DOI: 10.7860/JCDR/2023/65064.18650

Original Article

Pathology Section

Combined Cyclin D2 and Protein Convertase 2 Genes in Differentiating Various Follicular-patterned Lesions and Neoplasms of the Thyroid: A Cross-sectional Study

PRASANNA VENKADESA PERUMAL[1], NEELAIAH SIDDARAJU[2], SUNIL KUMAR SAXENA[3], SOUNDRAVALLY RAJENDIRAN[4], RAMACHANDRA V BHAT[5]

ABSTRACT

Introduction: A pretherapeutic distinction between benign and malignant thyroid nodules is critical for the clinical management of patients presenting with thyroid nodules. However, certain follicular-patterned lesions, such as adenomatous nodules, follicular neoplasms comprising Follicular Adenoma (FA) and Follicular Carcinoma (FC), as well as the Follicular Variant of Papillary Thyroid Carcinoma (FVPTC), can pose a significant dilemma during pre-therapeutic Fine Needle Aspiration Cytology (FNAC) evaluation. The present (pilot) study explores the possible utility of messenger Ribonucleic Acid (mRNA) and the protein expression of the two relatively less-explored genes, Cyclin D2 (CCND2) and Protein Convertase 2 (PCSK2), in distinguishing various follicular-patterned thyroid lesions and neoplasms by testing these molecular markers initially on histopathological sections.

Aim: To assess the RNA and protein expressions of CCND2 and PCSK2 genes in differentiating follicular-patterned thyroid neoplasms.

Materials and Methods: This was a cross-sectional analytical study conducted over 6 years, from August 2014 to August 2020, at the Department of Pathology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) along with Immunohistochemistry (IHC) was performed on a total of 75 tissue samples from follicular-patterned thyroid lesions and neoplasms, including 19 Follicular Hyperplasias (FHs), 10 Nodular Goitres (NGs), 17 FAs, 8 FCs, and 12 FVPTCs, along with nine Conventional Papillary Thyroid Carcinomas (CPTCs). After confirming the RNA and protein expression levels in each of these lesions, Immunoreactive Scoring (IRS) was performed to assess their IHC expression. The Kruskal-Wallis and Analysis of Variance (ANOVA) tests were used to analyse the mRNA expression data, and Pearson analysis was conducted to correlate the IHC data between the study groups. A p-value of <0.05 was considered statistically significant.

Results: Among the 75 thyroid lesions studied, both NG and FA showed a relatively higher cyclinD2 mRNA expression, with fold changes of 1.21 and 1.46, respectively. This was also reflected in IHC, with moderate nuclear expression observed in these cases. The PCSK2 mRNA expression was similar to that of CCND2, with the only difference noted between FH and FA. On IHC, eight out of 75 cases had positive PCSK2 expression, including five FHs, two FVPTCs, and one FA, while the remaining 67 cases were negative.

Conclusion: The CCND2 and PCSK2 genes assessed in the present study, regarding their mRNA and protein expressions, were not found to be of any practical value in distinguishing benign and malignant follicular lesions or neoplasms of the thyroid.

Keywords: Adenoma, Carcinoma, Follicular pattern, Follicular variant, Immunohistochemistry, Messenger ribonucleic acid expression, Papillary carcinoma

INTRODUCTION

A variety of thyroid disorders are common worldwide. Thyroid Function Tests (TFT), imaging studies, and FNAC are the common investigative modalities employed in thyroid work-up. The role of FNAC in establishing the neoplastic and non neoplastic nature of a thyroid nodule is well-established. The Bethesda System of Reporting Thyroid Cytopathology (TBSRTC) is the universally accepted pattern for reporting thyroid FNAC. Although FNAC is highly effective in detecting malignancies such as conventional papillary carcinoma, medullary carcinoma, anaplastic carcinoma, and high-grade Non-Hodgkin's Lymphoma (NHL), it cannot distinguish between a FA and FC, which are categorised as 'follicular neoplasm' or 'suspicious of follicular neoplasm' under TBSRTC. Furthermore, lesions like FH/adenomatous nodules and FVPTC often cause significant diagnostic difficulty, creating a major hurdle in deciding the nature of treatment or surgery [1-3].

Distinguishing various follicular-patterned thyroid lesions is not a problem confined to cytology alone. A significant diagnostic dilemma is encountered even in certain instances of histopathology. IHC markers like galectin-3, CBP/p300 Interacting Transactivator with Glu/Asp Rich Carboxy-Terminal Domain-1 (CITED), Hector Battifora Mesothelial Cell-1 (HBME-1), and cytokeratin-19 (CK-19), which were considered 'promising' in earlier studies, were subsequently shown to be of limited practical value [4-6]. Notably, the revised American Thyroid Association (ATA) in 2015 recommended the incorporation of 'molecular testing' in the pre-therapeutic follow-up of doubtful thyroid follicular lesions, a recommendation that has been accepted by TBSRTC-2017. Currently, molecular assays like Affirma gene classifier and ThyroSeq® are being applied to FNAC samples. However, the currently available molecular tests are not 100% effective in identifying the precise nature of doubtful thyroid nodules, which is why the search for further markers is ongoing [7]. There has been significant emphasis on certain less-explored genes like CCND2 and PCSK2 [8-11].

