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Dysregulation of KRT19, TIMP1, and CLDN1 gene expression is associated with thyroid cancer

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ABSTRACT

Thyroid nodules are the main indicators of thyroid cancer, their malignancy is evaluated by cytological analysis and imaging technology, however, there are still cases where the result is not enough to classify thyroid cancer. Therefore, there is a necessity for accurate molecular biomarkers to collaborate in the diagnosis. Here, we analyzed the mRNA relative expression of CLDN1, TIMP1, and KRT19 genes in FNA of malignant (n = 48) and benign (n = 49) thyroid nodules by RT-qPCR analysis to assess their predictive value as cancer biomarkers. We identified a significant overexpression of the three transcripts in malignant nodules, therefore, the evaluation of their predictive capacity to distinguish between benign and malignant nodule as individual biomarkers were evaluated by logistic regression tests, obtaining promising prediction results to rule out cancer; later by random forest to create a stronger model, we included expression results with clinicopathological characteristics, the best model consists of the three-mRNA level expression with patient's history of cancer (AUC = 0.821, accuracy = 85.4% and sensitivity of 81.1%). These results demonstrate a dysregulated expression of CLDN1, KRT19 and TIMP1 in thyroid cancer, thus, represent a promising panel of biomarkers to be evaluated in indeterminate thyroid nodules.

1. Introduction

The prevalence of thyroid cancer has increased in the last decades, reaching up to 7.4% of all malignant neoplasms in México [1]. Thyroid nodules are in most cases, the first indicators of thyroid cancer [2], and the fine needle aspiration (FNA) biopsies guided by high-resolution ultrasound (US) have significantly increased its identification [3]. The Thyroid Imaging Reporting and Data System (TI-RADS) is a standardized grading method to evaluate thyroid nodules based on imaging features, it plays an important role in the diagnosis, monitoring, and therapeutic decisions of thyroid disease; however, it's not enough to classify cases with thyroid cancer [2,4]. The cytological evaluation of the nodule is currently the principal analysis to distinguish benign from malignant nodules, though, there is also a risk of undetermined results, therefore, an accurate diagnosis is still needed to avoid unnecessary surgery in patients with benign nodules [5].

The development of thyroid carcinoma is strongly influenced by a series of molecular alterations, some specific to this pathology [6]. Analyzing the mRNA expression profile in FNA leads to identifying

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phenotypic signatures associated with specific lesions [7]. *KRT19, TIMP1*, and *CLDN1* are epithelial biomarkers highly dysregulated in the tumorigenesis process [8–11], mainly in the regulation of cell proliferation, invasiveness, and metastasis [12–14]; also, it is hypothesized that somatic mutations in the activators of MAPK and FAK-PI3K/Akt signaling pathway are an early event independent in common in the upregulation of these genes [14–16].

In this study, we aimed to investigate the mRNA relative expression of TIMP1, KRT19 and CLDN1 in FNA biopsies and its malignancy's association to assess their predictive value as cancer biomarkers.

2. Materials and methods

2.1. Sample collection

We studied thyroid nodule FNAs submitted to Alvarez & Arrazola Radiólogos clinic, in Sinaloa, México, from June 2018 to July 2021. The samples were collected by ultrasound guidance, stabilized in RNA protect Cell Reagent (Qiagen, Hilden, DE), and stored at −20 °C until RNA extraction. The Bethesda system of the cytology report was considered to categorize the biopsies in study groups [5].

2.2. Study population

Our study sample consisted of 97 patients: 48 patients were diagnosed with malignant lesions and 49 patients with benign lesions. All patients follow the next criteria: being Mexican with a confirmed cytology diagnosis of Bethesda II (benign group) or V-VI (malignant group) realized by a certified pathologist and who had not received radiotherapy. Clinicopathological risk characteristics were collected through a direct questionnaire. Patients were approved by signing an informed consent, previously reviewed and approved by the Ethics Committee of Alvarez & Arrazola Radiólogos, adhering to laws and regulations described in the Declaration of Helsinki [17].

2.3. Molecular analysis

Thyroid nodule FNAs were collected, processed, and tested for relative expression of *CLDN1, TIMP1*, and *KRT19* genes. According to the manufacturer's protocol, total RNA from 97 FNAs was extracted with the RNeasy Plus-Mini Kit (Qiagen, Hilden, DE). Reverse transcription reactions were performed in a final volume of 20 μL by using 100 ng from FNA with the GoTaq 2-step RT-qPCR system Probe (Promega, Madison, WI) following the manufacturer's instructions.

