Original Article

Immunohistochemical analysis of thyroid follicular neoplasms and BRAF mutation correlation

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Abstract

BACKGROUND: The accurate diagnosis of benign and malign thyroid tumors is very important for the clinical management of patients. The distinction of thyroid papillary carcinoma follicular variant and follicular adenoma can be difficult. **AIM:** To investigate the alternative methods like immunohistochemistry and exon 15 in the BRAF gene 1799 T/A mutation analyses for distinguishing thyroid tumors. **MATERIALS AND METHODS:** We applied immunohistochemical markers; CK19, HMWCK, Galectin-3, HBME-1 and Fibronectin and mutant allele-specific PCR amplification technique was used to determine 1799 T/A mutation within the BRAF gene. Formalin-fixed parafin embedded tissues from 45 surgically total resected thyroids, included 26 thyroid papillary carcinoma follicular variant (FV-TPC), 8 Follicular Adenoma (FA), 6 Minimal invasive follicular carcinoma (MIFC) and 5 Follicular Carcinoma (FC). **STATISTICAL ANALYSES USED:** Pearson Chi-Square and Kruskal Wallis tests were performed. **RESULTS:** There was a positive correlation between FV-TPC and HMWCK, CK 19, HBME1, Galectin 3, fibronectin ($P < 0.05$), but there was no correlation with FV-TPC and BRAF gene mutation ($P > 0.05$). HBME-1 and CK 19 stained strong and diffuse positive in FV-TPCs but weak and focal in FAs. **CONCLUSION:** Our study suggests that morphologic features combined with immunohistochemical panel of HMWCK, CK19, HBME-1, Galectin-3 and fibronectin can help to distinguish benign and malign thyroid neoplasms and FV-TPC from follicular adenomas. BRAF gene 1799 T/A mutation has been non-specific but its detection can be a useful tool combined with immunohistochemistry for diagnosing FV-TPC.

Key Words: BRAF, Immunohistochemistry, papillary carcinoma follicular variant, thyroid

Introduction

The prognosis and management of thyroid nodules are mostly related to their diagnosis.The main diagnostic gold standard is pathologic evaluation with routine hematoxylin and eosin (H and E).[1,2]

There are often morphologic similarities between benign and malignant lesions and follicular and papillary architectures may be seen in both benign and malignant lesions.[1,3] Some important features of malignancy, like; pale nuclei, vesicule nucleus for papillary thyroid carcinoma are open to subjective interpretations and there are disagreements among pathologists.[1,2]

Papillary thyroid carcinoma (PTC) consitutes the majority of all thyroid malignant neoplasms and the follicular variant (FV-TPC) is the most common among the subtypes. Histopathology is characterized by follicular growth patern and nuclear features identical to usual type.[4,5] Some cases of FV-TPC are surrounded by fibrous capsule.[5] There are diagnostic problems when the characteristic nuclear features are not diffusely distributed throughout the lesion.[2,4,5] Such lesions can be diagnosed as benign follicular nodule or if they show capsular or vascular invasion as in follicular carcinoma.[4-6]

The alternative methods like immunohistochemistry and genetic mutation analysies has become available for distinguishing these lesions.

The different expression patterns of cytokeratins has been investigated in various thyroid lesions.[3,7] CK 19 is a low molecular weight keratin widely present in simple epihelial cells and is a minor component of stratified epithelia such as basal cell layer.[4,8,9] CK 19 has been used to distinguish PTC from other benign lesions.[2,3,5,7,10,11] Besides CK 19, galectin 3, HMBE-1 and fibronectin are useful markers.[6,9] HBME-1 is positive in papillary carcinomas showing follicular or papillary differentation and originates from mesothelial cells.[2,11,12] HBME-1 has low sensitivity of that limits its value but it has high specifity for borderline lesions.[12] It indicates malignancy but not papillary differentiation.[2]

Galectin 3 has not been a reliable marker and also stained normal, hyperplastic and inflamed thyroid tissue, but positivity in malignancies has been more diffuse and strong.[12]

