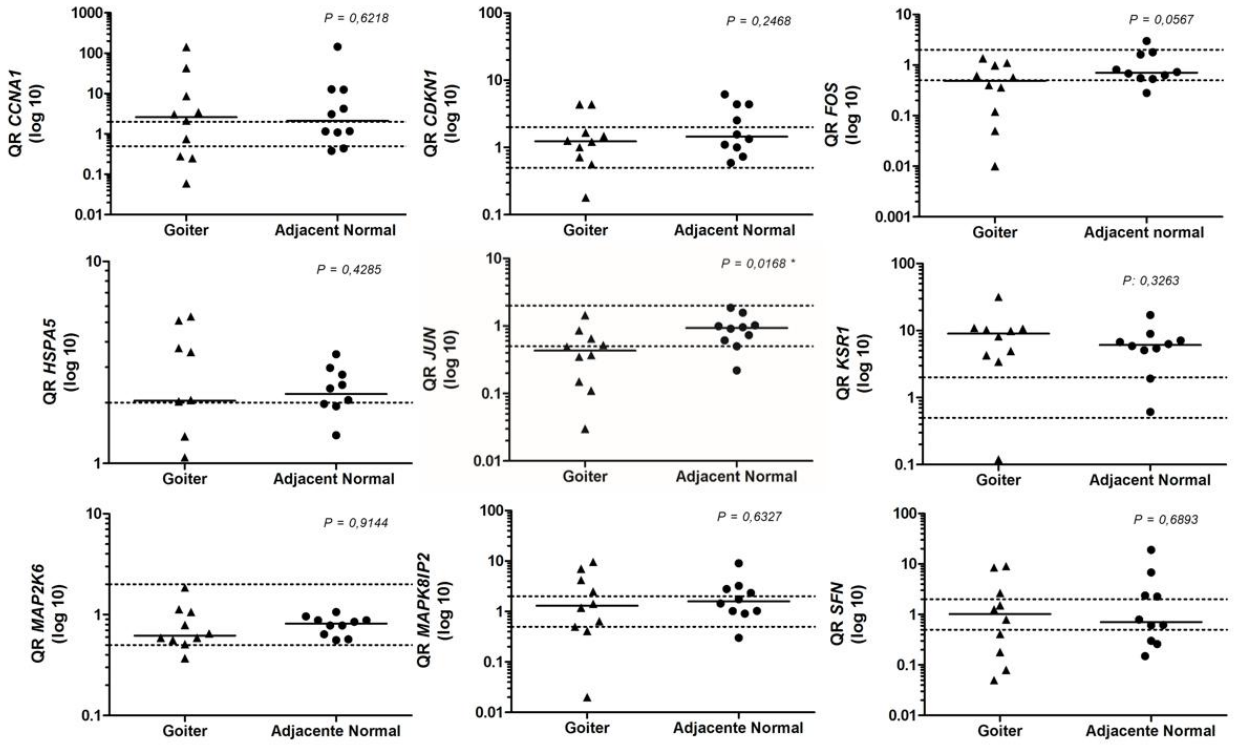
Comparing PTC tissues with normal adjacent thyroid tissues (15 samples) (control group), the FOS (P = 0.0477), JUN (P = 0.0242), CDKN1C (P = 0.0459) and MAP2K6 (P = 0.0188) genes showed reduced expression in PTC samples when compared to the control group (Figure 3). When compared the goiter group with normal adjacent thyroid tissues (10 samples) (control group), the JUN gene (P = 0.0168) showed reduced expression in the goiter samples in relation to the control group (Figure 4). In the fifth analysis, performed between PTC and goiter samples (control group), was observed over expression for the SFN (P = 0.0002) and CCNA1 (P = 0.0043) genes and down expression for MAP2K6 (P = 0.0326) gene in the PTC samples in relation to the control group (Figure 5).

Figure 3. Comparison between medians of gene expression levels by the RT-qPCR from CCNA1, FOS, JUN, KSR1, MAP2K6, MAPK8IP2, HSPA5, SFN and CDKN1C genes in of PTC and adjacent normal group (control group).