The quantitation of gene expression was accomplished by using PrimeTime qPCR Probes (Integrated DNA Technologies, Coralville, IA) with sequence-specific oligonucleotide primers combined with reporter-dye, performed on a Step One Plus system (Applied Biosystems, Foster City, CA). Each sample was assayed in duplicates with the following reaction conditions: total volume of 10 μL; 5 μL of 2× PrimeTime Gene expression Master Mix previously prepared with dye stock solution (Integrated DNA Technologies, Coralville, IA) and 1× final concentration of each PrimeTime qPCR probe in duplex assays. The PCR protocol was as follows: polymerase activation for 3 min at 95 °C, then 45 cycles of a 2-step PCR protocol, a denaturation step at 95 °C for 5 s, and an annealing-extension step at 60 °C for 30 s. Negative controls were included in each set of reactions.

Relative quantitation of gene expression was calculated by using the method described by Taylor [18] using β-actin as a reference gene to normalize gene expression. Normalized data were then log2 transformed for further analysis.

2.4. Statistical analysis

Percentages and frequencies of clinical data were calculated. Contingency tables by groups and their relationship to epidemiological variables were analyzed using Pearson chi-square and Odds ratio (OR) with confidence intervals (CI) of 95%. Quantitative analysis using relative expression was performed using log-transformed relative normalized expression. The Mann-Whitney *U* test and Kruskal-Wallis were used to compare differences between continuous variables. Bivariate correlations were analyzed by the Spearman test for mRNA level expression and age. Logistic regression generated Receiver Operating Characteristic (ROC) curves on each mRNA relative expression and age to distinguish benign and malignant groups. Statistical analysis was performed using SPSS v20 (SPSS UK, Ltd, Woking, UK). Predictive models integrating different variables were carried out through random forest using the Orange program, v3.27.1. We tried other models with 100–500 trees, and 20-fold cross-validation performance was used to tune the model. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Clinicopathological characteristics

The malignant nodules patients had an average age of 47.5 ± 15.2 years, and the benign group of 51.3 ± 14.5 years ($p = 0.138$). Performing the age ROC curve, an area under the curve (AUC) of 0.412 was observed ($p = 0.138$), indicating age has a poor discrimination capacity to discriminate between groups. Concerning the family history of cancer, we identified that 62% of our malignant group had relatives with cancer, 32.25% of them with thyroid cancer. However, the analysis showed no relation between family history and the presence of thyroid cancer, despite in the benign group 55.1% had no relatives with cancer ($p = 0.176$).

Our study revealed that previous cancer increased more than nine times the risk of developing malignant lesions ($p = 0.016$; OR 9.14, CI 95% 1.10–76.15); no other statistical associations were found between groups and risk factors. A summarization of the relationship between clinical parameters and groups is shown in Table S1.

3.2. Relative mRNA expression

To determine whether mRNA of *CLDN1, TIMP1*, and *KRT19* levels are clinically correlated with malignant thyroid nodules, its relative expression was determined in 97 FNAs (malignant, n = 48). As demonstrated in Fig. 1, increased expression levels of the three genes were statistically differentially expressed in the malignant FNAs compared with benign nodules ($p < 0.0001$). The mean mRNA expression level for *CLDN1, TIMP1*, and *KRT19* was 41.315 ± 0.554, 4.465 ± 0.301, and 11.64 ± 0.424 -fold higher, respectively.

We performed correlation analysis to establish relationships between the relative expression of the mRNAs, identifying a strong positive relationship between the relative expression of the three genes ($p < 0.0001$), correlation coefficients between genes are shown in Fig. 2. Also, correlations were evaluated between age and gene expression, identifying a statistically significant correlation with mRNA level of *KRT19* (Rho = −0.310; $p = 0.036$) and *CLDN1* (Rho = −0.341; $p = 0.022$).