Overexpression of fibronectin by thyroid tumors was first reported in 1988 but there has not been any data about its expression in hyperplastic nodules and comparision of other thyroid malignancy markers.[13]

BRAF is a protooncogene and codes a serine/threonine kinase which transduces regulatory signals by Ras-Raf-MEK-ERK cascade and has been identified to have mutations in several human cancers.[14-16] The V600E mutation (T1799) constitutes most of the BRAF mutation in PTC.[17]

Detection of BRAF gene mutation has been reported to be spesific for PTC but not for follicular carcinomas or benign thyroid neoplasms. But it could serve as a a potential diagnostic tool for the evaluation of thyroid nodules with indeterminate histopathologic findings.

Materials and Methods

This was a retrospective archive study including 36 female and 10 male patients who were diagnosed as FA, FC, MIFC and FV-TPC between 2005-2009. Only patients that underwent total thyroidectomy procedure were included in this study. The ethical committee on human research at our institution approved the protocol for all human research and this study was financially supported by Scientific Research Committee (Grant No: 03M0123).

Routine hematoxylin-eosin staining, HMWCK, CK 19, HBME1, Galectin 3, Fibronectin immunoperoxidase tecniques and DNA extraction tecnique for the BRAF

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gene mutation were performed on parafin-embedded tissues [Figures 1 and 6].

Immunohistochemical staining was carried out by deparaffinization, dehydration and incubation in citrate buffer. A labelled streptoavidin-biotin-peroxidase (immunoenzymatic) antigen detection system and AEC chromogen were used.

The antigen staining was performed with Mouse Monoclonal Antibody, Cell marque Corp. USA for Mesothelial cell (HBME-1), Cytokeratin 19 and Galectin-3, with Mouse Monoclonal Antibody, Novacastra-Leica Biosystems, Newcastle, United Kingdom for HMWCK (RTU-CK 34BE12) and with Rabbit Polyclonal Antibody, Gene Tex, Inc. CA, USA for Fibronectin.

For the evaluation of HMWCK, CK 19, HBME1, Galectin 3, fibronectin, we counted the positive stained cells per field of at least 5 dense stained fields, at a magnification of ×400 by an Olympus B×53 light microscope.

Immunoreactivity was scored by a semiquantitative scoring method. By evaluating both the heterogeneous positive distribution and the differing intensity of the staining simultaneously, we classified all cases in 5 categories according to Savin *et al.*:[18] Group 0 less than 5% positive epithelial cells, Group 1 from 5% to 20% positive, Group 2 from 20% to 50% positive, Group 3 from 50% to 80% positive, and Group 4 more than 80% positive cells.

DNA extraction

Genomic DNA was extracted from 3 to 4 (10-µm thick) sequential sections for each of the 21 archivel formaline fixed paraffine embedded (FFPE) tissues. Paraffine was removed from the specimens by adding 1 ml xylen to a 1.5 ml eppendorf tube containing 3-4 sections of FFPE tissue, and incubating at 90°C for 30 min to dissolve the paraffine. Genomic DNA was then extracted with a commercially available kit, QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Germany), using the manufacturer's instructions.

Mutant allele specific PCR amplification

Exon 15 in the BRAF gene 1799 T/A mutation in thyroid papillary carcinomas was detected by using mutant allele specific PCR amplification (MASA) technique according to Sapio *et al.*[19] Two different forward primers (5'-GTGATTTTGGTCTAGCTACAGT- 3' and 5'-GTGATTTTGGTCTAGCTACAGA- 3') were used to amplify the wild-type or mutant alleles

