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Original Article

Pathology Section

Expression of B-type RAF V600E in Thyroid Carcinoma and its Association with Histological Type

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ABSTRACT

Introduction: Thyroid cancer is one of the most common cancers amongst all endocrine cancers. Incidence of thyroid malignancy is about 3-4% of all malignancy in India and 80% of thyroid malignancy belongs to Papillary Thyroid Carcinoma (PTC). Factors affecting the prognosis of PTC include patient's gender, age, histological findings, tumour size, lymph node metastasis, extrathyroidal extension, and remote metastasis. Presence of B-type RAF V600E (BRAF V600E) mutation in thyroid carcinoma patients tends to present with more aggressive clinicopathological behaviours of PTC, prompting more aggressive radioiodine treatment.

Aim: To find out the frequency of occurrence and expression of BRAF V600E mutation by Immunohistochemistry (IHC) in thyroid cancer and its association with histological type and Tumour Nodes Metastases (TNM) Staging.

Materials and Methods: The present observational retrospective study included patients treated for thyroid carcinoma between January 2014 to February 2019 at RL Jalappa hospital and Research centre, Kolar, Karnataka, India. The IHC was done with rabbit monoclonal anti-BRAF V600E antibody IgG Clone RM8 (VE1). Clinical records, Fine Needle Aspiration Cytology (FNAC) diagnosis were analysed for 45 thyroid carcinoma cases. Immunopositivity was scored positive when unambiguous clear cytoplasmic staining for the antibody was observed in tumour cells. Categorical data was presented in the form of frequencies and proportions and continuous data was presented as mean and standard deviation. The t-test were applied to find out the difference in means among the groups. The p-value <0.05 was considered statistically significant.

Results: Out of total 45 cases, 18 were classical PTC, 18 were Follicular Variant of Papillary Thyroid Cancer (FV-PTC), 4 were micropapillary carcinomas, 3 were Follicular carcinomas, 1 was Oncocytic variant of PTC and 1 undifferentiated thyroid carcinoma. Out of total 45 cases, 33 cases (73%) were found to be BRAF V600E positive and 12 (26%) were negative for BRAF V600E. Out of the total 33 BRAF V600E positive cases, 19 cases showed strong staining, 14 cases showed moderately positive staining.

Conclusion: The PTC is the most frequent type of thyroid carcinoma amongst all the sub-types. BRAF V600E expression is commonly seen in higher tumour size (T3, T4) and classical PTC. Tumours with extrathyroidal extension and capsular invasion showed strong positivity.

Keywords: Histopathology, Immunohistochemistry, Molecular pathology

INTRODUCTION

Thyroid cancer is one of the most common cancers amongst all endocrine cancers. Incidence of thyroid malignancy is about 3-4% of all malignancy in India and 80% of thyroid malignancy belongs to PTC [1]. Factors affecting the prognosis of PTC include patient's gender, age, histological findings, tumour size, lymph node metastasis, extrathyroidal extension and remote metastasis. There are numerous variants of Raf kinase. The most periodically found genetic aberration in PTC is BRAF V600E mutation. Literature states that BRAF gene mutations affect the factors that predict extrathyroidal extension, lymph node metastasis, disease recurrence, higher tumour stages 3 and 4 [2,3]. Role of BRAF mutations in the pathogenesis of tumours in malignant melanoma, ovarian tumours and colorectal carcinoma has been studied [2,4].

The BRAF V600E mutation has a vital role in infancy stage of tumour where it induces high proliferation of the tumour cells. It is also involved in the aggressiveness and tumour undifferentiation [5]. Retrospectively analysis of patients who were on higher doses of radioiodine treatments were found to be positive for BRAF V600E [6]. This likely reflects that BRAF V600E patients tends to present with more aggressive clinicopathological behaviours of PTC, prompting more aggressive radioiodine treatment. BRAF studies are reported mainly from western countries and only limited publications are from India [3,7]. Gold standard for detection of BRAF V600E mutation detection is molecular genetics study using Polymerase Chain Reaction (PCR) and Deoxyribonucleic Acid (DNA) sequencing. Only few IHC identification methods of BRAF V600E mutation using formalin fixed paraffin embedded tissue sections is reported in the literature [8-13] in which the histological type was not correlated with BRAF expression.

