BRAF V600E Immunoexpression in Papillary Thyroid Carcinoma and Its Association with Prognostic Factors and Histopathologic Variant

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Abstract

Aim: to provide additional information regarding the clinicopathological characteristics of Papillary Thyroid Carcinoma (PTC). Methods: Fifty patient with PTC were reviewed to determine prognostic factors such as age, gender, size of tumor and histologic variant. BRAF V600E mutation was detected by immunohistochemical staining and assessed with H score. Result: BRAF V600E mutations were detected in 17 (34%) cases. There were seven cases with extrathyroidal extension (ETE) p 0,04, 11 cases with lymph node metastasis (LNM) p < 0,001, and 8 cases with tall cell variant p 0,047.The cases with positive BRAF V600E mutation had mean age of 44.71 years, and the size of the tumor between 0.1-4cm. Six cases of them are male and 11 female.

Conclusion: There were significant relationships between BRAF V600E mutation with ETE, LNM, and tall cell variant. There was no significant relationship between BRAF V600E mutation, either with age, gender, or size of the tumor. BRAF V600E immunohistochemical examination can be performed as additional investigation for PTC patients.

Introduction

Thyroid carcinoma is the most common malignancy in endocrine organs. Incidence rate has increased worldwide and including in Indonesia.[1,2,3] Papillary thyroid carcinoma (PTC) is the most common type, which comprises 80-90% of all thyroid malignancies .[4,5] BRAF gene mutation is mutation that often found in the PTC, which is about 20-80% of all thyroid carcinoma. More than 90% of BRAF mutation involves changes of thymine to adenine at nucleotide 1799 (T1799A) in exon 15 resulting in the substitution of valine into glutamine at the point mutation of the amino acid position 600 (BRAF V600E).[2,6,7]
More than 30 studies have been conducted to determine the relation between the clinicopathologic characters of PTC with BRAF V600E mutation. Most of the studies indicated BRAF V600E mutation was associated with advanced disease stage, tumor aggressiveness, high recurrence rate and increased mortality of the patients.[8,9] Some studies have also suggested that the BRAF V600E mutation was associated with age, gender, tumor size, extrathyroidal extension (ETE), and lymph node metastasis (LNM).[2,10-13] Other studies have also shown a significant relationship between BRAF V600E mutation with histopathological variants of PTC such as classical variant, tall cell, and oncocytic.[2,10-13] Our study using immunohistochemical staining with BRAF V600E antibody was expected to provide an additional information regarding the clinicopathological characteristics of PTC in Indonesia

Materials and Methods

Samples: We collected all cases of PTC in the Department of Anatomic Pathology, Faculty of Medicine Universitas Indonesia - Ciptomangunkusumo Hospital (Jakarta, Indonesia) in the period of January 2014 to April 2015. Exclusion criteria were cases with inadequate slides, paraffin blocks were not found and PTC cases with other components, such as Hashimoto's thyroiditis, Anaplastic thyroid carcinoma and Hürtle cell carcinoma. The age and sex were noted based on medical records. The size of the tumors was noted based on medical or macroscopic records and microscopic assessment.

Histology: PTC is defined as a malignant tumor of the thyroid follicular cells marked with pseudo-inclusion, ground-glass appearance, and nuclear grooves.[4,14] Classic variant consist of papillary pattern with fibrovascular stalk, follicular variant consist of follicular growth pattern of> 50% tumor area, follicles with irregular shapes small to medium sized. Tall cell variant is composed of cells with a height of at least 3 times the width of cell constitute cover >50% tumor area and microcarcinoma variant with a diameter of 1 cm or less.[4,14,15] Extrathyroidal extension was assessed by microscopic examination which was an extension to the fatty tissue, muscle, or nerve around the thyroid gland. Lymph node metastatic tumor cells were characterized by the presence of PTC corresponding primary tumor in the lymph node in microscopic examination.

Immunohistochemistry: Sections of 4µm thick paraffin blocks were incubated overnight with primary antibody mouse monoclonal anti-human BRAF V600E (Spring Bioscience®) with a dilution of 1: 200. Positive control is a case of PTC with BRAF V600E mutation detected by Real-Time Polymerase Chain Reaction (PCR). Negative control is from each cases.