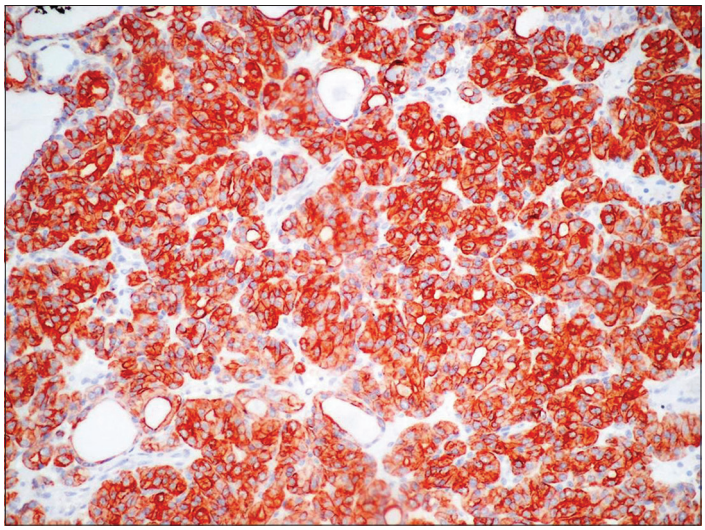

Figure 1: Strong and diffuse positivity of CK19 in TPCFV (H and E, ×200)

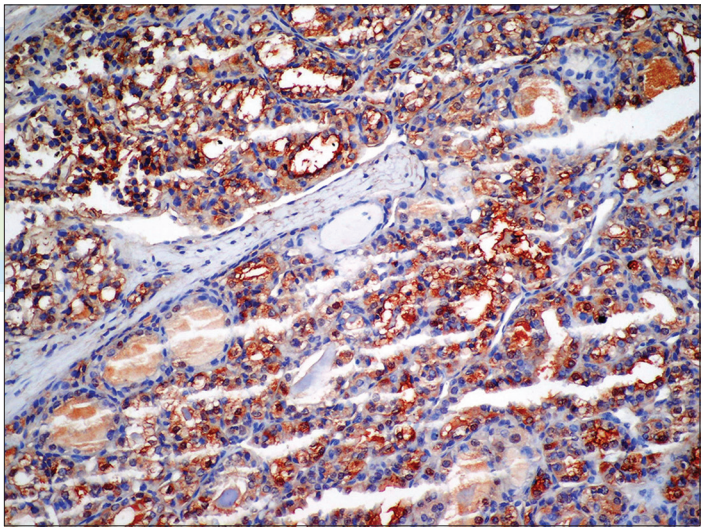

Figure 2: Strong and diffuse positivity of Galectin-3 in TPCFV (H and E, ×200)

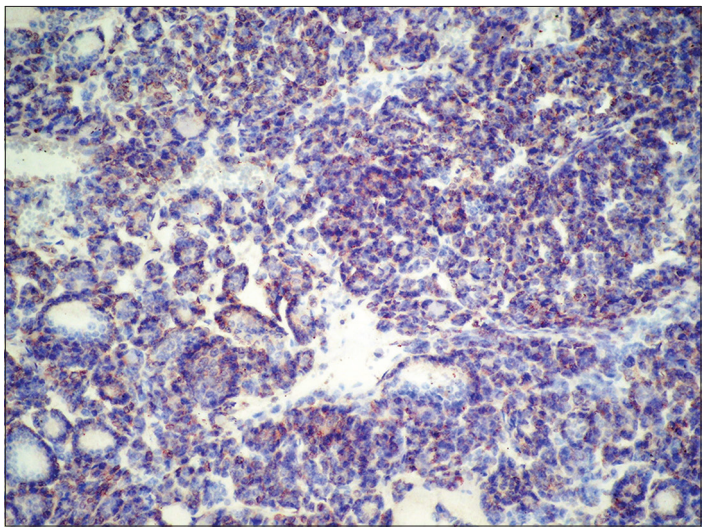

Figure 3: Weak and focal positivity of HMWCK in TPCFV (H and E, ×200)