This study was conducted to assess the frequency of occurrence and expression of BRAF V600E mutation by IHC in thyroid cancer and its association with histological type.

MATERIALS AND METHODS

The present observational retrospective study was conducted at RL Jalappa Hospital and Research Centre, Kolar, Karnataka, India from January 2014 to February 2019. Case analysis was done between May to July 2019.

All the methods in the study were in accordance with the standards of the Ethical Committee on human experimentation with Ethical clearance number SDUMC/KLR/IEC/44/2019-2020 for starting the study. Ethical clearance was also obtained for publishing this study with the Ethical clearance number SDUMC/KLR/IEC/81/2020-2021.

Sample size calculation: The sample size was calculated using the following formula:

$$n = \frac{Z^2 (p \times q)}{d^2}$$

Where, Z=1.96; it is standard deviation score for 95% set interval

p= assumed or estimated proportion (77%)=0.77 [14].

q=1-P (1-0.77)=0.23

d=allowable error (20% of P)=0.15

The sample size came to be 31 subjects at 95% confidence limit and 20% allowable error after assuming the percentage of BRAF positive expression in thyroid carcinoma in 77% of subjects in a similar study done by AbdElmageed ZY et al., [14]. However, in this study 45 cases were studied.

Inclusion criteria: All patients who underwent thyroidectomy from January 2014 to February 2019 with or without therapeutic neck dissection for thyroid carcinoma were subjected to histopathological examination.

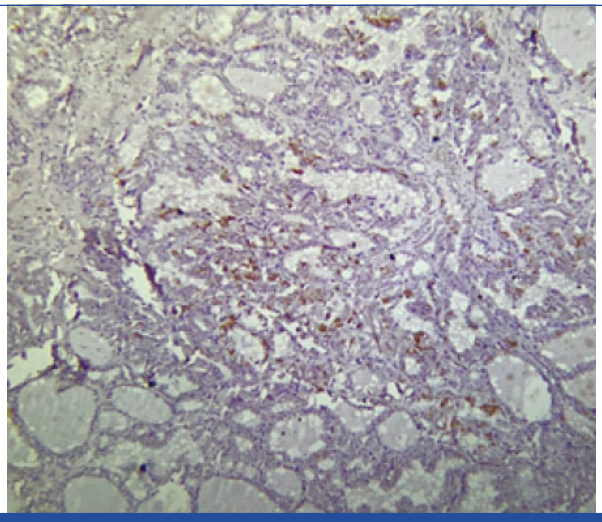
Exclusion criteria: Cases showing extensive tumour necrosis without sufficient viable tumour cells and patients who received chemotherapy were excluded from the study.