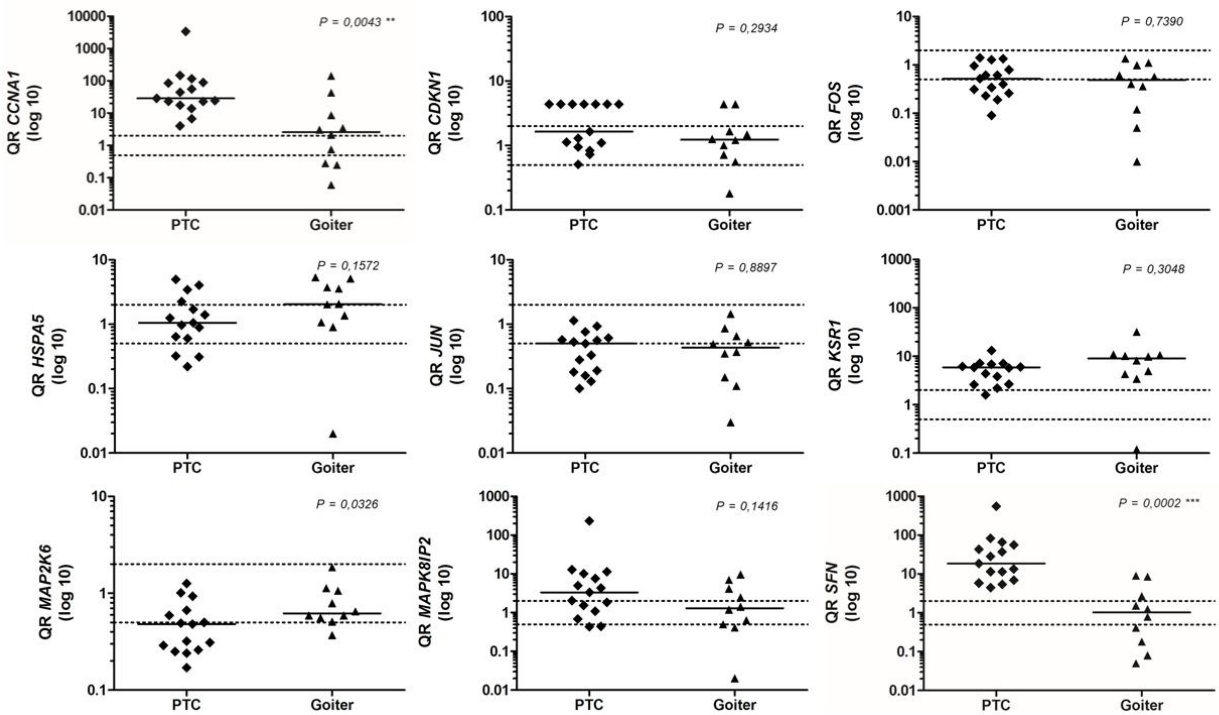
FOS, JUN, CDKN1C and MAP2K6 genes showed reduced expression in PTC samples when compared to the control group. QR: relative quantification of the gene. PTC: papillary thyroid carcinoma. Adjacent normal: thyroid tissue without histopathologically identified lesion. * P value significant. Source: Authors.

Figure 4. Comparison between medians of gene expression levels by the RT-qPCR from CCNA1, FOS, JUN, KSR1, MAP2K6, MAPK8IP2, HSPA5, SFN and CDKN1C genes in goiter and adjacent normal group (control group) samples.



JUN gene showed reduced expression in the goiter samples in relation to the control group. QR: relative quantification of the gene. Goiter: goiter samples. Adjacent normal: thyroid tissue without histopathologically identified lesion. * P value significant. Source: Authors.

Figure 5. Comparison between medians of gene expression levels by the RT-qPCR from CCNA1, FOS, JUN, KSR1, MAP2K6, MAPK8IP2, HSPA5, SFN and CDKN1C genes in samples of PTC and goiter (control group).



