BRAF^{V600E} Mutation: Has It a Role in Cervical Lymph Node Metastasis of Papillary Thyroid Cancer?

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Key Words

BRAF^{V600E} · Papillary thyroid cancer · Metastatic lymph node

Abstract

Background: The BRAF^{V600E} mutation is common in papillary thyroid cancer (PTC). Lymph node metastasis (LNM) may be associated with poor prognosis. However, the LNM mechanism remains unclear. **Objectives:** Our aim was to evaluate the prevalence of the BRAF^{V600E} mutation in primary tumors and accompanying LNM at the time of diagnosis. **Methods:** This retrospective study included 51 PTC patients (40 women, 11 men; mean age 40.0 ± 16.5 years; range 6–81) who underwent total thyroidectomy accompanied by a lateral neck dissection due to preoperatively detected LNM. Real-time PCR was used for the detection of the BRAF^{V600E} mutation in specimens from primary thyroid tumors and metastatic lymph node tumors. **Results:** The prevalence of the BRAF^{V600E} mutation was 64.7% (n = 33) in primary tumors and 47.1% (n = 24) in metastatic lymph nodes. Of 33 patients with BRAF^{V600E}-positive primary tumors, 18 (54.5%) had BRAF^{V600E}-positive metastatic lymph nodes. Of 18 patients with BRAF^{V600E}-negative primary tumors, 6 (33.3%) had BRAF^{V600E}-positive metastatic lymph nodes. The presence of the BRAF^{V600E} mutation in the primary tumor did not affect

the tumor size, but the diameter of metastatic lymph nodes significantly increased (by nearly 3 mm) with the presence of BRAF^{V600E} in LNM (p = 0.01). **Conclusions:** In our study, the BRAF^{V600E} mutation did not show a one-to-one correspondence. This indicates that the presence of BRAF^{V600E} in the primary tumor is not clonal and addresses the role of intra-tumor heterogeneity in PTC tumorigenesis. This supports the theses that mutations occur in the later stages of tumorigenesis, might be subclonal, and develop de novo, or that some other factors may be involved in the development of metastasis.

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Introduction

Papillary thyroid cancer (PTC) is the most common type of endocrine cancer [1]. It originates from follicular epithelial cells and usually has a slow progression with a good prognosis. However, of note, it presents with a high rate of lymph node metastasis (LNM; 45%) at the time of diagnosis [2]. The basic mechanism underlying PTC tumorigenesis is the MAP kinase/ERK pathway involved in cell division, proliferation, differentiation, adhesion,

Table 1. BRAF^{V600E} mutation and clinicopathological features in primary tumors and LNM

Clinicopathological features	BRAF ^{V600E} in primary tumor		p value	BRAF ^{V600E} in LNM		p value
	(+)	(-)		(+)	(-)	
Mean tumor ^a /metastatic focus ^b size, mm	19.06±13.8 ^a	16.5±8.7 ^a	0.7	15.4±5.9 ^b	12.9±12.07 ^b	0.01
Gender						
Female (n = 40)	24 (60)	16 (40)	0.18	18 (45)	22 (55)	0.57
Male (n = 11)	9 (81.8)	2 (18.2)		6 (54.5)	5 (45.5)	
Age at diagnosis						
<45 years	27 (69.2)	12 (30.8)	0.22	19 (48.7)	20 (51.3)	0.66
≥45 years	6 (50)	6 (50)		5 (41.7)	7 (58.3)	
Histological type						
Classical	31 (68.9)	14 (31.1)	0.08	21 (46.7)	24 (53.3)	0.87
Subtype	2 (33.3)	4 (66.7)		3 (50)	3 (50)	
Lymphocytic thyroiditis						
Yes	21 (65.6)	11 (34.4)	0.85	15 (46.9)	17 (53.1)	0.97
No	12 (63.2)	7 (36.8)		9 (47.4)	10 (52.6)	

Values are n (%) unless otherwise indicated. p < 0.05, statistically significant.

^a Primary tumor size. ^b Metastatic focus size in LNM.

migration, and apoptosis. Somatic point mutations that occur in the BRAF gene, which encodes serine/threonine protein kinase involved in this cascade, represent a significant genetic event in PTC [3]. Among these, the most common BRAF alteration is the V600E mutation (BRAF^{V600E}), which involves transversion of thymine to adenine at position 1799 in exon 15 and, subsequently, valine(V)-to-glutamate(E) substitution at residue 600 [4]. This mutation turns BRAF into an oncogene that strongly activates the MAPK pathway. It is known that the presence of BRAF^{V600E} results in the suppression of tumor suppressor genes and increases the number of tumor-facilitating molecules [5]. Controversy exists in the literature about the prognostic nature of the BRAF^{V600E} mutation, with some studies having reported an association with poor prognostic characteristics [6, 7] and some having failed to show such an association [8]. Discrepancies in the results of these studies may be due to variations in the evaluation of clinical data or to genetic and geographic diversity.