Categorizing patients by pathological subtype, the papillary subtype (worst prognosis) group consistently exhibits the highest expression levels in each case ($p < 0.0001$). The mainly observed cell types in thyroid lesions in our study, macrophages ($p = 0.216$), multinucleated giant cells ($p = 0.849$), lymphocytes ($p = 0.556$) and

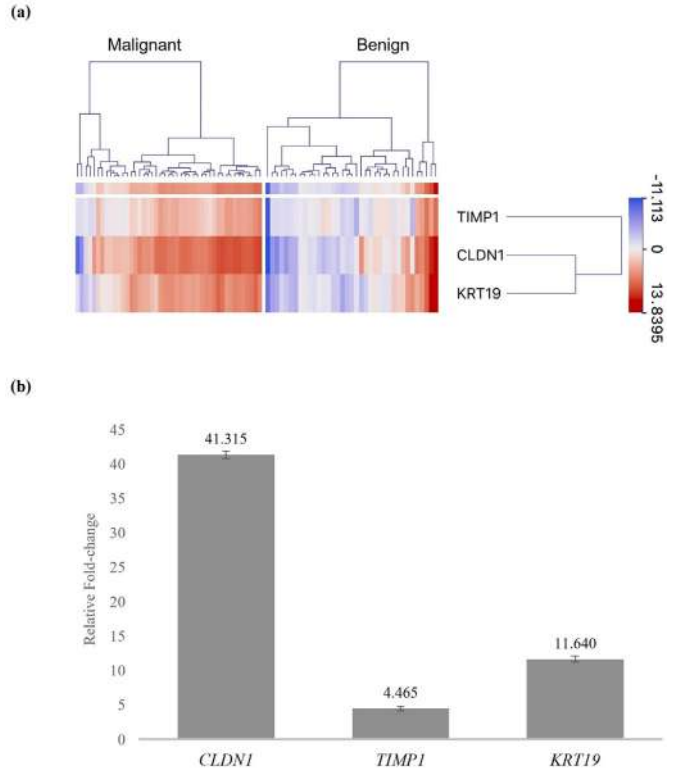

Fig. 1. Relative mRNA expression. (a) Heatmap representing mRNA expression in *TIMP1, CLND1* and *KRT19*. The color bar on the right side demonstrates the log2 fold changes from comparison of benign nodules versus malignant nodules. Analysis generated by Orange program v3.27.1. (b) Relative expression level in malignant group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