Methods of Validation:
Immunohistochemical staining were assessed by two independent observers and then assessed the suitability between the two observers. Semiquantitative scoring were done using the modified H score system.[16] This system includes percentage (%) of positive cells in 1000 tumor cells and also we assessed the staining intensities: 0: negative, 1: weak positive, 2: moderate positive, 3: strong positive. H score for each sample was calculated with the formula of H score = $\boldsymbol{H\ score = \Sigma\ Pi\ (i + 1)}$; Pi is the percentage of tumor cells stained (0-100%) and i is intensity of the staining (0,1,2,3).

Statistical analysis: The data was analized statistically Using IBM SPSS Statistics 20, with Chi-square test, or Fisher's test. The numerical data was analyzed using unpaired t or Mann-Whitney test.

Result

BRAF V600E Immunoexpression

The range H score BRAF V600E is 100-400. We defined the cut-off point to divide the BRAF V600E positive and negative H score with the curve of the receiver operating characteristic (ROC) and obtained the value of area under the curve (AUC) was 0.805 (95% CI 0.625 to 0.984). Cutting point of balance between sensitivity and specificity is 78% in H score of 326.5. H score $\geq$ 326.5 was determined as positive BRAF V600E mutation and < 326.5 as negative BRAF V600E mutation. BRAF V600E mutation was found in 17 (34%) cases by immunohistochemistry. Images of BRAF V600E immuno-expression can be seen in Figure 1.

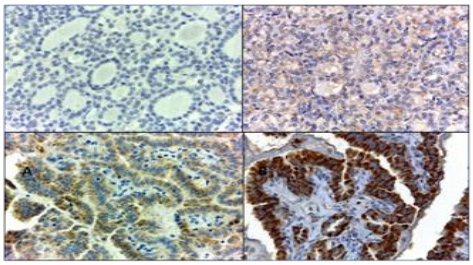

Figure 1 Immunohistochemical staining results BRAF V600E A.Negative (0). B. Weak (+1) C.Moderate (+2) D.Strong (+3).

We found significant relationship between BRAF V600E mutation with ETE and LNM. (Table 1) There were no significant relationships between BRAF V600E mutation either with age, gender, or size of the tumor. Histopathologic variants in this study were follicular, tall cell, classic, and microcarcinoma. These variants were further categorized into 2 groups: tall cell and non tall cell. There were significant relationships between BRAF V600E mutation, both with tall cell and non-tall cell variants.

BRAF V600E Mutation Analysis

Table 1. BRAF V600E Mutation in PTC at Ciptomangunkusumo Hospital, Jakarta, Indonesia

Factors	**BRAF V600E positive**	**BRAF V600E negative**	**p**	**Odds Ratio (OR)**	**95% CI**
Age					
Mean (SD)	44,71 (15.090)	41.58 (15.839)	0,505[a]		
Tumor size (min-max cm)	17 (0,1-4)	33 (0,1-9)	0,134[b]		
Gender					
Male	6	10	0,720[c]		
Female	11	23			
ETE					
Present	7	2	0,04[d]	10,85	1.932 - 60.930
Absent	10	31			
LNM					
Present	11	5	<0,001[c]	10,267	2.592 - 40.669
Absent	6	28			
Variant					
Tall cell	8	6	0,047[c]		
Non Tall cell	9	27			
Total	17	33			

a= Unpair t test; b= Mann-Whitney; c=Chi square; d=Fisher's exact

Discussion

BRAF V600E Mutation on PTC

Many studies have been done to detect BRAF V600E mutation by immunohistochemical staining which is a simple and in-expensive method..[6,11,17,18] Zagzag et al. detected mutations in BRAF V600E, using specific antibody clone VE1 and showed positive results in 89% of cases with a specificity of 100% and sensitivity of 89%.[11]