Initially, the BRAF^{V600E} mutation was thought to occur in the early stages of thyroid carcinogenesis. However, thanks to the molecular dissection of the genome, intratumor heterogeneity has been increasingly understood and shown to take part in thyroid tumorigenesis; however, the question of how this heterogeneity develops has yet to be clarified. It has been suggested that heterogeneity might originate from certain groups of tumor cells

rather than from clonal growth, and thus mutations might exhibit intratumor heterogeneity [9]. Tumor heterogeneity may be associated with thyroid tumor micro-environment, thus some cells provide a basis for mutation while others do not harbor mutations. This may also be associated with other genetic factors or defects in DNA repair mechanisms [10, 11]. Moreover, despite our current knowledge of BRAF^{V600E} having a role in PTC tumorigenesis, it is still to be clarified why this mutation is not present in all cases [12, 13]. It is likely that the broad spectrum of PTC encompassing the latent microcarcinoma classic or aggressive variants is based on intratumor heterogeneity. The BRAF^{V600E} mutation and tumor heterogeneity have been thought to have a particular role in the development of LNM detected even in the early stages of tumor growth. The present study was designed to evaluate the prevalence of the BRAF^{V600E} mutation in primary tumors and accompanying metastases in PTC patients who were detected to have cervical LNM at the time of diagnosis.

Materials and Methods

This retrospective study included 51 patients (40 women, 11 men) who presented to our thyroid disease department between January 2012 and December 2014 and were diagnosed as having PTC. All the patients underwent total thyroidectomy accompanied by a lateral neck dissection due to preoperatively detected

LNM. The mean age of the patients was 40.0 ± 16.5 years (range 6–81), with 39 patients (76.5%) being younger than 45 years. The clinicopathological characteristics of the patients are shown in table 1. All the patients were operated on by the same surgical team. Tissue samples were processed by the same group of pathologists. Samples from primary tumors and metastatic lymph nodes were fixed in paraffin for molecular pathology examination. Histological diagnoses were made based on the criteria of the UICC/AJCC (Union for International Cancer Control/American Joint Committee on Cancer) TNM staging system [14]. The presence of the BRAF^{V600E} mutation was sought in primary tumors and accompanying neck lymph node metastases. From paraffin-fixed and hematoxylin-eosin-stained specimens of the primary thyroid tumors and metastatic lymph node tumors, morphologically well-preserved areas with adequate numbers of tumor cells were marked for real-time PCR analysis. Five 5- μ m-thick sections were then obtained from these areas and treated with paraffin. Then, DNA isolation was performed using the Cobas DNA Sample Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), and DNA quantification was made on a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, N.C., USA). All the samples studied were greater than 2 ng/ml. V600E mutation analysis of the BRAF gene was performed using the Light Cycler 480 Instrument and the Cobas 4800 BRAF V600 Mutation Test (Roche Diagnostics GmbH). Validation of each measurement was checked using mutation-positive and mutation-negative controls.

Statistical Analysis

Data are presented as frequency, percentage, and arithmetic mean. Statistical analyses were made with the use of the χ^2 , Mann-Whitney U, and Spearman correlation tests. A p value of less than 0.05 was considered statistically significant.

Results

The mean primary tumor size was 18.2 ± 12.2 mm (range 5–75), and the mean metastatic lymph node diameter was 14.2 ± 9.7 mm (range 1–50). The prevalence of the BRAF^{V600E} mutation was 64.7% (n = 33) in primary tumors. This prevalence was 39.4% in our previous study [6]. We think that the reason for high prevalence in the current study is the fact that most of the patients had LNM. We have actually previously shown that the risk of LNM increases in the presence of BRAF mutation. The prevalence was 47.1% (n = 24) in metastatic lymph nodes. The presence of the BRAF^{V600E} mutation in primary tumors did not directly match its presence in metastatic lymph nodes (p = 0.14). The relationship of BRAF^{V600E} between primary tumors and LNM is summarized in table 2. Of 33 patients with BRAF^{V600E}-positive primary tumors, 18 (54.5%) had BRAF^{V600E}-positive metastatic lymph nodes. In the remaining 15 patients without BRAF^{V600E}-positive metastatic lymph nodes, there were 7 unifocal and 8 multifocal primary tumors. Eighteen pa-

Table 2. The relationship of BRAF^{V600E} in primary tumors and accompanying metastatic lymph nodes

Primary tumors (n = 51)	BRAF ^{V600E} in LNM	
	positive (n = 24, 47.1%)	negative (n = 27, 52.9%)
BRAF ^{V600E} positive (n = 33)		
Unifocal	4	7
Multifocal	14	8
BRAF ^{V600E} negative (n = 18)		
Unifocal	1	7
Multifocal	5	5

tients had BRAF^{V600E}-negative primary tumors. In this group, BRAF^{V600E}-positive metastatic lymph nodes were detected in 6 patients (33.3%) whose primary tumors were multifocal in 5 patients and unifocal in 1 patient.