The CCND2 is one of the members of the D-type cyclin family, the others being CCND1 and cyclin D3. The D-type cyclins exhibit a

similar amino acid sequence, and their major function is in cell cycle regulation, especially in G1/S transition. They are also involved in cellular differentiation and oncogenic transformation. CCND2 was the first to be discovered among them and has been extensively studied, while CCND1 is relatively less explored. CCND2, located on chromosome 12q13, is usually expressed in B-lymphocytes [12]. It has been shown to increase phosphorylation of the retinoblastoma (Rb) protein and promote cell cycle progression, cell proliferation, and tumourigenesis [13]. Studies have documented its overexpression in gastric [12] and thyroid cancers [11].

The PCSK2 gene encodes a member of the subtilisin-like proPCSK family. A total of nine mammalian PCSK proteins have been discovered so far and have been classified based on their cleavage preferences. PCSK2 is located on chromosome number 20b and p11.2, consisting of 12 exons. The PCSK2 protein, synthesised as an inactive form (72kDa) in the endoplasmic reticulum, is transported to the Golgi complex, where it transforms into mature secretory granules (64kDa) [14]. The protein functions as a mediator of prohormones and pro-peptides in neuroendocrine cells [15]. Studies have highlighted its role as an IHC marker for the detection of a variety of metastatic neuroendocrine tumours [13].

Molecular methods to diagnose and distinguish follicular-patterned thyroid neoplasms have not been widely used in India. The present study assessed the utility of mRNA and protein expression of two important but less emphasised genes, PCSK2 and CCND2, on tissue samples using RT-qPCR and IHC techniques to address the long-standing diagnostic dilemma of differentiating follicular thyroid tumours.

MATERIALS AND METHODS

This cross-sectional analytical study was conducted over a six-year period, from August 2014 to August 2020, at the Department of Pathology, JIPMER, Puducherry, India, in collaboration with the Departments of Biochemistry, JIPMER, Puducherry, and the Department of Pathology, Indira Gandhi Medical College and Research Institute (IGMC&RI), Puducherry, India. The study commenced after obtaining approval from the Research Monitoring and Ethics Committees (IEC NO: JIP/IEC/SC/2015/19/783) of JIPMER.

Inclusion criteria: The study included all well-circumscribed or encapsulated lesions with histopathological diagnosis of adenomatoid nodule/FH, FA, FC, FVPTC, which had preoperative FNAC interpretations/diagnosis of NG, adenomatous nodule/FH, Follicular Lesion of Undetermined Significance (FLUS), Follicular Neoplasm/Suspicious for FN (FN/SFN), or FVPTC.

Exclusion criteria: Cases without a preoperative FNAC interpretation/diagnosis were excluded from the study.

Sample size calculation: The sample size was estimated based on the area under the curve of 0.725 in ROC with a negative-positive ratio of 0.666 (derived from our previous hospital records) and with an alpha error of 0.05 and a beta error of 0.20 using Medcalc 15.6.1 Software.

Study Procedure

Relevant clinical and laboratory results, including imaging findings, were gathered for all 75 cases included in the study. All cases had preoperative FNAC diagnosis, and thyroidectomy was performed based on the FNAC diagnosis or strong clinical suspicion of malignancy. Histopathological diagnosis was considered the gold standard. Strict precautionary measures were followed during the collection of tissue samples from the pathological lesions for molecular studies. Normal thyroid tissue collected from the surrounding pathological lesions served as the control. Fresh tissue from the lesional area of the thyroidectomy specimen was transferred into RNA later solution and stored at -80°C. RNA was isolated from the frozen tissue specimens using the RNA extraction kit (Roche Life Science). The extracted RNA was converted into cDNA using the cDNA synthesis kit (Roche Life Sciences). RT-qPCR was conducted to study the mRNA expression of the candidate genes using the Universal Probe Library Assay kits supplied by Roche Life Sciences. RT-qPCR was performed using the UPL probe numbers 49 (CCND2) and 21 (PCSK2) supplied by Roche Life Sciences. For the CCND2 gene, the Forward Primer-CCGCAGTGCTCCTACTTCAA and the Reverse Primer-GCCAAGAAACGGTCCAGGTA were used. For the PCSK2 gene, the Forward Primer-CGTGCAGGACCCTGAGAAAA and the Reverse Primer-GTCTCCTCTTCTGGTTGCGT were used. The β-actin (probe Number-9) served as the housekeeping gene (positive control). IHC for both PCSK2 and CCND2 proteins was performed in all 75 cases. The antibody kits for PCSK2 and CCND2 were obtained from ORIGENE Technologies (US) and Biorbyt Ltd (UK), respectively. Antibody dilution was done according to the manufacturer's protocol. The working condition of both antibodies was tested using the control tissues recommended by the manufacturers. For each tissue sample, 3 μm thick sections were prepared from the routinely processed and paraffin-embedded tissue blocks, which were placed on poly-L-lysine coated slides for immunostaining. The slides were treated with 3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidase. Antigen retrieval was done using the pressure cooker method with citrate buffer (pH 6.4). Primary antibody incubation was performed for 2 hours, followed by polymer horseradish peroxidase incubation for 40 minutes. Diaminobenzidine (DAB) was used as the chromogen. The IHC slides were examined, and an IRS was performed by the Pathologist (NS) [16]. The scoring pattern is elaborated in [Table/Fig-1].

