BRAF^{V600E} Mutation and p27^{kip1} Expression in Papillary Carcinomas of the Thyroid ≤1 cm and Their Paired Lymph Node Metastases

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BACKGROUND. *BRAF*^{V600E} mutation and p27^{kip1} expression have been introduced as novel indicators that may predict prognosis in different tumors, as well as in papillary thyroid carcinomas.

METHODS. Tissue samples from 214 consecutive patients who underwent total or near-total thyroidectomy with histological diagnosis of papillary thyroid carcinoma (PTC) ≤1 cm were analyzed for *BRAF*^{V600E} mutation by a real-time, allele-specific amplification and for p27^{kip1} expression by immunohistochemistry.

RESULTS. The *BRAF*^{V600E} mutation was detected in 88 of the tumors examined, with significant differences between groups with and without lymph node (LN) metastases; the mean age of patients with *BRAF*^{V600E} mutation was significantly higher than that of patients without mutations. A significant association was found between low p27^{Kip1} protein expression and multifocality, bilaterality, and extrathyroidal extension, in addition to LN metastasis. In 42 cases with LN metastases, 23 harbored the *BRAF*^{V600E} mutation in the metastatic tumor and presented a wider diameter of the largest metastatic area, a higher number of involved LNs, and a higher percentage of metastatic lesions with extracapsular extension of LN (ECE-LN). A significantly lower mean value of p27^{Kip1} was observed in LNs harboring the *BRAF*^{V600E} mutation and in ECE-LN; an inverse correlation was found between p27^{Kip1} and the number of metastatic LNs, as well as the diameter of the largest metastatic area in LN.

CONCLUSIONS. The authors' data suggested that *BRAF*^{V600E} mutation and p27^{Kip1} down-regulation in cancer cells of PTC ≤1 cm may be factors that facilitate tumor-cell growth and progression once these are seeded in the LNs. ***Cancer*** **2007;110:1218–26.** © *2007 American Cancer Society.*

KEYWORDS: papillary thyroid carcinoma, *BRAF*, p27, cell cycle.

It is a well-known fact that papillary thyroid carcinomas (PTC) often comprise relatively small tumors compared with other histological types of thyroid tumors. In the last few years, it has been estimated that as the use of thyroid ultrasound and other neck-imaging modalities has increased, nodules that are not large enough to be palpated are discovered and identified with greater frequency.[1,2] Because they are often found in thyroids resected for other lesions, small PTCs that measure ≤1.0 cm are often described as "microcarcinoma," "occult," or "incidental".

Small PTCs usually have an excellent clinical outcome, but their prognostic factors have not yet been fully established. In a recent retrospective chart-review study of patients treated and followed for small (<1.5 cm) papillary thyroid cancers, nonincidental thyroid

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cancer and lymph node (LN) metastases at presentation were found to be associated with persistent and/or recurrent disease.[3]

BRAF^{V600E} mutation and p27^{kip1} expression have been introduced as novel indicators that may also predict a poorer prognosis in various tumors, as well as in PTC. *BRAF* is a member of the RAF kinase family and promotes signaling through the RAS-MAP kinase signal-transduction cascade.[4] Mutations of the *BRAF* gene have been found in a variety of human cancers, most notably in melanomas and thyroid cancer. The most common *BRAF* mutation is the T1799A transversion mutation (formerly known as *BRAF* T1796A mutation) in exon 15 of the gene. This mutation causes a V600E (formerly known as V599E) amino acid substitution in the protein and confers the kinase oncogenic function through constitutive activation of the MAPK signaling pathway.[4]

The p27^{Kip1} protein is an important cdk inhibitor and inhibits the formation of cyclin D1/cdk complexes during G0 and early G1 phases of the cell cycle, and its decrease is closely related to cell-cycle progression in many cancers.[5]

The aim of the present study was to determine the frequency of *BRAF*^{V600E} mutation and the expression of p27^{Kip1} protein in a series of primary PTCs $\leq$1.0 cm and their paired LN metastases.

MATERIALS AND METHODS

Patients

We considered a group of 214 consecutive patients who underwent total or near-total thyroidectomy during the period from January 1990 to December 1999, with histological diagnosis of PTC $\leq$1 cm, and who were followed in our endocrinology clinic. This retrospective study was performed in accordance with the rules of the institutional review board at the Faculty of Medicine (University of Palermo). Forty-two patients had regional LN metastases, 36 with nonincidental and 6 with incidental carcinomas.

