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The Different Expression of BRAF^{V600E} Mutation in Patients with Papillary Thyroid Carcinomas Coexisting with or without Benign Thyroid Nodules

Young-Pyo Kim, M.D., **Sung-Jeep Kim,** M.D., **Young Ae Kim,** Ph.D., **Keehwan Kim,** M.D., **Chang Hyuck An,** M.D., **Woochan Park,** M.D. and **Jeong-Soo Kim,** M.D.

Department of Surgery, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Uijeongbu, Korea

Purpose: BRAF^{V600E} mutation is the most common genetic alteration in papillary thyroid cancer (PTC) and has been associated with poor prognostic factors. The purpose of the present study is to investigate the frequency of the BRAF mutation in PTC with and without benign thyroid nodules (BN).
Methods: 98 DNA samples were extracted from frozen tissues of 51 PTC and 47 BN specimens of 70 patients and were divided into four group: PTC with BN, PTC alone, BN with PTC and BN alone group. We investigated the BRAF mutation by sequencing and clinicopathologic characteristics.
Results: Total positive rate of BRAF mutation was 23.5% in the two PTC groups. That rate of the PTC with BN group was 10.7% and the PTC alone group was 39.1%. Positive rate in the PTC with BN group was lesser than the PTC alone group and had statistically difference (P=0.02). The positive rate of BRAF mutation was 7.1% in the BN with PTC group and 5.3% in the BN alone group. Positive rate in these two group was not statistically different (P=0.80).
Conclusion: The frequency of BRAF mutation in PTC with concurrent BN was lower than in PTC alone. This result suggests that the effect of BRAF mutation is lesser associated with PTCs with BN than PTC alone group. **(Korean J Endocrine Surg 2012;12:11-15)**

Key Words: Papillary thyroid carcinoma, Benign thyroid nodule, BRAF mutation, PCR

Correspondence: Jeong-Soo Kim, Department of Surgery, Uijeongbu St. Mary's Hospital, The Catholic University of Korea, 65-1, Geumo-dong, Uijeongbu 480-717, Korea
Tel: +82-31-820-3048, Fax: +82-31-847-2127
E-mail: drbreast@catholic.ac.kr
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INTRODUCTION

The thyroid cancer is the most prevalent endocrine malignancy and the papillary thyroid carcinoma (PTC) is the most common thyroid cancer.(*1*) Nowadays thyroid nodules are common and are being increasingly detected. Most thyroid nodules are asymptomatic and benign, and only about 5 percent of all palpable nodules are found to be malignant.(*2*) Many tests have been employed to separate benign from malignant thyroid nodule, but differentiating benign from malignant lesions is not always possible using current imaging and cytological techniques.(*2,3*)

The ratio of PTC among thyroid cancer is about 93% in Korea, while the ratio of PTC in western countries is about 80%.(*4*) Many genetic and epigenetic changes, environmental factors are involved in tumorigenesis of PTC. Since the initial discovery of BRAF^{V600E} mutation in human cancer,(*5*) many studies about the effect of B-isoform of the Raf kinase (BRAF) mutation on human cancers have been done extensively and found that BRAF mutation occur in a broad range of human cancers including thyroid cancer and it is the most frequent genetic alteration in approximately 29% to 83% of PTCs.(*1,3*) It has a fundamental role in thyroid tumorigenesis through aberrant overactivation of the mitogen-activated protein kinase (MAPK) signaling pathway(*3,6,7*) which induces excessive proliferation and differentiation into cancer cell.(*8,9*)

The BRAF mutation was also detected in microcarcinoma or in a very early stage of PTC and this fact suggests that BRAF mutation can play a role not only in predisposing to another genetic alteration but also in the initiation of PTC tumorigenesis associated with poor prognosis.(*8,9*) This mutation is also much more frequently in the tumors with larger size, lymphovascular invasion or metastases, high mortality, and may play a role in the progression of PTC to anaplastic thyroid cancer.(*7,8*) The prevalence of

BRAF mutation is much higher (73～86%) in the Korea than that in Western countries (29～83%) in PTC.(*3,10*)

Another characteristic of thyroid cancer is common coexistence with BNs. BRAF mutation is highly specific for PTC among primary other thyroid cancer and has not been observed in benign thyroid lesions.(*11*) But, the influence of coexisting BN on BRAF mutation status and tumorigenesis effect in PTC with BN are not clear and have not yet evaluated. So we studied the status of BRAF mutation in four groups; PTC with BN, PTC alone, BN with PTC and BN alone and evaluated that whether BRAF mutation is more associated with influencing on development of PTC coexisting BNs than PTC alone or not.