Study Procedure

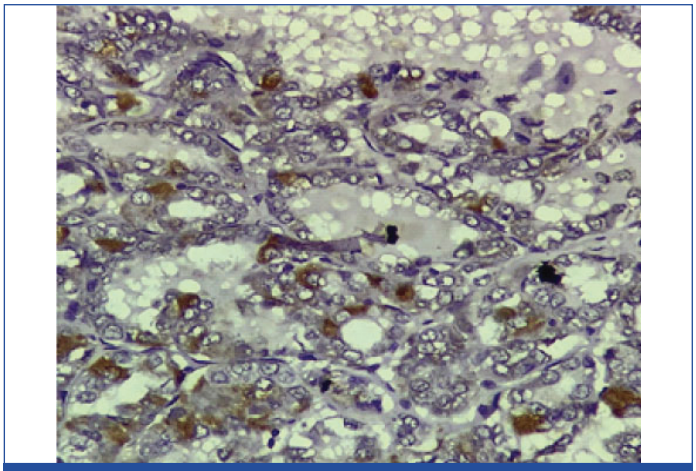
The IHC for the BRAF VE1 antibody was performed on formalin-fixed, paraffin-embedded 5 µm thick sections which were mounted on super frost slides and dried in an oven at 60°C for one hour to ensure optimal adhesion. Melanoma and colorectal carcinoma slides were used as positive control. Non biotin polymer-based Horseradish Peroxidase (HRP) detection system was used. Antigen retrieval was performed in microwave at power 10 for 6 minutes in Citrate buffer. Endogenous peroxidase was inhibited by adding 0.3% H_2O_2 to slides and incubating for 10 minutes. Sections were incubated with primary antibody (Rabbit antihuman BRAF V600E monoclonal antibody; clone VE1 Bio SB, California USA) diluted at 1:70 for 120 minutes in a humid chamber followed by three washes with Tris-Buffered Saline (TBS) at pH 7.6, and then incubated for 120 minutes with prediluted secondary antibody. Next, the slides were washed again with TBS buffer. Finally, 3,3'-diaminobenzidine chromogenic solution (enhancer) was applied for three minutes, and the slides were counter stained with haematoxylin for five seconds. IHC staining assessment was done as per Spring bioscience method [Table/Fig-1] [10,14]. Weak (1+) or no staining was considered as negative [Table/Fig-2]. Positive staining was considered when cytoplasmic staining of the tumour cells was moderate 2+ [Table/Fig-3] to strong 3+ [Table/Fig-4].

Score	BRAF V600E assessment	Staining pattern
1+	Weak (Negative staining)	Faint and incomplete cytoplasmic positivity
2+	Moderate (Moderately positive staining)	Moderate and complete/incomplete cytoplasmic positivity
3+	Strong (Strong positive staining)	Strong and complete membrane positivity

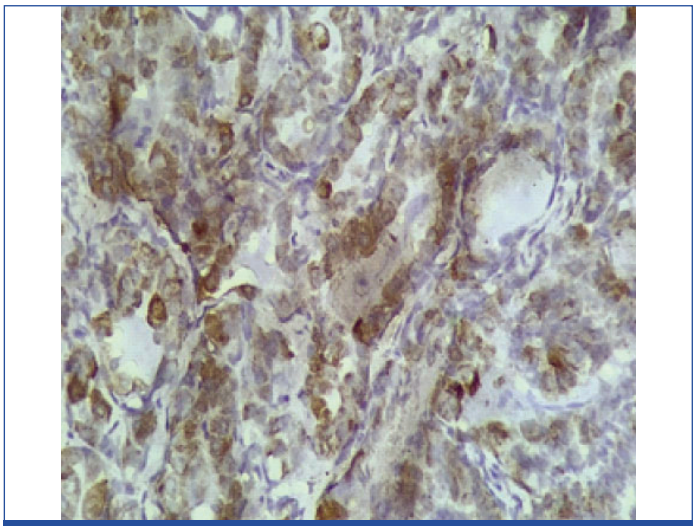
[Table/Fig-1]: Staining of BRAF V600E assessment was done according to spring bioscience method [10,14].



[Table/Fig-2]: Showing 1+ BRAF V600E IHC staining of tumour cells (40x).



[Table/Fig-3]: Showing 2+ BRAF V600E IHC staining of tumour cells (40x).



[Table/Fig-4]: Showing 3+ BRAF V600E IHC staining of tumour cells (40x).

STATISTICAL ANALYSIS

Categorical data was represented in the form of frequencies and proportions. Continuous data was represented as mean and standard deviation. Chi-square test was used as test of significance. The p-value <0.05 was considered statistically significant. Data was analysed after entering into Microsoft Excel sheet using IBM Statistical Package for Social Sciences (SPSS) (trial version 23) and PRIMER statistical software.