MAP2K6 gene showed down expression in the PTC samples in relation to the control group. QR: relative quantification of the gene. Goiter: goiter samples. Adjacent normal: thyroid tissue without histopathologically identified lesion. * P value significant. Source: Authors.

Of the nine genes proposed as diagnostic markers in our previous study by PCR array analysis of the MAP Kinase Signaling Pathway (Silva et al., 2020), FOS, JUN, MAP2K6, CCNA1 and SFN genes were effective in the accurate identification of thyroid lesions with potential to be tumor markers. FOS and JUN are proposed molecular markers with potential to identify thyroid lesions, as shown by differential expression among PTC and goiter tissues with normal thyroid tissues, confirmed by differential expression of these genes in the comparison with normal adjacent thyroid tissues. MAP2K6, CCNA1 and SFN genes are proposed molecular markers with potential for the identification of PTC samples, as shown by differential expression between PTC with normal and goiter samples groups. Besides the molecular pathway MAPK/KINASE is directly involved in the development of the thyroid cancer, the regulatory role that the genes of this pathway have in the cell proliferation, motility, apoptosis, transcription, translation, among others reinforce the importance of molecular studies in the MAP/KINASE pathway (Katz et al., 2007; Zaballos & Santisteban, 2017; Silva et al. 2020).

The reduced expression observed in our study for FOS and JUN genes in PTC and goiter samples when compared with normal thyroid tissues and normal adjacent thyroid tissues indicates that these genes are promising molecular markers for the diagnosis of thyroid malignancies. The differential expression of these genes in goiter samples suggests a malignant potential in these tissues, so clinical follow-up of these patients is necessary. FOS and JUN are oncogenes and encodes an important transcription factor in the MAPK/KINASE pathway called AP-1, which participates in cellular processes that include cell growth, differentiation and apoptosis (Xiao et al., 2019; Ameri et al., 2021). The FOS-JUN dimer is the most common form of the AP-1 protein in human cells, and when inappropriately expressed it is related to angiogenesis and metastasis processes (Xiao et al., 2019). The role of this protein is described in the signaling pathway of T cell receptors (Ameri et al., 2021), breast cancer (Ivanova et al., 2011), however, little explored in PTC (Xiao et al., 2019). In thyroid carcinomas, Liu et al. (1999) observed the under expression of the FOS gene in PTC by the qRT-PCR technique, noting its power as a marker in differentiation and playing a role in thyroid maintenance. However, Xiao et al (2019) observed over-expression of the AP-1 protein in PTC, relating protein expression with tumor size and prognostic predictor, which may be an important diagnostic and prognostic molecular marker of PTC.

Likewise, was observed reduced expression for MAP2K6 genes in PTC and goiter samples in relation to the normal thyroid tissues and normal adjacent thyroid tissues. Belonging to MAPK pathway, this gene is knowing for activate by phosphorylation the p38 pathway, that is related to transduce cellular stress-cell death (Han et al., 1996; Rasmussem et al., 2016; Kumar et al., 2021). Its increased expression is related as possible diagnostic or prognostic molecular marker to several types of cancer such as esophageal, colorectal and the its reduction expression with glioblastoma (Parray et al., 2014; Rasmussem et al., 2016; Liu et al., 2018). Rasmussem et al., (2016) show that the MAP2K6 gene is a target to miR-625-3p activator and when this gene was down regulated promote a resistance to Oxaplatin. Liu et al. (2018) reported that MAP2K6 under expression is significantly associated whit overall poor survivor in glioblastoma, because this gene is identified as a tumor suppressor in this type of cancer. In thyroid lesions our study was the first to show the decrease of MA2K6 expression in PTC, indicating that this gene is promising molecular marker for the diagnosis of thyroid lesions, and, more studies should be carried out to elucidate the real mechanism of this gene in PTC lesions.