METHODS

1) Patients

This study involved 98 samples collected from 70 patients with PTC or BN who were undergone surgery between December 2004 and June 2010. 28 patients had PTC with BNs (PTC samples were 28, BN samples were 28), 23 patients had PTC alone (PTC samples were 23) and the rest 19 patients had BNs alone (BN samples were 19). We divided 98 samples to four groups; PTC with BN, PTC alone, BN with PTC and BN alone group (Fig. 1). The medical records of these patients were retrospectively reviewed and the following data were collected: patient demographics, surgical procedures, tumor size, lymph node and distant metastases, TNM staging and histologic type. We collected tissue specimens that were immediate treated with liquid nitrogen after sampled from cancer and BN lesions in operating room. And it was confirmed by frozen biopsy. This study approval was obtained from the institutional review board.

2) Paraffin-embedded tissue, DNA isolation, and identification of $BRAF^{V600E}$

For the tumor resections, archival formalin-fixed and paraffin-embedded tissues were sectioned, the sections were reviewed by two endocrine pathologists to confirm the PTC and histologic type of BNs.

DNA was extracted from the paraffin sections with a commercially available kit (QIAamp DNA Formalin-Fixed, Paraffin-Embedded [FFPE] Tissue Kit), according to the manufacturer's instructions. A region of the BRAF coding sequence encompassing the V600E mutational site was amplified using HotStar Tag DNA polymerase (Qiagen) with forward 5'-AATGCTTGCTGATAGGAAAAT and reverse 5'-TAATCAGTGGAAAAATAGCCTC primers.

PCR amplification was performed using 100 mg of tumor sample DNA as template or an aliquot of 1～3 μl from the tissue samples. The PCR reactions were carried out in a 96-well thermocycler. Cycling conditions were as follows: a denaturation step at 95°C for 5 min was followed by 1 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, primer extension at 68°C for 30 sec, 38 cycles of annealing and primer extension, and final extension at 68°C for 5 min. After PCR products were purificated by using Labopass PCR kit, we sent the PCR products to COSMO Genetech, Int. to analyze of the BRAF mutation.

3) Statistical analysis

Correlation of clinicopathologic characteristics with each four group and BRAF mutation in PTC with BN compared with PTC alone and BN alone was assessed with the χ^2 test or Fisher's exact test, where appropriate, with SPSS statistical software version 13.0. A P value of less than 0.05 was considered statistically significant.

RESULTS

PTC with BN group had 28 patients, male and female were 3 and 25, mean age was 50.74, mean tumor size was 1.19±0.80 cm, mean total and metastatic LN was 5.96±3.82 and 1.06±1.49 respectively. Histologic types of PTCs were classified with classic type (18 patients), follicular type (5 patients) and mixed type (5 patients). According to the TNM staging, stage I and III were 17 patients and 11 patients. T1 (17 patients) and N0 (15 patients) was most common. All patients were M0. PTC alone group had

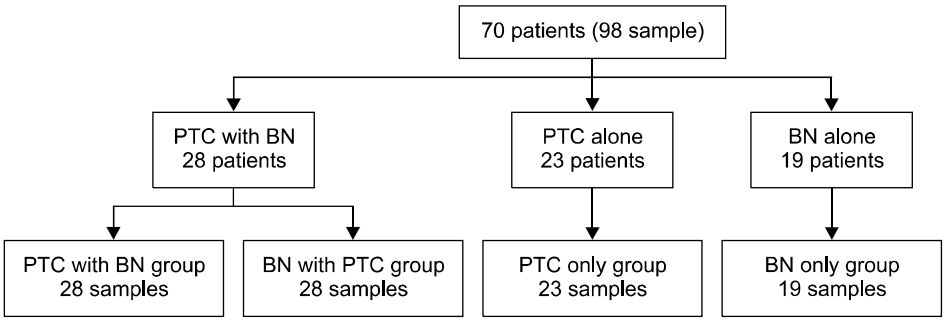

Fig. 1. Four groups of samples. PTC = papillary thyroid cancer; BN = benign lnodule.