A (Percentage of positive cells)	B (Intensity of staining)	IRS Score (Multiplication of A and B)
0= no positive cells	0=no colour reaction	0-1=negative
1=<10% of positive cells	1=mild reaction	2-3=mild
2=10-50% positive cells	2=moderate reaction	4-8=moderate
3=51-80% positive cells	3=intense reaction	9-12=strongly positive
4=>80% positive cells	Final IRS score (A×B):0-12	

[Table/Fig-1]: Immunoreactive Scoring (IRS) System (16).

STATISTICAL ANALYSIS

For multiple comparisons between the study groups, the Kruskal-Wallis test was used for unpaired data, while a one-way ANOVA was used for paired data. Pearson's correlation analysis was performed to assess the correlation of IHC expression in each study group. All statistical tests were conducted using GraphPad Instat Version 3.06, and a p-value of <0.05 was considered statistically significant.

RESULTS

The patients' age ranged from 19 years to 75 years, with a median of 56 years. There were 60 women and 15 men, resulting in an M:F ratio of 1:4. The preoperative cytological diagnosis and their histopathological correlation with the mean age of the patients are provided in [Table/Fig-2].

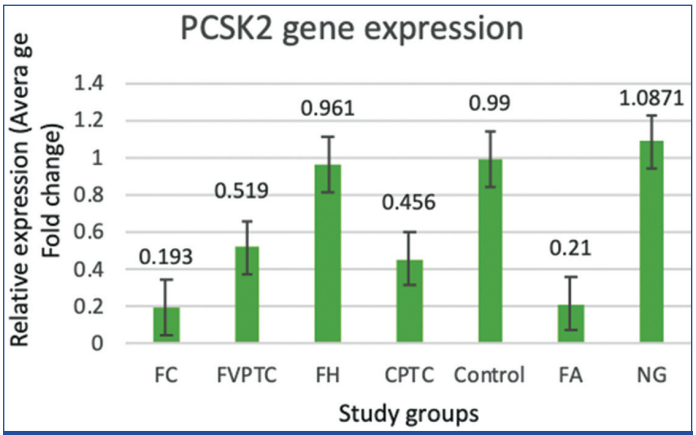
mRNA expression: The PCSK2 mRNA expression showed no significant change in any of the study groups, except for the FH group, which exhibited a significantly elevated level compared to FA and CPTC (p-value <0.001) [Table/Fig-3]. Among the 75 cases, eight showed PCSK2 gene mRNA expression, including five FHs and one case each of FVPTC, FA, and NG, while the remaining 67 cases had a similar expression pattern [Table/Fig-4]. Relative PCSK2 gene expression did not yield significant results for differentiating between FC and FA (p-value 0.17). The CCND2 mRNA expression levels remained unchanged in the FC group compared to the FA, FH, and NG groups [Table/Fig-5]. The CCND2 gene expression did not show significant results for differentiating between FC and FA (p-value 0.44) [Table/Fig-6]. The expression pattern of CCND2 was

FNA diagnoses: TBSRTC Category (/%)	Histopathological diagnoses (n/%)	Mean age (years)
TBSRTC-I (Non diagnostic): 01 (1.3)	Nodular Goitre (NG):10 (13.3)	37.2
TBSRTC-II (benign): 37 (49.5)	Follicular Hyperplasia (FH):19 (25.4)	36.6
TBSRTC-III (AUS/FLUS): 06 (8.0)	Follicular Adenoma (FA):17 (22.7)	38.9
TBSRTC-IV (FN/SFN): 11 (14.6)	Follicular Carcinoma (FC): 08 (10.6)	50.3
TBSRTC-V (SM): 12 (16)	Follicular Variant of Papillary Thyroid Carcinoma (FVPTC): 12 (16)	38.8
TBSRTC-VI (Malignant): 08 (10.6)	Conventional PTC:09 (12)	44.6
Total: 75 (100%)	Total: 75 (100%)	

[Table/Fig-2]: Cytohistopathological correlation with the mean age of study participants.
TBSRTC: The Bethesda system of reporting thyroid cytopathology; AUS: Atypia of undetermined significance; FLUS: Follicular lesion of undetermined significance; FN: Follicular neoplasm; FN/SFN: Follicular neoplasm/suspicious of follicular neoplasm; SM: Suspicious of malignancy. In Subheading, Nodular Goitre should be Nodular Goitre