Previous research stated that the BRAF V600E mutation in the PTC might be heterogeneous, which was proved by specific antibodies.[18,19] Majority of cases in this study demonstrated non homogeneous staining, so we use a scoring system to determine the positivity. Distribution of tumor cells that had mutations in the positive cases also varied. The strongly positive stained cells varies from 34-100% of tumor cells in positive case 9 of which stained > 80%. This finding is in line with research conducted by de Biase et al. and heterogeneous staining was not due to preservation or poor tissue fixation.[19]

Other meta-analysis studies have demonstrated that BRAF V600E mutation in the PTC was an independent prognostic marker associated with poor survival and high recurrence rate.[20] Kim et al. in meta-analysis study conducted in 2012 stated that the PTC with BRAF V600E mutation have a risk of 1, 5 to 2.1-fold to undergo ETE, LNM, and recurrent.[21] Our study involved 50 PTC cases showed positive result in 17 (34%) cases. The analysis showed a significant association between V600E BRAF mutation and prognostic factors, for example, ETE, LNM, and the tall cell variant.

BRAF protein is a central regulator in the MAPK pathway, which in turn activates BRAF mutant protein and causes the MEK ERK protein phosphorylation. Active ERK protein moves into the cell nucleus and induce transcription factors and cellular transformation.[2] MAPK pathway dysregulation and / or BRAF mutations can increase transcription of MET gene and will increase the expression of Met as a receptor protein tyrosine kinase which in turn activated by ligand hepatocyte growth factor (HGF) so that the tumor cells are able to migrate and invade the capsule and lymphatic vascular structures.[22,23] Nardone et al 2003 study also expressed high Met protein expression in tall cell variant, and is related to tumor aggressiveness.[24] Some studies have also suggested that the BRAF V600E mutation associated with increased expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), which increases tumor invasion ability.[25,26] MET gene transcription may also be caused by a mutation or dysregulation of others, namely RAS oncogenes or RET / PTC.[27,28] Similarly to the case study that is not mutated BRAF V600E but can also occur ETE and LNM. So that needs to be further investigated regarding other pathways that play a role in the pathogenesis of PTC such as RAS or RET / PTC.

Adeniran research reported 97 cases of PTC , 42% of them experienced a BRAF mutation, 18% RET / PTC, and 15% RAS mutation.[29] Cases with BRAF mutations generally occur in older patients with classic or tall cell variant, more advanced stage and ETE. Cases of mutated RET / PTC were reported at a younger age and the more numbers of LNM. While the case with exclusively RAS mutations occur in follicular variant of PTC with less LNM.[29]

Some targeted therapies that inhibit BRAF selectively or non-selectively have been approved by the FDA effective and well tolerated by patients with mutations in BRAF V600E.[2,30] It can be administered to PTC patients with advanced stage, have experienced metastasis, and resistance to radiation therapy.[31,32] However, a review by Alonso-Gardoa et al. 2015, said that it is still needed further research as a stable treatment results and benefits of the combination therapy of several therapeutic targets.[30]

Association of BRAF V600E immunoexpression with Age

Meta-analysis study conducted by Lassalle et all[33] in 2010, showed 12 studies that found significant relationship between BRAF V600E mutation with age. Other studies have found no association between age and the V600E BRAF mutation.[11,20,34] Our study did not gain significant relationship between BRAF V600E mutation with age. There is a case study that found mutated BRAF V600E are more found at the age of 45 years or more as many as 12 cases with the oldest 68 years of age. Meanwhile, at the age less than 45 years, only five cases of mutated BRAF V600E. This shows the V600E BRAF mutation is more common in older age. Research Ciampi and Nikiforov in 2007 stated that BRAF V600E mutation is more common in old age, while at a young age the mutations in RET / PTC are more often found.[35]

Association of BRAF V600E immunoexpression with Gender

PTC can occur in women and men, where women are more often in the ratio 2:1 to 4:1.[4,36,37-42] Male gender said to be a poor prognostic factor in the PTC as related to high frequency of tumor recurrences.[43] The study included 16 men, 7 of them with LNM and 6 of them with the ETE. Four cases with both ETE and LNM. Lymph node metastases and ETE also associated with tumor recurrence.43 Several studies stated BRAF V600E mutation linked with male gender.[20,44,45] However, in this study we found no such link. Seventeen cases with BRAF V600E mutation in this study only six (35%) waas male.