The presence of the BRAF^{V600E} mutation was not correlated with age and gender. Lymphocytic infiltration in nontumoral tissue was detected in 32 patients (62.7%). Despite not being statistically significant, this rate was considered to be high. The majority of the patients (n = 45, 88.2%) had conventional papillary thyroid carcinoma, but this did not show a statistically significant difference. The presence of the BRAF^{V600E} mutation in the primary tumor was not correlated with tumor size or with the diameter of the metastatic lymph node. Conversely, metastatic lymph nodes showed a significant relationship between BRAF^{V600E} positivity and the diameter of metastatic lymph nodes (p = 0.01). BRAF^{V600E}-positive metastatic lymph nodes were nearly 3 mm greater in diameter than those without the BRAF^{V600E} mutation.

Discussion

Despite considerable improvements in our understanding of the molecular mechanism of PTC, its LNM mechanism remains unclear. The frequent detection of the BRAF^{V600E} mutation in PTC, along with its absence in other thyroid cancers, suggests that this mutation may play a role in the early stages of PTC tumorigenesis and thus may exist in tumor metastases [15]. Recent studies have shown that wild-type BRAF and mutated BRAF (BRAF^{V600E}) genetic alterations coexist in PTC, forming intratumor heterogeneity. Mutational differences between primary tumors and accompanying lymph node metastases are thought to be a result of tumor heterogene-

ity [9, 10, 16]. Therefore, a BRAF-mutated primary tumor may cause LNM to have either a mutant or wild-type variation. On the other hand, a primary tumor with wild-type BRAF may give rise to LNM of mutated BRAF [17, 18]. However, it is clear that if LNM was characterized by BRAF mutation, mutation-bearing cells would form a prevailing majority over wild-type BRAF in the primary tumor and LNM. On the other hand, Jovanovic et al. [19] evaluated microsatellite allelic imbalance in multifocal PTC and demonstrated that not all foci harbored the BRAF^{V600E} mutation. This suggests that the BRAF^{V600E} mutation may be a subclonal event in the development of PTC, providing further support to the view that the BRAF^{V600E} mutation may not be the initiator of PTC tumorigenesis.

Our study group was composed of PTC patients with LNM, which enabled us to make a more refined evaluation to understand the relationship of the BRAF^{V600E} mutation with primary tumors and accompanying LNM. Primary tumors of 33 patients (64.7%) were BRAF^{V600E} positive. The overall BRAF^{V600E} positivity for LNM (with and without primary tumor positivity) was 47.1% (n = 24). Our finding that the rate of accompanying LNM mutations was lower than that seen in primary tumors seems to contradict the view advocated by the clonal evolution model that most tumor cells would harbor mutations. Rather, it provides support for the theses that mutations occur in the later stages of tumorigenesis, might be subclonal, or develop de novo, or that some other factors may be involved in the development of metastasis [9, 17]. Specifically, in those with primary tumor mutations, the rate of accompanying LNM mutations was 54.5%. This shows that primary tumor mutations do not necessarily correspond to LNM mutations and that other factors may take part in LNM. Of note, some reported rates of accompanying LNM mutations are remarkably higher than that found in our study, being 73.9% [20], 81.0% [17], and even 96.0% [21] in three studies. Discrepancies in the reported rates of LNM mutations may be related to tumoral multifocality. The natural environment of lymph nodes and growth factors may provide a favorable settlement and harbor for PTC cells carrying BRAF^{V600E}. Moreover, access of mutated cells to lymph nodes may bring about some biological changes, with eventual proliferation of cancer cells [22]. The latter may account for how even small tumor foci may cause larger LNM. In particular, multifocal PTC may present as a heterogeneous BRAF^{V600E} distribution, which is thought to result from independent clonal growth in diverse tumor foci [23]. On the other hand, it is not clear whether multifocal tumors