S. No.	Comparison among groups	Mean rank difference between groups	p-value	Remarks
1	FA vs. FC	-2.331	0.1724	NS
2	FVPTC vs. FC	-0.1235	>0.9999	NS
3	CPTC vs. FC	-1.540	0.7120	NS
4	FH vs. FC	0.2527	0.998	NS
5	NG vs. FC	1.243	0.7694	NS
6	FVPTC vs. FA	2.208	0.1203	NS
7	CPTC vs. FA	0.7916	0.9580	NS
8	NG vs. FA	2.584	0.0194	*
9	FH vs. FA	3.575	0.0005	***
10	CPTC vs. FVPTC	1.416	0.7002	NS
11	FH vs.FVPTC	0.3671	0.9976	NS
12	NG vs.FVPTC	1.367	0.6098	NS
13	FH vs. CPTC	1.792	0.3784	NS
14	NG vs. CPTC	2.783	0.0454	*
15	NG vs. FH	0.9906	0.8067	NS

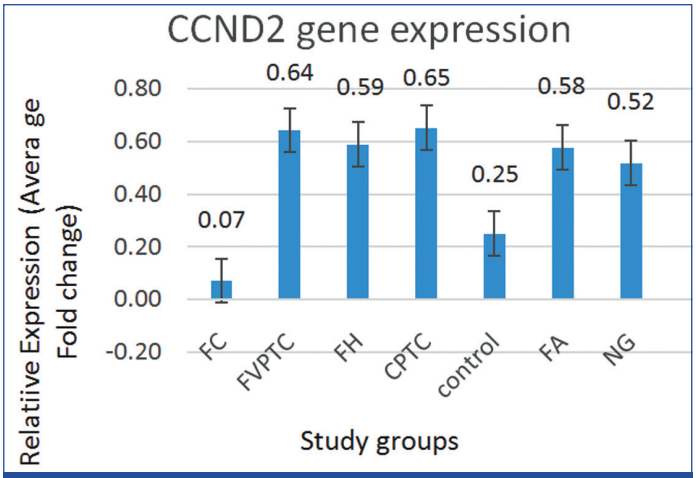
[Table/Fig-3]: PCSK2 gene expression in thyroid tumours.
PCSK2: Protein convertase2; FC: Follicular carcinoma; FA: Follicular adenoma; FVPTC: Follicular variant of papillary thyroid carcinoma; CPTC: Conventional papillary thyroid carcinoma; FH: Follicular hyperplasia, NG: Nodular goitre, *Significant p-value; ***Highly significant



[Table/Fig-4]: The relative PCSK2 gene expression among the study groups.

similar in all study groups, except the FC group, which exhibited a lesser fold change. Therefore, there was no statistical evidence to support a significant difference among the various study groups.

IHC expression: Among the 75 study cases, only one case each of FA and NG exhibited positive expression (moderate) of CCND2 [Table/Fig-7]. None of the malignant cases expressed the marker. A high percentage of FAs (94.2%) and NGs (91.6%) were negative for



[Table/Fig-5]: The relative gene expression of CCND2 in the present study groups.

S. No.	Comparison among groups	Mean rank difference between groups	p-value	Remarks
1	FA vs. FC	-0.934	0.4494	NS
2	FVPTC vs. FC	-0.776	0.7396	NS
3	CPTC vs. FC	-0.471	0.9651	NS
4	FH vs. FC	-0.199	0.9840	NS
5	NG vs. FC	-0.516	0.9603	NS
6	FVPTC vs. FA	0.158	0.9842	NS
7	CPTC vs. FA	0.463	0.9603	NS
8	FH vs. FA	0.735	0.4719	NS
9	NG vs. FA	0.417	0.9732	NS
10	CPTC vs. FVPTC	0.304	0.9840	NS
11	FH vs. FVPTC	0.576	0.8275	NS
12	NG vs. FVPTC	0.259	0.9840	NS
13	FH vs. CPTC	0.271	0.9489	NS
14	NG vs. CPTC	-0.045	0.9840	NS
15	NG vs. FH	-0.317	0.9681	NS

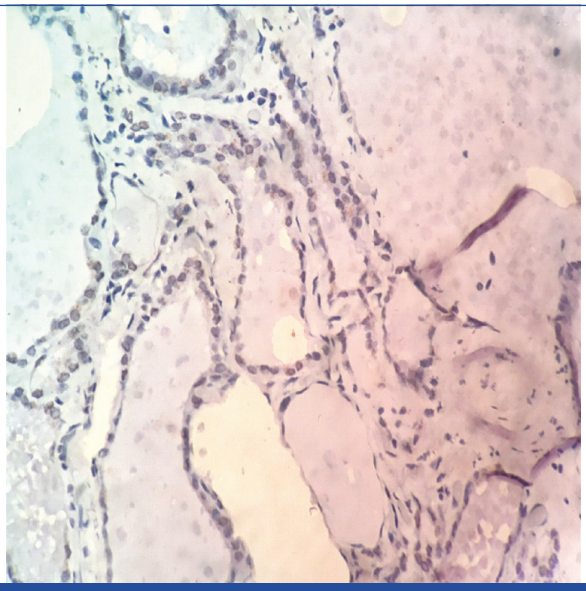
[Table/Fig-6]: CCND2 gene expression in various study groups.
CCND2: CyclinD2; FC: Follicular carcinoma; FA: Follicular adenoma; FVPTC: Follicular variant of papillary thyroid carcinoma; CPTC: Conventional papillary thyroid carcinoma; FH: Follicular hyperplasia; NG: Nodular goitre; NS: Not significant