RESULTS

Majority of the patients were females with a male to female ratio of 1:5 having a mean age of 40.56±11.86 years (ranging from 19 to 67 years). Majority of the cases 38 (84.4%) were BETHESDA category V on FNAC, while 7 (15.5%) cases belonged to BETHESDA category IV. Clinically, majority of the nodules 34 (75%) were solitary thyroid swellings, rest of the cases presented with diffuse swellings. Out of total 45 cases, 18 (40%) were classical PTC, 18 (40%) were FV-PTC, 4 (8.89%) were micropapillary carcinomas, 3 (6.67%) were follicular carcinomas, 1 (2.22%) was oncocytic variant of PTC and 1 (2.22%) undifferentiated thyroid carcinoma. Out of total 45 cases studied; 33 cases were positive for BRAF V600E antibody and 12 cases were negative. BRAF positivity is seen more frequently in classical PTC that is 17 out of 18 cases (94%), as compared to FV-PTC that is 10 out of 18 (55%) cases [Table/Fig-5]. Out of total 33 BRAF positive cases, strong expression of BRAF (3+ staining) was observed in 19 (58%) cases [Table/Fig-4] while 14 (42%) cases showed 2+ staining [Table/Fig-3]. Among BRAF positive cases, it was found that most of the patients (55%) were females of the age group of 40 years and above.

Majority of positive cases i.e., 22 (66.67%) were T3 Stage. All 4 cases of T4 showed BRAF positivity [Table/Fig-6].

S No.	Histological type	BRAF positive (N=33)	BRAF negative (N=12)	Total	p-value
1	Classical variant TC	17	01	18	<0.023
2	Follicular variant-PTC	10	08	18	<0.063
3	Micropapillary carcinoma PTC	03	01	04	<0.608
4	Oncocytic variant-PTC	01	--	01	<0.59
5	Follicular carcinoma	01	02	03	<0.34
6	Undifferentiated carcinoma	01	---	01	<0.59

[Table/Fig-5]: Showing association of BRAF expression with histological type.
Statistical test used-Chi-square; p<0.05 significant

Tumour stage	No. of BRAF positive cases		No. of BRAF negative cases		p-value
	No	%	No	%	
T1	2	6.06	0	0	p=0.223
T2	5	15.15	5	41.67	
T3	22	66.67	7	58.33	
T4	4	12.12	0	0	
Total	33	100	12	100	

[Table/Fig-6]: Showing association of BRAF expression with T stage.
Chi-square=5.061 with 3 degrees of freedom; p=0.223

Extrathyroidal extension/capsular invasion was seen in six cases. Out of these six cases, 5 (83%) showed 3+ BRAF staining. Cervical lymph node dissection was done for 9 (20%) cases out of which 7 (15%) cases showed lymph node metastasis. Out of 7 cases with lymph node metastasis, 6 (86%) showed strong BRAF V600E (3+) staining in thyroid tumour tissue and 1 case (14%) was negative for BRAF staining.

DISCUSSION

The most common genetic alteration reported in PTC is BRAF V600E mutation and found to be associated with an aggressive phenotype. BRAF mutations reported in PTC ranges from 5-90% [2]. This variation may be due to temporal differences in PTC pathogenesis and these differences may be due to different procedures (frozen tissue versus paraffin embedded tissue) and different techniques of detection (microdissection, direct sequencing, pyro sequencing, and microdissection) and different sensitivity and specificity. IHC staining is a technique that is widely used in diagnostic pathology laboratories and in contrast to molecular techniques, it is rapid and economical. It also provides the ability to easily quantify the BRAF V600E mutation and has the added advantage of allowing visualisation of individual antigen-bearing tumour cells and assessing for tumour homogeneity and heterogeneity.

Mutated BRAF protein occurs early in carcinogenesis of PTC with no significant difference between macro-carcinomas and micro-carcinomas [15]. It is also associated with diverse aggressive subtypes of PTC, more commonly seen in tall cell variants, oncocytic variants including Warthin tumour like variant [2,3,6,7]. In contrast, an exclusively follicular growth was predictive for the absence of mutated BRAF protein. Similarly, in present study BRAF positivity is seen more frequently in classical PTC cases (94%) as compared to FV-PTC (55%). Researchers found that solid variants and even focal presence of solid growth pattern is inversely related to BRAF protein expression because of different BRAF mutation in solid tumours being on exon 15 of the BRAF gene which is different from the typical T1799A BRAF mutation [16].