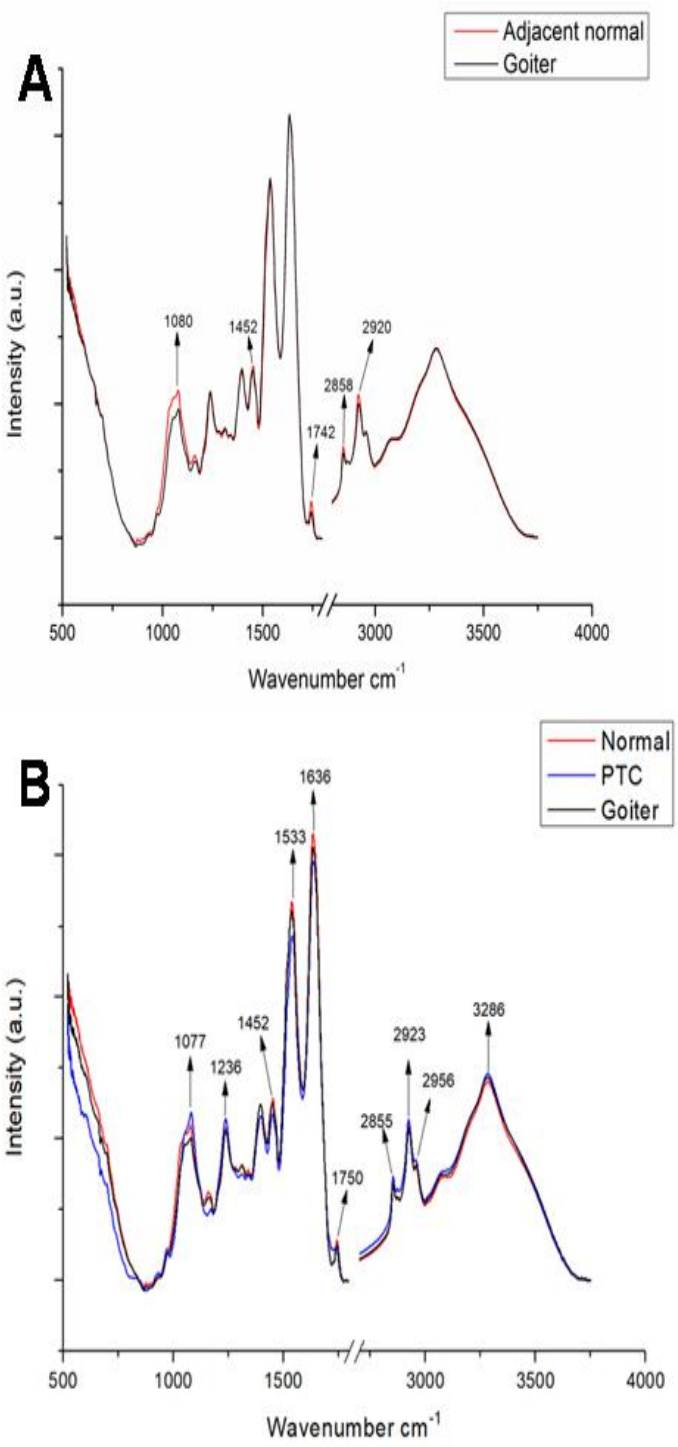
The differential expression observed in the CCNA1 gene in the comparison between PTC with goiter and normal samples, showing over-expression of this gene in PTC samples when compared to both groups, allows us to relate it with role diagnosis in PTC. The CCNA1 gene plays an important role in the M phase of the meiotic cell cycle and it promotes cell cycle progression by activating CDK1 and CDK2 (Khaja et al., 2013; Munari et al., 2015). In our previous study, the potential diagnosis of this gene and Cyclin A1 protein in PTC primary tumors has already been highlighted (Silva et al., 2020). In the literature, CCNA1 is described with its strong relationship with tumor progression and invasion (Khaja et al., 2013; Munari et al., 2015). Khaja et al. (2013) observed the high expression of the CCNA1 gene in primary breast tumors and lymph nodes

when compared to the adjacent normal tissue of these same patients. Munari et al. (2015) in order to understand the molecular role of Cyclin A1 in the progression of urothelial bladder carcinoma observe the high expression of this protein in these carcinomas. We emphasize the importance of new studies with the CCNA1 gene in thyroid carcinomas in order to affirm the role of this gene in thyroid carcinogenesis.