CCND2, indicating its poor concordance even among non malignant thyroid lesions [Table/Fig-7]. None of the FH cases showed CCND2 positivity, suggesting that the marker was not specific for either benign neoplastic or non neoplastic lesions. The majority of cases exhibited a negative staining pattern for CCND2, with focal nuclear positivity observed in one case each of FA and NG [Table/Fig-8]. Immunohistochemically, PCSK2 expression was observed only in a few cases of FVPTC, FA, and FH, while the remaining study groups were all negative (100%) [Table/Fig-9]. Out of 12 FVPTCs, only 2 were PCSK2 positive, with one showing moderate expression and the other showing strong expressions. Among 19 FHs, 5 expressed

Histopathology diagnosis	Number of cases	CCND2 expression			
		Negative (0-1)	Mild (2-3)	Moderate (4-8)	Strong (9-12)
FC	8	8	0	0	0
FA	17	16	0	1	0
FVPTC	12	12	0	0	0
CPTC	9	9	0	0	0
FH	19	19	0	0	0
NG	10	9	0	1	0

[Table/Fig-7]: The scoring of IHC expression of CCND2 by using the IRS scoring system.
CCND2: CyclinD2; IRS: Immunoreactive score; FC: Follicular carcinoma; FA: Follicular adenoma; FVPTC: Follicular variant of papillary thyroid carcinoma; CPTC: Conventional papillary thyroid carcinoma; FH: Follicular hyperplasia; NG: Nodular goitre

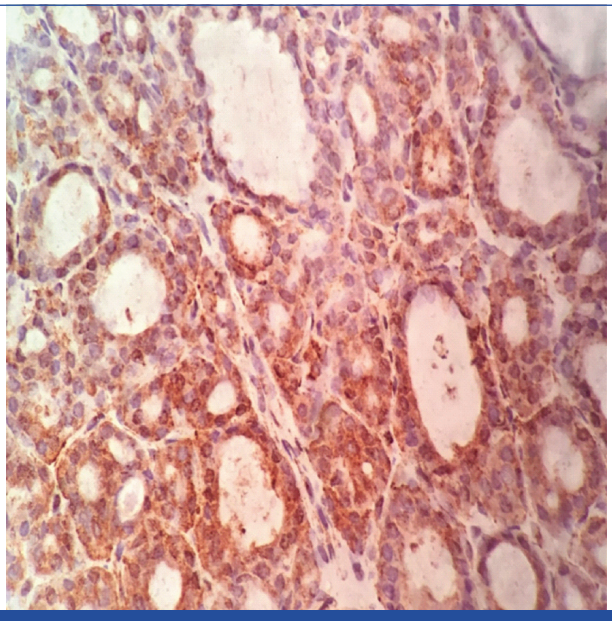
the marker, with three exhibiting moderate expressions and the remaining two showing mild and strong expressions, respectively [Table/Fig-10]. Pearson correlation analysis for PCSK2 IHC expression showed no correlation between FA vs. FVPTC (p-value 0.771) and FH vs. FVPTC (p-value 0.585), with R-values of 0.07 and 0.13, respectively. The correlation between FA and FH showed a moderate correlation with an R-value of 0.47 and a p-value of 0.052 [Table/Fig-11].



[Table/Fig-8]: MNG showing a moderate nuclear CCND2 expression (IHC, 40x, DAB chromogen).

Histopathology diagnosis	Number of cases	PCSK2 expression			
		Negative (0-1)	Mild (2-3)	Moderate (4-8)	Strong (9-12)
FC	8	8	0	0	0
FA	17	16	1	0	0
FVPTC	12	10	0	1	1
CPTC	9	9	0	0	0
FH	19	14	1	3	1
NG	10	10	0	0	0

[Table/Fig-9]: The scoring of IHC expression of PCSK2 by using the IRS scoring system.
PCSK2: Protein convertase2; IRS: Immunoreactive score; FC: Follicular carcinoma; FA: Follicular adenoma; FVPTC: Follicular variant of papillary thyroid carcinoma; CPTC: Conventional papillary thyroid carcinoma; FH: Follicular hyperplasia; NG: Nodular goitre



[Table/Fig-10]: Follicular Hyperplasia (FH) showing a strong granular cytoplasmic PCSK2 expression in hyperplastic cells (IHC, 40x, DAB chromogen).

S. No.	Study groups	R-value	p-value	Correlation
1	FA vs FVPTC	0.07	0.771	Weak
2	FA vs FH	0.47	0.052	Moderate
3	FH vs FVPTC	0.13	0.585	Weak

[Table/Fig-11]: Pearson correlation of PCSK2 IHC expression.
PCSK2: Protein convertase2; IHC: Immunohistochemistry; FA: Follicular adenoma; FVPTC: Follicular variant of papillary thyroid carcinoma; FH: Follicular hyperplasia

The majority of cases exhibited negative protein expression for CCND2 on IHC (73 negatives and 2 positives), so correlation analysis was not performed. Overall, both CCND2 and PCSK2 were not deemed suitable for routine application in differentiating various follicular-patterned neoplasms/lesions based on their immunohistochemical results.