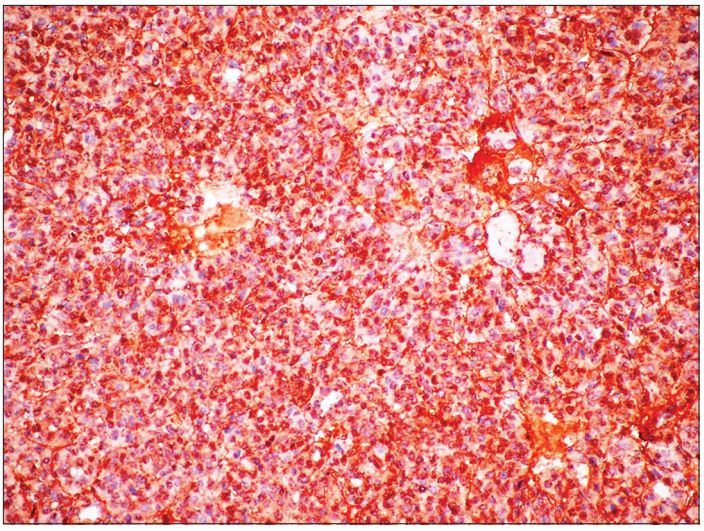

Figure 4: Strong and focal positivity of Fibronectin in TPCFV (HEX200)

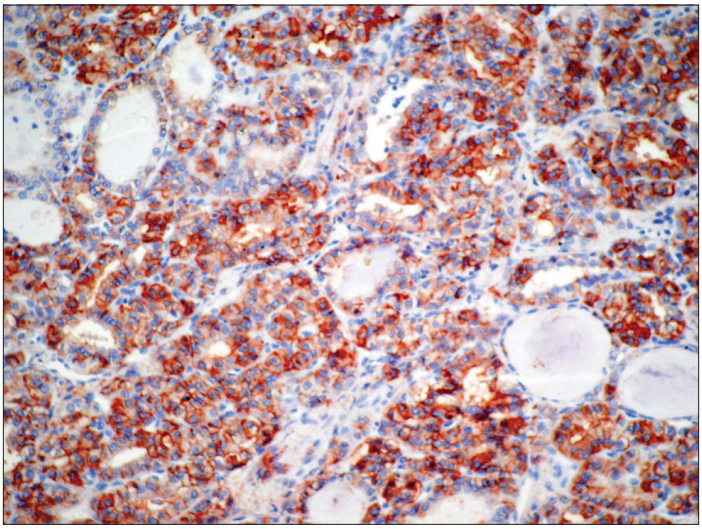

Figure 5: Strong and diffuse positivity of HBME-1 in TPCFV (H and E, ×200)

respectively. The sequence of the reverse primer was 5' GGCCAAAATTTAATCAGTGGA-3'. PCR was performed in a total volume of 25 µl, containing 100 ng of genomic DNA, 12.5 µl of 2X reaction buffer (GML SeqFinder Sequencing System, Switzerland), 5 pmol of each primer (Biomers, Germany). After an initial denaturation step at 94°C for 2 minutes (min), samples were subjected to 35 cycles of PCR at 94°C for 30 seconds (s), at 60°C for 30s, and at 72°C for 30s, with a final extension step at 72°C for 2 min. Amplified products were verified by electrophoresis using 2% agarose gel, showing a 129 bp PCR fragment [Figure 6]. All samples were used at least twice for mutation.

Statystical analysis

Statistical evaluations were performed using the "SPSS 13,0 for Windows" packet program and $P < 0.05$ was considered statistically significant. For the comparison of the findings, Pearson Chi-Square and Kruskal Wallis tests were performed.

Results

Descriptive statistics of histopathological diagnose and HMWCK, CK 19, HBME1, Galectin-3, fibronectin are shown in Table 1.

Most of the carcinomas and follicular neoplasms were positive for CK 19, HMWCK, Galectin-3, HBME-1, Fibronectin in varying intensity and wide spread [Figures 1-5]. One FC for Galectin 3 and one FA for CK19 was out of evaluation due to non-specific staining.

CK 19 was Group 4 in 57.7% (15) of FV-TPC cases, Group 0 in 85.7% (6) of FA cases. FC cases stained in Group 0 and 1 patern in 60% (7) of cases.