SFN gene showed increased expression in PTC samples compared to goiter and normal tissues, putting it as a potential diagnostic marker in PTC. This gene encodes a mitotic translation regulatory protein that can interrupt cell cycle progression in the G2/M phase, playing an inhibitory role in DNA errors in mitosis (Jiang et al., 2018; Hu et al., 2019). In the literature, the SFN gene is described by the regulation of key genes that influence tumor initiation and progression (Jiang et al., 2018). In several cancers the high expression of this gene has been described, being important biomarker in adenocarcinomas (Shiba-Ishii et al., 2015), breast cancer (Boudreau et al., 2013) and esophageal cancer (Ren et al., 2010). In thyroid carcinomas, Ito et al. (2003) finding over expression of this gene in aggressive PTC and anaplastic carcinoma. The authors suggest that SFN gene is associated with greater aggressiveness of these carcinomas, being a biomarker of worse prognosis in PTC. Our results confirm the findings of Ito et al (2003) and suggest the SFN gene as a diagnostic marker of this tumor histological type, however, we emphasize the importance of further studies in the Brazilian population.

FTIR spectral analysis showed differences in intensity of nucleic acid bands (1077cm^{-1}, 1080 cm^{-1},1236 cm^{-1}), proteins (1452 cm^{-1},1533 cm^{-1},1636 cm^{-1}) and lipids (1742 cm^{-1},1750 cm^{-1}, 2855 cm^{-1}, 2858 cm^{-1}, 2920 cm^{-1}, 2923 cm^{-1},2956 cm^{-1}, 3286 cm $^{-1}$) (Movasaghi et al., 2008; Zhang et al., 2015; Wu et al. 2016; Depciuch et al. 2018) (Table 1). Increase in intensity was observed in the bands related to nucleic acid (1077cm^{-1}, 1236 cm^{-1}) in PTC samples when compared with goiter and its adjacent normal samples (Figure 6).

Figure 6. (A) Average spectrum of sample of goiter and adjacent normals assessed by FTIR. (B) Sample mean spectra of PTC, goiter and adjacent normal assessed by FTIR.



PTC: papillary thyroid carcinoma. Goiter: goiter. Adjacent normal or normal: adjacent normal to the goiter. Source: Authors.