DISCUSSION

The follicular-patterned thyroid lesions pose significant diagnostic challenges, both in cytology and histopathology, resulting in a “grey zone” of uncertainty. As a result, there is a continuous search for reliable Immunohistochemical (IHC) markers and molecular assays. In the present study, we examined 75 cases with histopathological confirmation, of which 66 were follicular-patterned lesions including NG, FH, FA, FC, and FVPTC. The significant difference in the number of non neoplastic and neoplastic follicular-patterned lesions between cytological and histopathological interpretations highlights the gravity of the problem faced by cytopathologists. In the present study, authors aimed to evaluate the effectiveness of CCND2 and PCSK2 genes, along with their corresponding proteins, in distinguishing among various follicular-patterned lesions and neoplasms.

The CCND2 and PCSK2 genes encode proteins belonging to the Cyclin-D and subtilisin-like proPCSK families, respectively. According to the literature, CCND2 is frequently downregulated in cancer conditions, although upregulation has been reported in malignancies such as breast and prostate cancers [17]. Takano Y et al., investigated the role of CCND2 and CCND1 genes in gastric and breast cancers using IHC and Western blotting methods [12]. They found that CCND2 overexpression was significantly associated with cancer invasion and lymph node metastasis. Overexpression of CCND2 was also observed in ovarian granulosa cell tumours [18]. Sarkar S et al., studied CCND2 expression in colorectal cancer and found that positive expression was associated with tumour progression [19]. In another significant study, three candidate genes including CCND2, PCSK2, and UbcH10 were analysed in 84 follicular neoplasms. The mRNA expression of all three genes was compared, and it was found that only UbcH10 yielded significant results. CCND2 and PCSK2 expressions were more frequently observed in benign thyroid tumours [20].

However, an analysis of CCND2 gene expression in follicular tumours by Prabakaran I et al., did not reveal any significant difference between benign and malignant follicular tumours [21]. They did notice relatively higher expression of the CCND2 gene in normal and benign thyroid tissues compared to malignant follicular tumours. Krause K et al., studied the utility of multiple genes for differentiating between benign and malignant thyroid tumours [22]. PCSK2 was one of the genes included in their study; however, according to their results, PCSK2 alone was not able to differentiate between benign and malignant thyroid neoplasms. The possibility of an altered mechanism of pro-protein processing involved in the tumour progression of thyroid neoplasms has remained elusive for most researchers. Nejjari M et al., showed that PCSK2 dysfunction causes malignant cell migration and metastasis in colonic cancer [23]. According to the study by Weber F et al., PCSK2 was found to be effective in differentiating follicular thyroid tumours, although it failed to provide similar results in the present study [17]. Weber F et al., observed a significant downregulation of both CCND2 and PCSK2 mRNA in FC compared to FA, with PCSK2 showing

a fold difference downregulation of 263 times [17]. The CCND2 and PCSK2 mRNA results of the present study with some of the important studies documented in the literature has been presented in [Table/Fig-12] [11,21].

S. No.	Studies	Study place	PCSK2 Fold change		CCND2 Fold change	
			Benign	Malignant	Benign	Malignant
1	Present study	Puducherry, India	0.75	0.4	0.55	0.48
2	Yuan S et al., 2023 [11]	Hebei, China	Not studied	Not studied	1.12 Upregulated	2.52 Upregulated
3	Prabakaran I et al., 2010 [21]	Pennsylvania, USA	-	-22.4 Down regulated	Not significant	Not significant

[Table/Fig-12]: Comparison of our study results with other studies [11,21].
PCSK2: Protein convertase2; CCND2: CyclinD2

Cerutti J et al., conducted a study on multiple gene expression patterns using Serial Analysis of Gene Expression (SAGE) analysis. In their study, six of the genes analysed were unable to distinguish between FA and FC [24]. PCSK2 was one of the genes included in the SAGE analysis, and it showed mRNA expression of 30 to 69% in FC and 30 to 40% in FA. Therefore, PCSK2 expression may be rarely observed in thyroid follicular-patterned neoplasms. While some studies have demonstrated significant downregulation of CCND2 and PCSK2 in FC [17,22], our present study did not find any difference in their expression between FC and FA. Both Weber F et al., and Krause K et al., used Beta-actin as the common housekeeping gene in their studies [17,22]. Similarly, the present study also employed Beta-actin as the common housekeeping gene, but authors were unable to demonstrate the diagnostic utility of CCND2 and PCSK2 gene assays in distinguishing follicular-patterned neoplasms of the thyroid.

There has been significant literature on the role of IHC in differentiating benign and malignant thyroid nodules [4-6]. Various IHC panels, including markers such as galectin-3, HBME-1, CITED-1, CK-19, Thyroid Peroxidase (TPO), and Thyroglobulin (TG), have been extensively studied [4]. While these markers have shown high sensitivity (>80%) and specificity (>80%), some of them are also known to be expressed in a considerable number of benign thyroid nodules and even in normal thyroid tissue [4-6]. More recently, markers such as anti-BRAFV600E (VE1), Trophoblast Cell-surface Antigen-2 (TROP-2), CD56, and Ki67 (a proliferative marker) have been found to be useful as IHC markers for distinguishing benign and malignant thyroid nodules, as well as various follicular-patterned lesions including Non Invasive Follicular Tumour with Papillary (NIFTP)-like nuclear features [5,6].