Galectin-3 was Group 4 in 30.8% (8) of FV-TPC cases, Group 0 in 62.5 (5) of FA cases. FC cases stained in Group 0 in 50% (2) and MIFC stained Group 1 in 50% (3) of cases.

HMWCK was Group 1 in 38.5% (10) of FV-TPC cases, Group 0 in 75% (6) of FA cases. FC cases stained in Group 1 in 60% (3) and MIFC stained Group 1 in 50% (3) of cases.

Fibronectin was Group 1 in 34.6% (9) of FV-TPC cases, Group 0 in 75% (6) of FA cases. FC cases stained in Group 1 in 60% (3) and MIFC stained Group 0 and 1 in 33.4% (4) of cases.

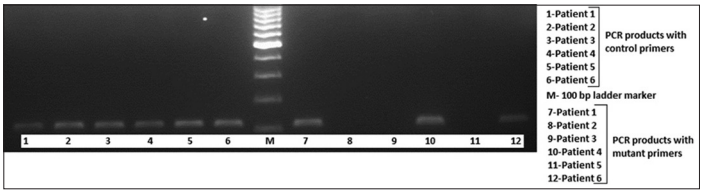

Figure 6: Genotyping of BRAF gene T1799A mutation by MASA-PCR technique

Table 1: Expressions of CK 19, HBME-1, HMCWK, Galectin-3, Fibronectin in thyroid neoplasms

	Group 0		Group 1		Group 2		Group 3		Group 4	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
CK19										
TPCFV	0	0	3	11.5	5	19.2	3	11.5	15	57.7
FA	5	71.4	1	14.3	0	0	1	14.3	0	0
FC	2	40	1	20	1	20	0	0	1	20
MIFC	2	33.3	2	33.3	1	16.7	1	16.7	0	0
HBME-1										
TPCFV	1	3.8	8	33.3	2	7.7	6	23.1	9	34.6
FA	5	62.5	1	12.5	2	25	0	0	0	0
FC	1	20	1	20	1	20	1	20	1	20
MIFC	2	33.3	0	34.6	0	0	2	33.3	0	0
HMWCK										
TPCFV	3	11.5	10	38.5	3	23.1	6	23.1	4	15.4
FA	6	75	2	25	0	0	0	0	0	0
FC	2	40	3	60	0	0	0	0	0	0
MIFC	2	33.3	3	50	0	0	1	16.7	0	0
Galectin-3										
TPCFV	4	15.4	3	11.5	4	26.9	7	26.9	8	30.8
FA	5	62.5	1	12.5	1	12.5	1	12.5	0	0
FC	2	50	1	25	0	0	1	25	0	0
MIFC	1	16.7	3	50	0	0	2	33.3	0	0
Fibronectin										
TPCFV	5	19.2	9	34.6	5	19.2	4	15.4	3	11.5
FA	6	75	1	12.5	1	12.5	0	0	0	0
FC	1	20	3	60	0	0	0	0	1	20
MIFC	3	16.7	1	16.7	2	33.3	0	0	0	0

TPCFV=Thyroid papillary carcinoma follicular variant; FA=Follicular adenom; FC=Follicular carcinoma; MIFC=Minimal invasive follicular carcinoma

HBME-1 was Group 4 in 34.6 (9) of FV-TPC cases, Group 0 in 62.5% (5) of FA cases. FC cases stained in Group 0 in 20% (1) and MIFC stained Group 0 in 33% (2) of cases.

BRAF gene T1799A mutation in exon 15 was studied in 21 patients with PTC by MASA-PCR technique and heterozygous mutation was detected in 11 patients out of them [Figure 6].

There was a positive correlation between FV-TPC and HMWCK, CK 19, HBME1, Galectin-3, Fibronectin ($P < 0.05$). There was no relation with FV-TPC, FA, MIFC, FC cases and BRAF T1799A mutation ($P > 0.05$).