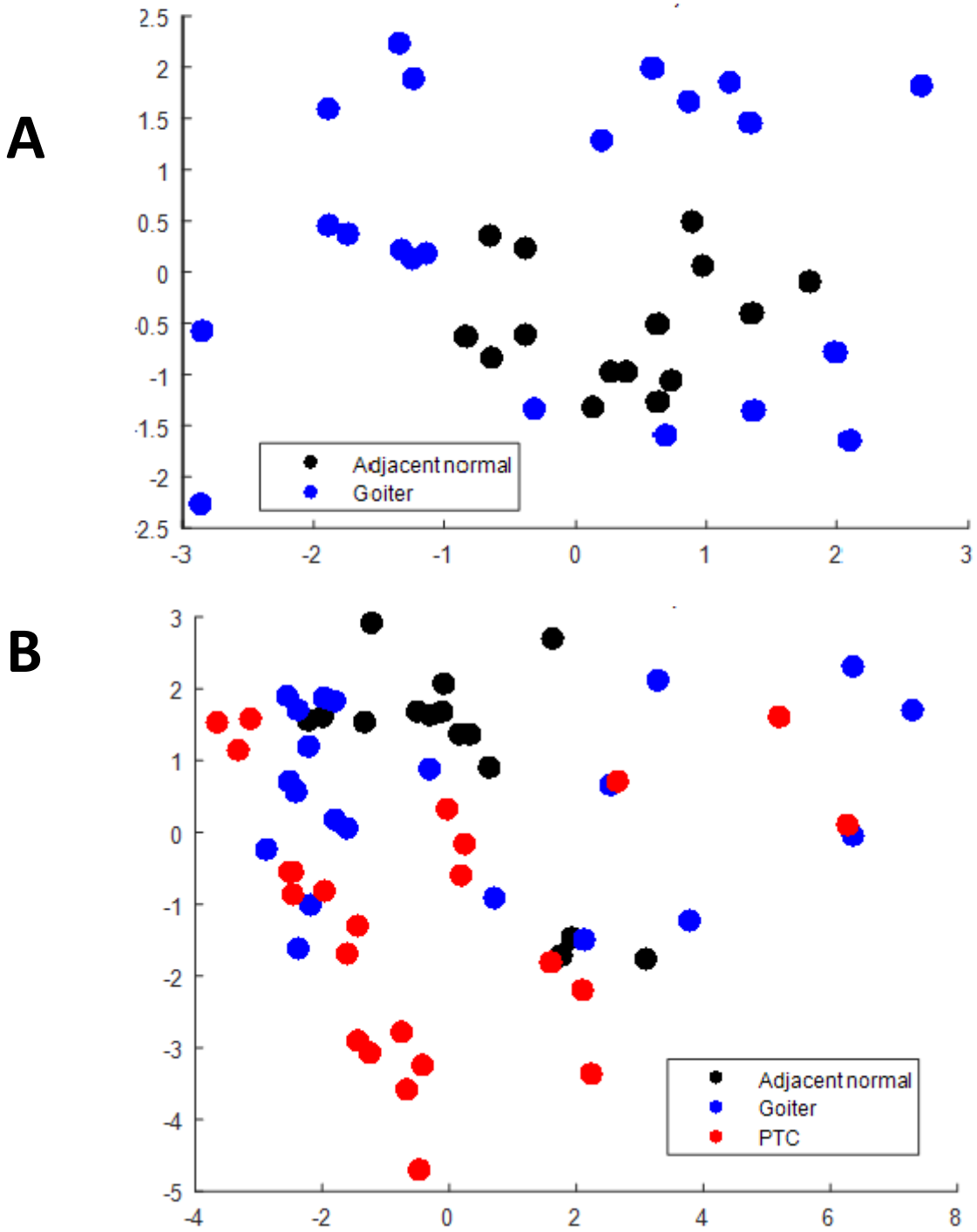
Table 1. Main vibrational modes detected in thyroid tissue samples (PTC, goiter and adjacent normal of goiter).

Peak position (cm^{-1})	Vibratons*	Substance
1077	$_{vs}PO_2^-$	Nucleic acid
1080	$_{vs}PO_2^-$	Nucleic acid
1236	Amida III and $_{vas}PO_2^-$	Nucleic acid
1452	$_{vas}CH3$	Protein
1533	Amida II	Protein
1636	Amida I	Protein
1742	C=O de ésteres	Lipids
1750	C=O de ésteres	Lipids
2855	$_{vas}CH_3$	Lipids
2858	$_{vas}CH_3$	Lipids
2920	$_{vas}CH_2$	Lipids
2923	$_{vas}CH_2$	Lipids
2956	$_{vas}CH_3$	Lipids
3286	$_{vs}O\text{-}H$	Lipids

*: vibrational mode, vs: symmetric stretch vibrational mode, vas: asymmetric stretch vibrational mode.
Source: Authors.

PCA was carried out for two groups: 1. goiter and adjacent normal, 2. Goiter, adjacent normal and PTC. Goiter and normal spectra showed some clustering, although there were overlaps. Similar trend was observed in PCA for normal, goiter, and PTC, although the overlap between PTC and goiter was higher. Since PCA results can be plotted only on two or three axes, PC-LDA analysis was performed, which takes n-dimensions into consideration. LOOCV was performed to validate the model. In both PCA analyses, clears clusters were not observed. However, PC-LDA was carried out to test whether classification can be improved and validated using LOOCV. LOOCV analysis of group 1 showed that 80% of the normal adjacent spectra could be distinguished from the spectra of the goiter samples. LOOCV of group 2 showed that 80.9 and 87.5% goiter and PTC could be distinguished correctly from normal tissues. Thus, results suggest possibility of distinguishing normal, benign and malignant thyroid tissues.