Limited literature is available on the IHC assessment of PCSK2 and CCND2 in thyroid neoplasms [17]. CCND2 has been well-studied as an IHC marker in gastric carcinomas, where it exhibits cytoplasmic, nuclear, or combined cytoplasmic and nuclear expression in certain normal tissues like the salivary gland and a variety of malignancies including Diffuse Large B-Cell Lymphoma (DLBCL), melanoma, prostatic and thyroid carcinomas [25]. Cytoplasmic expression of CCND2 in gastric carcinoma has been associated with poor prognosis [12]. PCSK2, which displays cytoplasmic expression, has been found to be useful in detecting neuroendocrine tumours of midgut, pulmonary, pheochromocytoma, and paraganglioma origin at metastatic sites [13].

Regarding their role in thyroid neoplasms, Weber F et al., performed IHC for CCND2 and PCSK2, both of which showed negative expression in FC, while FA had positive expression. They claimed that the results obtained with the combined PCSK2 and CCND2 markers were superior to other studies documented in the literature. None of their FC cases showed positive expression for PCSK2. Our PCSK2 findings are fairly similar to those of Weber F et al., with both studies showing negative PCSK2 expression in FC [17]. However, the same is not true for PCSK2 expression in our FA cases, which were all negative, in contrast to the Weber F et al., series where all FAs were PCSK2 positive [17]. In the present study, authors recorded positive PCSK2 expression in five FHs and a single FA. All these immunohistochemically positive PCSK2 cases also had a correspondingly increased PCSK2-mRNA level. Thus, both markers were not found to be of any practical benefit.

Limitation(s)

The authors could have arrived at more meaningful conclusions by assessing CCND2 and PCSK2 IHC as part of an immunopanel with other immunohistochemical markers that have already been tested with reasonably good sensitivity and specificity, as highlighted above. This is a limitation of the present study. However, the insignificant CCND2 and PCSK2 IHC results documented in the present study make them obviously inferior to the other markers highlighted in the literature.

CONCLUSION(S)

The preoperative distinction of various follicular-patterned thyroid nodules is diagnostically challenging for cytopathologists, although it is critical for patient management. These lesions often pose significant diagnostic dilemmas even upon histopathological examination. While sensitive molecular assays like Thyroseq and affirma gene classifier are currently being used on preoperative cytological samples, they are not completely specific, leading to a continued search for reliable molecular markers. In the present study, authors assessed the CCND2 and PCSK2 genes, based on their proven utility in certain malignancies, to distinguish between benign and malignant thyroid follicular lesions/neoplasms. However, authors did not find them to be of any practical value at both the mRNA and protein levels.

REFERENCES

[1] Han K, Ha J, Kong JS, Kim JS, Myung JK, Koh JS, et al. Cytological features that differentiate follicular neoplasm from mimicking lesions. J Pathol Transl Med. 2018;52(2):110-20.
[2] Balakrishnan K, Sumathi V, Elangovan RS, Hemalatha G. Follicular neoplasms cytohistomorphological aspects: A case study of 50 cases. Int J Sci study. 2016;4(4):225-30.
[3] Faquin WC. Diagnosis and reporting of follicular-patterned thyroid lesions by fine needle aspiration. Head Neck Pathol. 2009;3(1):82-85.
[4] Saleh H, Jin B, Barnwell J, Alzohaili O. Utility of immunohistochemical markers in differentiating benign from malignant follicular-derived thyroid nodules. Diagn Pathol. 2010;5:9.
[5] Chuang HW, Wang JS, Tsai JW, Hsu CT, Lin KJ. Immunohistochemistry helps to distinguish non-invasive follicular thyroid neoplasm with papillary-like nuclear features/non-invasive encapsulated follicular variant of papillary thyroid carcinoma with other follicular thyroid lesions. Medicina (Kaunas). 2021;57(11):1246.
[6] Dwivedi SS, Khandeparkar SGS, Joshi AR, Kulkarni MM, Bhayekan P, Jadhav A, et al. Study of immunohistochemical markers (CK-19, CD-56, Ki-67, p53) in differentiating benign and malignant solitary thyroid nodules with special reference to papillary thyroid carcinomas. J Clin Diagn Res. 2016;10(12):EC14-EC19.
[7] Tamhane S, Gharib H. Thyroid nodule update on diagnosis and management. Clin Diabetes Endocrinol. 2016;2:17.
[8] Jaaks P, Bernasconi M. The proprotein convertase furin in tumour progression: The PC furin in tumour progression. Int J Cancer. 2017;141(4):654-63.
[9] Shen Y, Dong S, Liu J, Zhang L, Zhang J, Zhou H, et al. Identification of potential biomarkers for thyroid cancer using bioinformatics strategy: A study based on GEO datasets. Biomed Res Int. 2020;2020:9710421.
[10] Leone V, Angelo D, Rubio I, Freitas PM, Fedrico A, Colamaio M, et al. MiR-1 is a tumor suppressor in thyroid carcinogenesis targeting CCND2, CXCR4, and SDF-1 alpha. J Clin Endocrinol Metab. 2011;96(9):E1388-98.
[11] Yuan S, Liu Z, Yu S, Wang X, Shi J. CCND2 and miR-206 as potential biomarkers in the clinical diagnosis of thyroid carcinoma by fine-needle aspiration cytology. World J Surg Oncol. 2023;21(1):22.
[12] Takano Y, Kato Y, van Diest PJ, Masuda M, Mitomi H, Okayasu I. CyclinD2 overexpression and lack of p27 correlate positively and cyclin E inversely with a poor prognosis in gastric cancer cases. Am J Pathol. 2000;156(2):585-94.
[13] Remes SM, Leijon H, Vesterinen T, Louhimo J, Pulkkinen V, Ezer S, et al. PCSK2 expression in neuroendocrine tumors points to a midgut, pulmonary or pheochromocytoma-paraganglioma origin. APMIS. 2020;128(11):563-72.