Discussion

FV-TPC is the most common variant of papillary carcinoma after the usual variant and was first defined by Lindsay

in 1960. Rosai and Chen later stated 'papillary carcinoma follicular variant 'term and described its biological similarities to the usual variant[4] A scheme for diagnosing these lesions has been structured by Rosai *et al*. and described that if a follicular paterned lesion displays focally well-developed nuclear features of papillary carcinoma then it sould be diagnosed as FV-TPC[4] Five of the most studied immunohistochemical markers are HMWCK, CK19, Galectin-3, HBME-1 and Fibronectin which have been studied for differential diagnosis of thyroid neoplasms.

The differential expression of cytokeratins has been investigated in various thyroid lesions.[3,7]

CK 19 has been obtained by Rapheal *et al*. as a useful marker for distinguishing papillary carcinoma from follicular neoplasms and hyperplastic nodules.[4] But this study has been designed on frozen sections of tissue sample.[4] Fonseca *et al*. planned the same study on paraffin-embedded material and found out that CK19 has been strongly expressed in papillary carcinoma and focally in follicular carcinoma so there could not be strict distinction between these two lesions. Miettinen *et al*. found out distinct differences of CK19 expression in papillary carcinoma and papillary hyperplasia in paraffin embedded thyroid tissues. But they observed positive expression of CK 19 in follicular neoplasms, too which meant the failure of CK 19 in distinguishing papillary from follicular neoplasms.[4] Beside these, the authors observed stronger CK19 expression in follicular carcinoma compared to follicular adenoma that the change of expression patern of cytokeratins could reflect the malignant transformation.[4] Zhu *et al*. described strong and diffuse expression of CK 19 in PTC but weak and focal in the papilla of tissue with benign diseases.[20] In our study; consistent with the literature CK 19 stained in all thyroid lesions but FV-TPC group stained strongly and more diffuse than the other groups, that it may be a clue for papillary differentiated encapsulated follicular lesions. In our follicular adenoma group, seven ofeight cases stained focally and weak (less than 5% and 20%) with CK19, there has been moderate staining (50-80%) in one case consistent with Erkılıç *et al*.'s study who found focal CK19 immunreactivity in their FA group.[21]

These findings let us suggest to take advantage of CK 19 in seperation of FAs from FV-TPCs in our daily practice.

HMWCK expression has been obtained to be increased in PCs and that it could be used for distinguishing benign and malign lesions in some studies.[22,23] Contrary to these Choi *et al*. investigated immunreactivity of HMWCK in thyroid carcinomas and found that it could be useful for FV-TPCs but not for classic papillary carcinomas.[24] Similar to this, we found stronger positive expression of HMWCK in FV-TPC cases compared to follicular carcinoma cases. So the utility of HMWCK in thyroid neoplasms is controversial and this might be due to the different biotin blocage methods used in different studies.

In routine pathology practice distinction of FAs from FV-TPC especially if papillary features are focal is difficult. Prasad *et al*. examined the utility of some markers including Galectin-3 in thyroid tumors and determined that Galectin-3 was the most sensitive and accurate marker for detecting carcinomas. Similarly, Saggiorato *et al*. found that Galectin-3 has been usually positive in malignant and negative in benign thyroid lesions.[1,25] But, there are definite problems about Galectin-3, because Galectin-3 expression has been detected in some non-thyroidal tissues, like histiocytes, squamous cells and fibroblasts which can be observed in degenerative changes of nodular hyperplasia. Some authors also showed Galectin-3 expression in nodular hyperplasia by mutipl techniques.[7,23,24] Beside these, in a study conducted by Park *et al*., Galectin-3 stained follicular carcinomas weakly.[7]

However in our study all the groups stained positive but FV-TPC group stained stronger than the other groups, on the other hand Galectin-3 is shown to be a sensitive marker for determining malign transformation and minimal invasive carcinoma.[1] So, the combination of multipl markers may improve the sensitivity of the diagnosis and these markers are important for follow-up and obtain biologic nature of these lesions even if they could not be useful for distinguishing benign and malign thyroid lesions.