Figure 7. PCA multivariate analysis. (A) Multivariate PCA analysis in a goiter sample and samples of its adjacent normals.



Goiter: spectrum of goiter samples. Adjacent normal: spectrum of normal adjacent samples of goiter. (B) Multivariate PCA analysis in PTC samples, goiter samples and samples of their adjacent normals. PTC: spectrum of papillary thyroid carcinoma specimens. Goiter: spectrum of goiter samples. Adjacent normal: spectrum of adjacent normal tissue to the goiter samples. Source: Authors.

Table 2. Principal linear component discriminant analysis (PC-LDA).

A			B			
	NA	**B**		**NA**	**B**	**PTC**
NA	15 (100%)	0	**NA**	14 (93.3%)	1 (6.6%)	0
B	0	21 (100%)	**B**	2(9.52%)	18 (85.71%)	1 (4.76%)
			PTC	0	1 (4.16%)	23 (95.83%)

In A: B (goiter) and NA (adjacent normal). In B: PTC (papillary thyroid carcinoma), B (goiter) and NA (adjacent normal). Source: The author.

Table 3. LOOCV. In A: B (goiter) and NA (normal adjacent).

A			B			
	NA	**B**		**NA**	**B**	**PTC**
NA	15 (100%)	0	**NA**	12 (80%)	3 (20%)	0
B	2 (9.52%)	19 (90.47%)	**B**	3 (14.28%)	17 (80.95%)	1 (4.76%)
			PTC	0	3 (12.5%)	21 (87.5%)

In B: PTC (papillary thyroid carcinoma), B (goiter) e NA (normal adjacent). Source: Authors.

Previous studies have shown biomedical applications of FTIR (Silva et al., 2020) (Sitole et al., 2014), including the diagnosis of thyroid lesions (Zeng et al., 2007; Zhang et al., 2015; Martinez-Marin et al., 2017). Zeng et al (2007) differentiated thyroid carcinomas from benign tissues using FTIR and observed that the malignant tissue has variation in the corresponding amide I and amide II bands due to the protein structural change and that the amount of lipids was reduced in the malignant tissues. The authors concluded that the FTIR spectroscopy analysis was a reliable and practical approach for diagnosing this malignancy. Zhang et al (2015) analyzed the preoperative spectral profile by FTIR spectroscopy in PCT samples with the aim of comparing and confirming the histological diagnosis. To carry out the study, the authors used 111 tissues from patients undergoing surgical operation (41 PTC, 70 of goiter and 50 samples of healthy volunteers) and observed spectral difference in the bands corresponding to lipids, DNA and proteins, discriminating PTC with of 88.8% accuracy. The results concluded that FTIR analysis is viable for rapid and non-invasive screening of these lesions, and may help in the diagnosis of these tumors. Martinez-Marin et al (2017) used FTIR spectroscopy to analyze in twenty patients the spectral difference of follicular thyroid carcinoma and follicular variant PTC and demonstrated the potential of this technique as a new tool to aid the histopathology in distinguishing follicular thyroid carcinoma and follicular variant PTC, in addition to stressing the careful importance for the selection of the area of interest in the analysis spectral, maximizing the power of the methodology, enabling it to be a focused and efficient approach when combined with the histopathological method. Our study adds to the evidence supporting development of FTIR as a thyroid cancer diagnostic tool, in addition to exploring precise molecular diagnostics for confirmation using RT-qPCR.

4. Conclusion

FOS and JUN genes were effective in accurately identifying thyroid lesions and MAP2K6, CCNA1 and SFN genes are proposed to be molecular markers with potential for the identification of PTC samples. The FTIR spectroscopy analysis was capable of distinguishing normal, benign goiter and malignant PTC with high sensitivity. Thus, the present study indicates that molecular analysis of the FOS, JUN, MAP2K6, CCNA1 and SFN genes and FTIR have potential for clinical applications in thyroid cancer diagnostics. Further studies with more samples are needed to elucidate the real potential of these genes and

FTIR spectroscopy in PTC.

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