[14] Benjannet S, Rondeau N, Paquet L, Boudreault A, Lazure C, Chrétien M, et al. Comparative biosynthesis, covalent post-translational modifications, and efficiency of pro-segment cleavage of the prohormone convertases PC1 andPC2: Glycosylation, sulphation, and identification of the intracellular site of prosegment cleavage of PC1 and PC2. Biochem J. 1993;294(3):735-43.
[15] Gyamera-Acheampong C, Mbikay M. Proprotein convertase subtilisin/kexin type4 in mammalian fertility: A review. Hum Reprod Update. 2009;15(2):237-47.
[16] Fedchenko N, Reifenrath J. Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue- A review. Diagn Pathol. 2014;9:221.
[17] Weber F, Shen L, Aldred MA, Morrison CD, Frilling A, Saji M, et al. Genetic classification of benign and malignant thyroid follicular neoplasia based on a three-gene combination. J Clin Endocrinol Metab. 2005;90(5):2512-21.
[18] Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, et al. Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature. 1996;384(6608):470-74.
[19] Sarkar S, Dey BK, Dutta A. MiR-322/424 and 503 are induced during muscle differentiation and promote cell cycle quiescence and differentiation by down-regulation of Cdc25A. Mol Biol Cell. 2010;21(13):2099-2366.
[20] Guerriero E, Ferraro A, Desiderio D, Pallante P, Berlingieri MT, Iaccarino A, et al. UbcH10 expression on thyroid fine-needle aspirates. Cancer Cytopathol. 2010;25;118(3):157-65.
[21] Prabakaran I, Grau J, Lewis R, Fraker D, Guvakova M. Rap2Al up regulated in invasive cells dissected from follicular thyroid cancer. J Thyroid Res. 2011;2011:979840.
[22] Krause K, Eszlinger M, Gimm O, Karger S, Engelhardt C, Dralle H, et al. TFF3-based candidate gene discrimination of benign and malignant thyroid tumors in a region with borderline iodine deficiency. J Clin Endocrinol Metab. 2008;93(4):1390-93.
[23] Nejjari M, Berthet V, Rigot V, Laforest S, Jacquier MF, Seidah NG, et al. Inhibition of proprotein convertases enhances cell migration and metastases development of human colon carcinoma cells in a rat model. Am J Pathol. 2004;164(6):1925-33.
[24] Cerutti J, Delcelo R, Amadei M, Nakabashi C, Maciel R, Peterson B, et al. A preoperative diagnostic test that distinguishes benign from malignant thyroid carcinoma based on gene expression. J Clin Invest. 2004;113(8):1234-42.
[25] Hans CP, Weisenburger DD, Greiner T, Chan WC, Aoun P, Cochran GT, et al. Expression of PKC-beta or cyclin D2 predicts for inferior survival in diffuse large B-cell lymphoma. Modern Pathol. 2005;18(10):1377-84.

PARTICULARS OF CONTRIBUTORS:
1. Laboratory Technician, Department of Microbiology, Jawaharlal Institute of Medical Education and Research (JIPMER), Puducherry, India.
2. Senior Professor, Department of Pathology, Jawaharlal Institute of Medical Education and Research (JIPMER), Puducherry, India.
3. Professor, Department of Otorhinolaryngology, Jawaharlal Institute of Medical Education and Research (JIPMER), Puducherry, India.
4. Professor, Department of Biochemistry, Jawaharlal Institute of Medical Education and Research (JIPMER), Puducherry, India.
5. Dean and Professor, Department of Pathology, Indira Gandhi Medical College and Research Institute (IGMC and RI), Puducherry, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Neelaiah Siddaraju,
Senior Professor, Department of Pathology, Jawaharlal Institute of Medical Education and Research (JIPMER), Puducherry-605006, India.
E-mail: rajusiddaraju@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Apr 29, 2023
- Manual Googling: Jul 13, 2023
- iThenticate Software: Aug 12, 2023 (8%)

ETYMOLOGY: Author Origin

EMENDATIONS: 8

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

Date of Submission: **Apr 27, 2023**
Date of Peer Review: **Jun 16, 2023**
Date of Acceptance: **Aug 14, 2023**
Date of Publishing: **Nov 01, 2023**