There are several studies reporting HBME-1 as an useful marker in the diagnosis of thyroid papillary carcinoma.[25,26] Cheung *et al*. showed positive immunoreactivity of HBME-1 in 55% of papillary carcinomas while no follicular adenomas were positive.[2] Rossi *et al*. declared that HBME-1 alone and combined with Galectin-3 and CK 19 can help to make correct distinction between malignant and benign thyroid neoplasms with high diagnostic accuracy.[27]

Similar to these studies Prasad *et al*. and Rorive *et al*. have found HBME-1 as the most specific marker for differentiating carcinomas from benign nodules.[1,28] Nakamura *et al*. also investigated immunohistochemical seperation of FV-TPC from FA and found that HBME-1 was useful.[6] In our study, HBME-1 has been strong positive in FV-TPCs while focal and weakeast staining has been determined in follicular adenomas. Our findings are not totally compatible with the literature but we can define that papillary carcinoma demonstrates more intense and diffuse positive staining which could help for differential diagnosis of these lesions.

Beside these we should know that none of the immune markers have 100% sensitivity alone.

Fibronectin; has been found to be upregulated in some thyroid cancers in some studies.[1,29]

Nakamura *et al*. found that most papillary carcinoma stained positive with Fibronectin, while most Follicular adenomas and carcinomas stained negative.[6] Prasad *et al*. showed the positive immunostaining of Fibronectin in papillary and follicular carcinomas and negative in normal thyroid tissues;[1] ın our study there was negativity in FV-TPCs (25%) as well as in follicular adenomas (75%).

Even those cases that were positive demonstrated weak and focal staining, there was significant background staining. Similar to our findings Nasr *et al*.[30] found negative staining

in 30% of papillary carcinomas and described non-specific findings as ours in their study which impairs the diagnostic utility of fibronectin in addition to our experience.

There are still conflicting results about the oncogenic mutation of BRAF gene. To date BRAF gene mutation has been reported to be confined to the thyroid tumors with papillary differentiation.[31]

There is a high frequency of BRAF gene mutations in exon 15 in large series of classical and tall cell variants of PTCs.[31-33] We have found T1799A mutation in the BRAF gene in 11 of our 25 FV-TPC, cases. This may depend on the heterogeneous series of tumors from different geographical areas of our country and the small, number of cases studied. Parellel to our study, Sapio *et al*. detected no mutations of BRAF in the normal thyroid tissues, follicular adenomas or follicular carcinomas but 19 of 43 papillary thyroid carcinomas showed BRAF gene T1799A mutation and 5 of the papillary carcinomas were follicular variant.[19] In addition to these, Frasca *et al*. evaluated BRAF mutation in a series of 323 PTCs and found in 38.6% of them with a strong association with residency in a specific part of the country.[34] They have suggested the possible link of BRAF mutation to the environmental carcinogens which might explain the heterogeneous results in our series too.

Conclusion

The accurate diagnosis is important for surgical planning and clinical management, For this reason the pathologist should use all the diagnostic tools, at this point immunohistochemical markers especially when used as a panel are the precise tools for the differential diagnosis. In some studies conducted by Saggirato *et al*. and Nakamura *et al*. Galectin 3 and HBME-1 when combined have been helpful in managing patients with follicular and papillary neoplasms.[6,25] However, BRAF mutation is a promising method, it should be used with combined panels of immunohistochemical markers. The discovery of more unique markers could help in the differential diagnosis of thyroid neoplasms.

In summary, our study suggests that morphologic features combined with immunohistochemical analysies might help to differentiate malignant thyroid neoplasms and FV-TPC from follicular adenomas and follicular carcinomas, but not as useful in seperating follicular adenomas from follicular carcinomas. However, further studies with larger series with immunohistochemistry and genetic analysies are needed to improve our understanding of these interesting neoplasms.

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