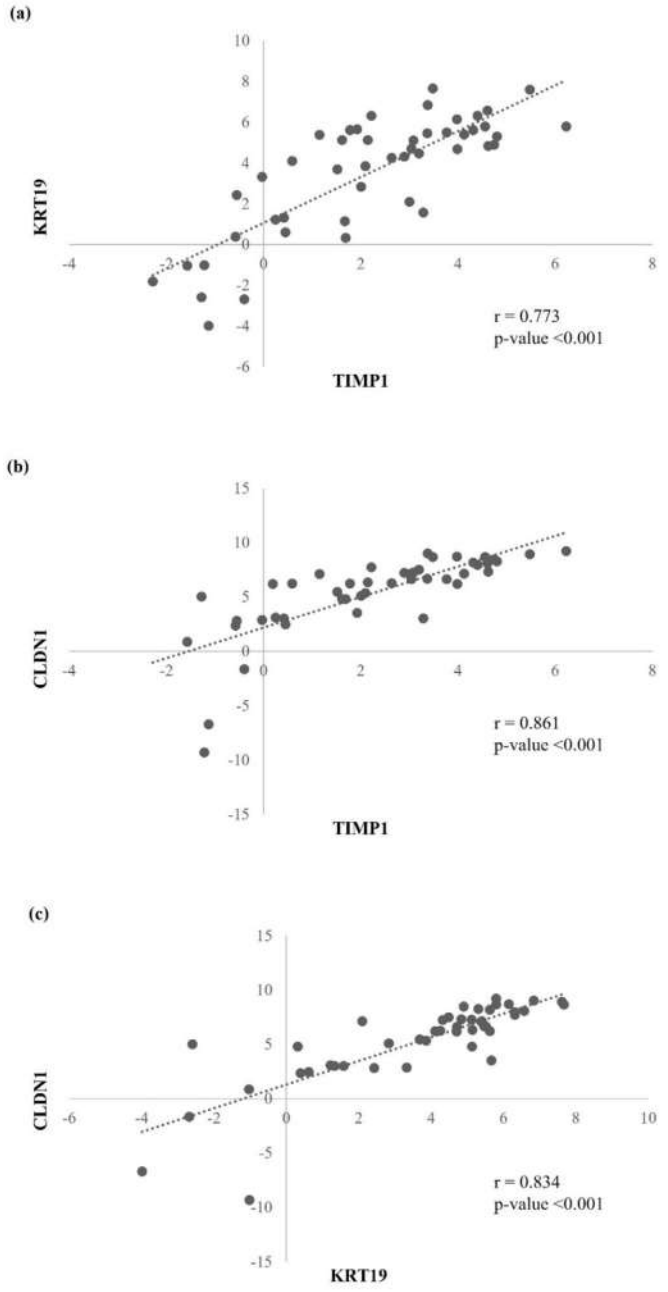

Fig. 2. Correlation analysis between different relative mRNA expressions in thyroid nodule samples. (a) KRT19 vs TIMP1; (b) CLDN1 vs TIMP1; (c) CLDN1 vs KRT19. The dots at each graph correspond to normalized log2 respective gene expression values.

macrophages with hemosiderin ($p = 0.393$) were identifying without difference between malignant and benign nodules. Correlation between these immune cells and mRNA levels was not observed.

3.3. ROC curves and predictive analysis

We generate a ROC curve for the predictive characteristics of the three genes expressed within our sample to discriminate between malign and benign nodules (Fig. 3). According to our results, the three genes are promising individual biomarkers for distinguishing benign from malignant lesions ($p < 0.001$). The AUC, CI, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each gene is shown in Table 1; the Youden index was used to select the most appropriate cut-off point for each biomarker. The best NPV is observed in *CLDN1* relative expression (95.8%). When performing the Fagan nomogram for each cut-off point, a positive likelihood ratio of 2.26, 3.82, and 2.28 were observed for *CLDN1, TIMP1*, and *KRT19*, respectively, going from a prior probability (odds) of 23.7% to a posterior probability of 41%, 54%, and 41%, in each case. The negative likelihood ratio was 0.15, 0.38, and 021, going to a posterior probability (odds) of 4%, 10%, and 6%, for *CLDN1, TIMP1*, and *KRT19*, correspondingly. These values for likelihood ratio are according to the prevalence of thyroid cancer in a second-level health institution and are not applicable for primary screening. However, strongly suggest the use of qPCR for *CLDN1, TIMP1*, and *KRT19* gene expression may be used to distinguish malignant from benign thyroid nodules in FNAs samples.

To evaluate the influence of risk factors and transcripts levels between groups, we compared the accuracy of the random forest model to rule out malignancy. The best model, using four input parameters (mRNA expression level of each gene and patient's history of cancer), had an accuracy of 85.4%, PPV 85.7%, AUC 0.821 and sensitivity of 81.1%; an improvement over the individual PPV of the null model seen in Fig. 3.

4. Discussion

In the present study, we identified higher expression of *CLDN1, TIMP1*, and *KRT19* transcripts in malignant thyroid nodules and is associated with a more aggressive subtype, suggesting a possible use in the detection of this pathology. Furthermore, when

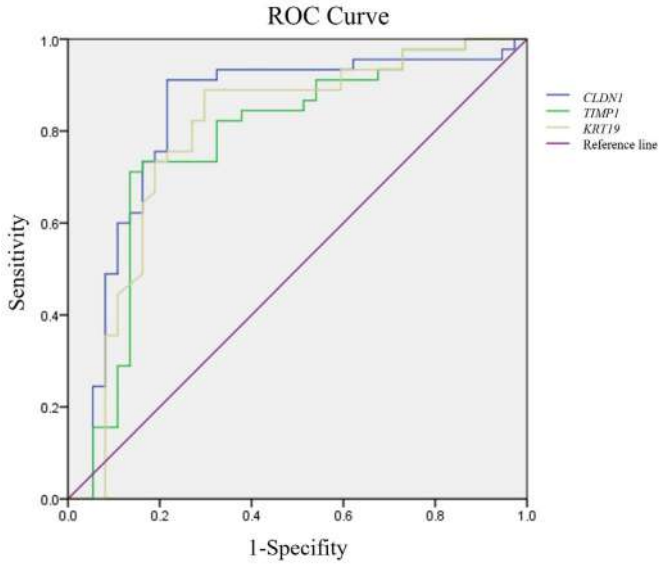

Fig. 3. Receiver operating characteristic (ROC) curve analysis *CLDN1, TIMP1* and *KRT19* to differentiate between groups.