23 patients. Its clinicopathologic characteristics were similar with PTC with BN group and no statistically difference (Table 1-1).

The clinicopathologic characteristics of BN with PTC were same as PTC with BN group except mean tumor size (1.26±0.43 cm) and histologic type (most common was nodular hyperplasia, 14 patients). BN alone group had 19 patients and all were female and mean age was 46.75. Common histologic type was nodular hyperplasia (18 patients) and two patients had multiple nodules that were different histologic types. Mean tumor size was 2.97± 1.71 cm and most common surgical procedure was lobectomy. These two clinicopathologic characteristics were statistically different between BN with PTC group and BN alone group ($P < 0.05$) (Table 1-2).

Total positive rate of BRAF mutation was 23.5% (12/51) in the two PTC groups. The positive rate of BRAF mutation was 10.7% (3/28) in the PTC with BN group and 39.1% (9/23) in the PTC only group and had statistically difference (P=0.02). The positive rate of BRAF mutation was 7.1% (2/28) in the BN with PTC group and 5.3% (1/19) in the BN only group. Positive rate in these two group was not statistically different (P=0.80) (Table 2).

The rate of BRAF mutation between PTC groups and BN groups was 23.5% (12/51) and 6.4% (3/47) respectively, This rate of PTC groups was higher than BN groups and statistically different (P=0.03) (Table 3).

Table 1-1. Clinicopathological characteristics between two PTC groups

		PTC with BN (n=28)	PTC alone (n=23)	P value
Age at operation				0.25
	<45	12	6	
	≥45	16	17	
Gender				1.00
	Male	3	3	
	Female	25	20	
Tumor size (mm)				0.77
	<10	10	10	
	≥10	18	13	
LN metastasis		13	9	0.78
Tumor stage				0.13
	I/II	17	19	
	III/IV	11	4	
BRAF positive		3	9	0.05
Histology type				0.52
	Classic	18	18	
	Follicular	5	2	
	Mixed	5 (17.9%)	3 (13.0%)	

PTC = papillary thyroid carcinoma; BN = benign thyroid nodule; LN = lymph node.

DISCUSSION

Benign tumors can share oncogenic mutation with their malignant counterparts and some are therefore considered to be pre-

Table 1-2. Clinicopathological characteristics between two BN groups

		BN with PTC (n=28)	BN alone (n=19)	P value
Sex	F	25	19	0.26
	M	3	0	
Age		50.74	46.75	0.57
Tumor size (cm)		1.26±0.43	2.97±1.71	0.00
Histologic type	Nodular hyperplasia	19	18	0.11
	Follicular adenoma	3	1	
	Lymphocytic thyroiditis	5	2	
Operation	TT	27	4	0.00
	STT	1	4	
	Near TT	0	1	
	Lobectomy	0	10	

TT = total thyroidectomy; STT = subtotal thyroidectomy.

Table 2. The numbers of BRAF mutation in four groups

		PTC with BN group	PTC only group	P value	BN with PTC group	BN only group	P value
BRAF mutation	+	3 (10.7%)	9 (39.1%)	0.02	2 (7.1%)	1 (5.3%)	0.80
	−	25	14		26	18	

Table 3. The numbers of BRAF mutation between PTC groups and BN groups

		PTC group	BN group	P value
BRAF mutation	+	12 (23.5%)	3 (6.4%)	0.03
	−	39	44	

cursor lesions with the potential to progress to malignancy (good example were melanocytic nevus and polyps of colon).(*5*) PTC also can occur in a background of normal thyroid tissue or in associated with benign thyroid disorders,(*12*) But usually benign thyroid nodules have little malignant potential but some BNs have malignant potential.(*13-15*) BN itself can be transformed into malignant thyroid carcinoma with low incidence, there were no obvious evidence that BN might lead high occurrence of coexcisting PTC and make worsening of prognosis.

In our study, there were three pathologic types (hyperplastic nodule, follicular adenoma, lymphocytic thyroiditis) and hyperpalstic nodule was most common in both BN groups and there were no stastically difference (P=0.11).