develop from one tumor focus by intratumoral spread or from diverse tumor foci. Intratumor heterogeneity of mutations may be associated with this, as well. It is not known whether multifocal tumors develop as independent foci (resulting from different progenitor cells) or arise from intrathyroidal metastasis. The dense lymphatic network in the thyroid suggests a higher likelihood of intratumoral spread. Some studies demonstrated increases in both intrathyroidal multifocality and LNM with increased intratumoral lymphogenesis [24, 25]. It is important to know how multifocality occurs, because if this condition occurs through intrathyroidal metastasis, then there is an increased likelihood of extrathyroidal metastasis. Park et al. [26] reported in patients with multifocal PTC that BRAF^{V600E} showed heterogeneity in 39.3% of the cases, that mutations were not present in all foci, and that mutations did not demonstrate clonal development. Some studies reported polyclonality as an uncommon entity for multifocal tumors [27]. Guerra et al. [9] assessed intratumor heterogeneity with the allelic percentage of the BRAF^{V600E} mutation and found mutations in less than 50% of the alleles, with the majority having wild-type BRAF. This intratumor heterogeneity raises the view that BRAF^{V600E} initiates tumorigenesis, but progression is mainly due to secondary genetic events. On the other hand, BRAF^{V600E} can be secondary in PTC tumorigenesis. In this case two questions remain unanswered: what initiates tumorigenesis if BRAF^{V600E} occurs secondarily, and what is the role of BRAF^{V600E} at this stage? Guerra et al. [16] reported that an allele percentage of more than 30% pointed to a poor prognosis and recommended the measurement of the allelic percentage of BRAF^{V600E} to evaluate the prognosis or the possibility of response to treatment with kinase inhibitors.

In our study, of 18 patients with BRAF^{V600E}-positive primary tumors, 14 had mutation-positive LNM despite multifocal primary tumors. It is likely that mutations in these patients were homogeneously transported. On the other hand, 15 patients had mutation-negative LNM despite primary tumor mutations. In 8 of these patients with multifocal tumors, metastasis spread was thought to develop from tumoral foci without mutations. Conversely, as reported in the study of Guerra et al. [9], these tumor foci consisted of numerous wild-type BRAF and a small number of mutant BRAF^{V600E}, and because the mutations were not clonal, mutant BRAF^{V600E} could not have a chance to proceed from the primary tumor to LNM.

Quantitative studies have demonstrated that BRAF^{V600E} is a subclonal or oligoclonal event rather than being clonal. Oncogen, distributed in some cells or groups

of cells within the tumor cells, constitutes intratumor heterogeneity. All these data suggest that the BRAF^{V600E} mutation is not an early event in PTC tumorigenesis, as previously thought, but rather an event occurring later, and that other genetic events are likely to take part in tumorigenesis and the metastatic process [9, 10].

Of 18 patients with BRAF^{V600E}-negative primary tumors, 12 also had BRAF^{V600E}-negative LNM, suggesting that non-BRAF^{V600E} mechanisms were involved in the metastatic process. Interestingly, in the remaining 6 patients, BRAF^{V600E}-positive metastatic lymph nodes were detected despite BRAF^{V600E}-negative primary tumors. This was at odds with the view that mutations initiate PTC tumorigenesis. Mutations in these patients either developed at late stages of tumorigenesis or were simply *de novo*. One of these patients had a unifocal primary tumor, suggesting a *de novo* mutation in the metastatic lymph node. Vasko et al. [17] also pointed to the possibility of *de novo* mutations in LNM. The detection of mutated LNM in 5 patients with multifocal primary tumors raises the possibility that mutations could be carried to LNM from a focus other than the dominant tumor. Apparently, this finding may challenge the approach of choosing the dominant focus for mutation analysis used in many studies.

Another important finding of our study is that mutation-positive LNM was significantly associated with a greater diameter of metastatic lymph nodes ($p = 0.01$). This is either because mutation itself accelerates cell growth in the lymph nodes or because lymph node microenvironment paves the way for the settlement of mutation through the growth of some nodes [22].

Although BRAF^{V600E} is considered to be a high-risk factor for aggressive behavior of tumors, it is not an independent risk factor for negative results. There are also

some reports which suggest that it may not be used as a poor prognostic factor [28]. The mechanism of BRAF^{V600E} and metastasis-based distribution characteristics of mutations in PTC will affect pharmaceutical mutation-directed studies. The subclonal nature of mutations may cause variations in metastasis-induced responses to BRAF inhibitors [29].

In conclusion, despite the high frequency rates of BRAF^{V600E} in PTC patients, the mutation does not show a one-to-one correspondence in terms of LNM mutations. This unmatched pattern of the mutation provides support for the view that intratumor heterogeneity plays a significant role in PTC tumorigenesis and that the mutation comes into effect at any stage rather than at the beginning of tumorigenesis. Another important point is that the metastatic lymph node harboring BRAF^{V600E} is enlarged most likely due to mutation-related causes, which may denote progression and thus should be borne in mind during treatment and follow-up of PTC patients with BRAF^{V600E}-positive metastatic lymph nodes. Additionally, knowing that the BRAF^{V600E} mutation presents as a subclonal or oligoclonal event resulting in intratumor heterogeneity can increase the response rates of novel mutation-directed drug therapies.

Acknowledgments

We thank S. Pinar Barut (molecular biology and genetic specialist) and Meltem Algan (biologist) for technical support in the BRAF^{V600E} mutation analysis.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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