Association of BRAF V600E immunoexpression with Tumor Size

Tumor size is an important variable in determining the prognosis of the patient, the larger the size of the tumor the worse the prognosis.[4,36,46-48] Several studies have shown a link between BRAF V600E mutation and tumor with larger size.[34,49-52] Other studies found no such association.[29,44] Our study found no significant association between BRAF V600E mutation with tumor size. However, from seventeen cases the mutated BRAF V600E in this study, we found ten (59%) cases measuring more than 2 cm or more.

Association of BRAF V600E immunoexpression with Histopathological Variant

In this study we found four variants, namely follicular, tall cell, microcarcinoma, and classic. Classic and tall cell variant and said to be related to the BRAF mutation V600E.[10-13] Research by Fernandez et al in 2013 showed BRAF V600E mutation in 72.2% of cases PTC tall cell variant, 77.4% of cases PTC classical variant, and 31.9% of cases PTC follicular variant.[53] Min et al in 2013 have positive results BRAF V600E mutation at 100% tall cell variant, classical variant 79.4%, and 47.6% follicular variant.[54] Study of Ghossein et al 2007 showed tall cell variant have significant association with poor prognostic factors such as older age, extrathyroidal extension, necrosis, and mitosis.[55] Calangiu et al 2014 study also states that PTC patient with tall cell variant and ETE, has a 5-year survival rate is lower than the patients with classic variant.[56] Moreover, tall cell variant generally associate with BRAF V600E mutation and also associated with aggressiveness.[18]

Our study showed a significant relationship between BRAF V600E mutation with histopathological tall cell variant and non tall cell (p 0.047). Fourteen cases with tall cell variant, 8 (57%) were mutated BRAF V600E. Meanwhile, eight cases with the classical variant, 6 (75%) were mutated BRAF V600E, and the follicular variant only 1 (6%) cases of mutated BRAF V600E. Two cases with microcarcinoma variant mutated BRAF V600E, one of them containing tall cell components more than 50%. There are 8 (16%) cases of non-tall cell mutated

BRAF V600E, one with follicular variant, six variants of the classic, and one variant microcarcinoma containing components classical variant. Of the seven cases of classical variant mutated BRAF V600E, five of which with ETE and LNM. This shows that the PTC with a classical variant can also be aggressive.

Association of BRAF V600E immunoexpression with ETE

Extrathyroidal extension is an important prognostic factor in patients with PTC since it is associated with high recurrence rates and mortality.[49,57] Our studies found there is relationship between the V600E BRAF mutation and ETE p 0.04 and OR 10.85 (95% CI 1.932 to 60.93). Nine subjects with ETE, 7 of them mutated BRAF V600E. Other studies also suggested a significant association between BRAF V600E mutation with ETE.[21,29,34,58-60]

Association of BRAF V600E immunoexpression with Lymph Node Metastasis

Lymph node metastasis in PTC associated with the occurrence of recurrency.[61] Our research shows there is a significant relationship between BRAF V600E mutation with LNM p <0.001 and OR 10.267 (95% CI 2.592 to 40.669). Sixteen cases with LN metastatis, 11 were mutated BRAF V600E. Several studies have also suggested an association between BRAF V600E mutation with LNM.[12,34,49,51,56,60] The association between BRAF V600E mutation with the incidence of metastatic lymph nodes is an indicator of recurrence. Several recent studies suggest that in PTC patient with BRAF V600E mutation total thyroidectomy should be performed with prophylactic lymph node dissection.[43,62]

Conclusion

BRAF mutation plays a fundamental role in the pathogenesis of PTC. The positivity of BRAF V600E immuno-expression in this study were 34%. Further research is needed to determine other pathways that play role in the PTC such as RET/PTC, RAS, and so on. There was no significant relationship between BRAF V600E mutation, either with age, gender, or tumor size. There were significant relationship between BRAF V600E mutation with ETE, LNM and histopathological tall cell and non-tall cell variants. Significant correlation between BRAF V600E mutation with LNM and ETE, showed that BRAF V600E immunohistochemical examination can also be performed to predict the prognosis of PTC patients.

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