In this study, BRAF mutation is more commonly seen in >40-year females (55%). In this study, total 33 (73%) of the cases were positive for BRAF V600E mutation. BRAF mutation was seen in aggressive tumour type and high BRAF V600E intensity staining in tumour with extrathyroidal extension in six cases, out of which 5 (83%) of cases showed strong 3+ staining. It was also found to be associated with vascular invasion in 36 (80%) of the cases similar to the findings by Zagzag J et al., [12]. Similar to other studies association of BRAF V600E positivity with higher tumour stage T3 and T4 was observed in this study [Table/Fig-7] [3,10-12,14,17].

The expression of BRAF mutation will be of high clinical interest especially in therapy-resistant cases of PTC, as targeted therapy inhibiting mutated BRAF V600E protein is under the clinical trial investigation as FDA approved BRAF inhibitor vemurafenib [18-20]. Other additional advantages of BRAF detection by IHC include usage on small tumours, micro-metastasis in a lymph node. BRAF tests have got a higher sensitivity of 81.25% when tested for preoperative immunocytochemistry. Hence, the potential of this diagnostic test can be utilised for indeterminate thyroid nodule [21]. An advantage of IHC over genetic analysis alone is that translated protein products and post-translational regulatory modifications such as activating phosphorylation of kinases can be qualitatively assessed [22].

Patients whose tumours displayed the molecular high-risk group of BRAF V600E positive IHC had quadruple the risk of recurrence compared to the low-risk group of BRAF V600E negative cases [22]. Meta-analysis showed BRAF independently predicted PTC recurrence [23].

Targeted therapy for BRAF positive thyroid carcinoma patients: Vemurafenib and dabrafenib are the BRAF V600E inhibitors that function as ATP-Competitive inhibitors. These targeted therapies are highly suggested in recurrent or metastatic radio-iodine refractory PTC patients to reduce the rate of progression of disease and increasing the disease free survival [20].

Parameter	Chakraborty A et al., 2012, Mumbai, India [3]	Koperek O et al., 2012, Vuenna, Austria [10]	McKelvie AP et al., 2013, Victoria, Australia [11]	Zagzag J et al., 2013, New York [12]	AbdElmageed ZY et al., 2017, Los Angeles [14]	Present study
Total no. of cases studied	140	144	77	37	130	45
Female/Male	1.6:1	1.6:1	2.3:1	2:1	3.3:1	5:1
Mean age in years	NA	49±10.6	46±8.6	43±8.2	45±9.6	48±6.8
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
No. of cases positive for BRAF	86 (61)	76 (53)	50 (64)	25 (67)	100 (77)	33 (73)
BRAF positivity among extrathyroidal extension cases	33 (81)	22 (16)	19 (68)	13 (52)	44 (44)	6 (13)
BRAF positivity among vascular invasion cases		14 (10)		19 (76)	27 (27)	36 (80)
Association of TNM stage T3 and T4 with positive BRAF expression	34 (74)	23 (16)	42 (84)		33 (33)	35 (78)

[Table/Fig-7]: Comparison of present study with similar studies [3,10-12,14].
NA: Not available; Data are presented as number of cases positive for BRAF V600 mutation and associated parameters with percentages in parentheses; Age and tumour size were assessed as continuous variables

Limitation(s)

In view of small sample size, multicentric study with large sample size is suggested for further validation of prognostic role of BRAF in thyroid carcinoma.

CONCLUSION(S)

The BRAF V600E expression is strongly associated with higher tumour size (T3, T4) and classical PTC. It is also associated with extrathyroidal extension and vascular invasion showing strong positivity. In view of strong BRAF expression in classical PTC, BRAF V600E immunostaining may be applied as "Rule In" diagnostic and prognostic marker in FNAC of suspicious thyroid nodules.

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