Hyperplastic nodules, characteristic of lesions seen in multinodular goiter (MNG), are due to follicular cell hyperplasia.(*13*) They are traditionally thought to be entirely benign disease.(*13*) But, in one study of 294 patients with MNG who had surgically removed, the incidence of malignancy was almost 11%(*15*) and some studies have shown that malignancy can be associated with nodular goiter.(*13,16*) Nevertheless, based on the clinical and experimental evidence available, hyperplastic nodules are not likely to have significant malignant potential and BRAF mutations have not been identified in hyperplastic nodules.(*13,17*)

Follicular adenomas (FAs) are solitary thyroid nodules that are well encapsulated and have follicular architecture. Kuma et al. (*18*) reported that malignant transformation of FA occurred in the 1% and Park et al.(*16*) reviewed the histopathology of 1,095 thyroid surgical specimens and found occult PTC in 4.3% of follicular adenoma. FAs are regarded as benign, indolent tumors that do not commonly transform or degenerated into carcinoma and do not have BRAF mutation.(*13*)

PTC can occur in association with lymphocytic thyroiditis. The coexistence of lymphocytic thyroiditis and PTC has been variously reported to range from 0.3% to 38%.(*19*) Loh et al.(*20*) reported a relatively common occurrence of lymphocytic thyroiditis in patients with PTC and they found that these PTC had a more favorable course and better prognosis. They hypothesized that lymphocytic infiltration developed mainly in response to tumor itself and this represents a form of immune reaction to control tumor growth and proliferation(*21,22*) and inflammatory infiltration could predispose to the development of PTC in patients with autoimmune lymphocytic thyroiditis.(*23*)

Pellegriti et al.(*24*) reported that Graves' disease patients with thyroid nodule have a 4.7% incidence of differentiated thyroid cancer, but thyroid nodules with autonomous function are less likely to harbor malignancy and this may be attributed to the suppressed TSH level in thyroid autonomy.

Although BRAF mutation involving V600E is highly specific for PTC among other primary thyroid neoplasm, it has not been observed in benign thyroid lesions.(*11,13,17*) But in our study, three BN were in positive of BRAF mutation. The pathology of two BNs was follicular adenoma and belonged to the group of BN with concurrent PTC. The pathology of one was a nodular hyperplasia and belonged to the group of BN alone. The reasons that BRAF mutations were found in these BN might be first due to contamination with PTC tissue during processing of sampling or preparation of slide or PCR. Second, it might be due to pathologic misleading of sample or mislabeled during laboratory examination. But these three samples were reviewed twice by another pathologist and there were no misleading.

The prevalence of BRAF mutation is much higher (73～86%) in the Korea than that in Western countries (29～83%) in PTC.(*3,10*) Our study showed that total positive rate of BRAF mutation was 23.5% (12/51) in the two PTC groups. That rate of the PTC with BN group was 10.7% (3/28) and the PTC only group was 39.1% (9/23). BRAF mutation rate of PTC with BN group was statistically lower than PTC alone group and very lower than reported rates of PTC in many literatures. We suggested that oncogenetic pathway of PTC concurrent with BN is different with PTC alone and another pathway may be involved in development of this PTC. On the other hand, it may be associated with faults in analysis of BRAF mutation. We sent our collected DNA to the company named COSMOGenetech and consulted for interpretation of frequency of BRAF mutation. They rechecked the BRAF mutation and sent same results to us. This result might be due to highly occurring of false negative rate in process of sequencing process. Also it could be occurred due to small numbers of sample and sampling errors. Inaccurate microdissection of tumor site from frozen section might cause underestimation of the BRAF mutation positive rate.

The presence of BRAF mutation has been associated with aggressive tumor characteristics, such as extrathyroidal extension, tumor recurrence and poor prognosis.(*8,9,11*) In our study, BRAF mutation rate was higher in PTC alone group than in PTC concurrent with BN and we might expect that PTC concurrent with BN is lesser aggressive and has better prognosis than PTC alone. But there were no different clinicopathologic features in

both PTC groups. We thought that this study is limited by the relatively small numbers of sample, frozen section sampling errors and must need more additional, extensive and long term follow up studies.

CONCLUSION

In this study, we found that the frequency of BRAF mutation in PTC with concurrent BN was lower than in PTC alone. This result suggests that the effect of BRAF mutation in tumorigenesis is lesser associated with PTCs with cocurrent BN than without concurrent BN. There may be different tumorigenetic pathways in PTCs with concurrent BN. we also didn't find that concurrent BN may affected to the poor clinocopathological features in PTC.

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