Table 1
Diagnostic accuracy parameters of *CLDN1, TIMP1* and *KRT19*.

Gene	AUC	p-value	95% CI	Sensitivity	Specificity	PPV	NPV
CLDN1	0.832	<0.001	0.733–0.932	92%	59.2%	41.1%	95.8
TIMP1	0.777	<0.001	0.669–0.886	70%	81.6%	54.2%	89.7%
KRT19	0.793	<0.001	0.687–0.900	88%	61.2%	41.4%	94.1%

AUC, Area under the curve; CI, Confidence interval; PPV, Positive predictive value; NVP, Negative predictive value.

performing analyzes to identify the predictive value of these biomarkers to distinguish between these two states, a high predictive negative value (NPV) indicated potential use as biomarkers to rule out cancer.

The dysregulation of *CLDN1* expression has been previously reported in epithelial tumors [10], which is generally mislocalized outside tight junctions [19]. Its upregulation increases paracellular permeability, favoring tumor progression by promoting cell migration and invasion, and metastasis [10,19]. In thyroid cancer, expression of *CLDN1* was demonstrated in papillary tumors and lymph node metastases [20]; our study showed the most prominent and highly significant alterations in *CLDN1*, indicating the best sensitivity and NPV value among transcripts.

In good agreement with previously reported immunohistochemically biomarkers for epithelial cancer [3,21], we also confirmed the overexpression of *KRT19* and *TIMP1* in malignant nodules. In the case of *TIMP1*, this behavior contrasts with its primary mechanism of action as an inhibitor of metalloproteinases (MMPs); but, the group of Song suggested an MMP-independent role by phosphorylation pathway in colon cancer, supporting the role in proliferation, tumor invasion, and metastasis [14,22].

KRT19 is an immunohistochemical marker frequently used to determine malignant carcinomas of the pancreas, breast, colon, high-grade serous, ovarian, urothelial, esophagus, and stomach [21]. Nevertheless, in the thyroid, its expression is not limited to malignant transformation [23]. Our results confirm the presence of the transcript in all types of lesions. However, its expression increased significantly only in the group with malignant nodules, with a sensitivity of 88%, so its potential use as an immunohistochemical marker for the diagnosis of thyroid cancer should be studied.

By performing a predictive analysis by ROC curves to determine the capacity of each gene in the differentiation between benign and malignant nodules, we identified that individually they present favorable results. Therefore, the collective ability of these markers, together with characteristics that have previously been identified as risk factors for the development of thyroid cancer [24,25], were included to evaluate these models. The results obtained by the random forest model showed as significant variables the expression of the three study genes together with a personal history of cancer, representing an AUC (0.821), with better precision and PPV (85.7%). Among the advantages of using these models is identifying risk variables that are not linearly associated with the study variable, but together with other markers can determine the direction of the diagnosis. Also, our results remark the importance of expression analysis and show an interesting potential as mRNA biomarkers for thyroid cancer detection.

Furthermore, the results demonstrated a strong correlation between the expression of these genes in thyroid cells. The observed correlation between *KRT19, TIMP1*, and *CLDN1* may suggest functional interactions between these gene products during thyroid cancer progression. It has been demonstrated genetic alteration of MAPK and FAK-PI3K/Akt pathway activators might affect their expression, leading to the oncogenic cell transformation, mostly favoring tumor proliferation and metastasis [12–16,26]. The connection should be further explored as it holds promise for preoperative diagnostic purposes and could provide new insights into our understanding of thyroid carcinoma's etiology.

5. Conclusions

CLDN1, KRT19 and TIMP1 were significantly overexpressed in malignant thyroid nodules and observed a positive correlation between their expressions. As individual biomarkers, the transcript level of each gene demonstrated a high predictive value to rule out cancer. Moreover, when combining the relative expression of the three genes with the patient's history of cancer in a random forest model, we obtained a stronger predictive model to rule out malignancy in FNA of thyroid nodules. These results allow testing of a discrimination panel for indeterminate FNAs results (Bethesda III-IV) at the same level of attention.

Data availability statement

The datasets generated and/or analyzed during the current study are not publicly available due to the privacy policies of the health institutions involved but are available from the corresponding author (N.G.M) on reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.05.093.

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