Histopathological Evaluation and Tumor Staging

Tumor specimens stored in the archives of the Institute of Pathologic Anatomy and Histology of the University of Palermo provided adequate histological material. For each case, all histological slides were reviewed by 2 pathologists (V.R. and D.C.), who were unaware of the clinical data, and diagnoses were reassessed according to the World Health Organization classification of thyroid malignancy.[6]

All primary tumors included in this study were classified as classical variants of PTC; tumors with morphological features of the follicular variant of PTC were not included. Tumors were staged according to the sixth edition of the Cancer Staging Manual,[7] where T (extent of the primary tumor) and N (regional LN metastasis) were determined on the basis of pathological data, and M (evidence of distant metastasis) was based on findings at the first postoperative ^{131}I whole-body scan.

All tissue samples were fixed in 10% buffered formalin, dehydrated in ethanol, and paraffin-embedded according to the routine technique. Hematoxylin & eosin-stained sections of 5 μm thickness were reassessed for intraglandular multifocality, bilaterality, tumor extension beyond the thyroid capsule or extrathyroidal invasion, vascular invasion, grade of sclerosis (sclerosing or nonsclerosing type) according to the amount of fibrous stroma coexisting with malignant cells, degree of encapsulation (encapsulated or nonencapsulated), and maximum diameter of the primary lesion ($<$5 mm or $\geq$5 mm.). Sections of LN metastases were reassessed for the number of involved LNs, extracapsular extension (ECE) of the metastatic lesion, and diameter of the largest metastatic area.

Immunohistochemistry

The avidin-biotin peroxidase complex technique was used on 5 μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization as previously described.[8] Briefly, the sections were reacted consecutively with the monoclonal antibody K25020 (Transduction Laboratories, Lexington, Ky) generated from mouse p27^{Kip1} protein at a concentration of 1:200. Thereafter, slides were washed in phosphate-buffered saline (PBS), and secondary incubations were carried out by using biotinylated alpha mouse IgG obtained from horse serum (ABC Kit; Vector Laboratories, Burlingame, Calif) for 30 minutes. Finally, 3,3-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark) in distilled water was used as the chromogen for 10 minutes, and sections were counterstained with Mayer hematoxylin.

The p27^{Kip1} protein expression in each tumor and matched LN metastases was evaluated according to its intensity. p27^{Kip1} expression was positive if the staining intensity of tumor cell nuclei was equal to or stronger than that of the adjacent lymphocytes and negative otherwise.[9–11] The staining of p27^{Kip1} protein in each tumor and matched LN metastases was evaluated under high-powered fields (final magnification, $\times$400) and reported as the percentage of expressor cells among the total number of counted cancer cells and used as a labeling index (LI). For

multifocal tumors and multiple LN metastases, the evaluations were assessed on different areas of all lesions. Twenty cases were evaluated separately by 2 different pathologists (D.C. and A.M.); because the variation was less than 5%, the first pathologist's data were used. Based on the median value (24.8%; range, 0%–90.1%) of $p27^{Kip1}$ protein expression in primary PTC of this series, $p27^{Kip1}$ protein status was classified as low ($p27^{Kip1}$ protein nuclear staining in <24.8% of tumor cells) or high ($p27^{Kip1}$ protein nuclear staining in ≥24.8% tumor cells).

DNA Extraction and Analysis

The DNA material was retrieved from paraffin blocks after careful microdissection performed by an experienced pathologist (A.M.). For tumors measuring <4 mm, DNA extraction was performed by using a laser pressure catapulting (LPC) laser microdissection system, based on a Zeiss inverted microscope PALM Laser Micro-Beam System UV laser at 337 nm (Carl Zeiss AG, Oberkochen, Germany), linked to a personal computer with the required software programs as previously described.[12] Genomic DNA was extracted by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. $BRAF^{V600E}$ mutation was detected by a real-time, allele-specific amplification essentially as described by Jarry et al.[13] Briefly, polymerase chain reaction (PCR) amplifications were performed by using QuantiTect SybrGreen PCR kit (Qiagen). The reaction mixture contained 5 μL of the supplied 2× master mix, 0.4 μL of each primer (0.4 μM each), and PCR-grade water to a volume of 7.9 μL. Capillaries were loaded with 7.9 μL of this master mix, and 2.1 μL of the extracted DNA template was added. Amplification was performed with a Lightcycler (Roche Diagnostics GmbH, Mannheim, Germany), and fluorescence was measured into the F1 channel. The cycling conditions were as follows: denaturation for 15 minutes at 95°C; amplification for 45 cycles, with denaturation for 10 seconds at 95°C; annealing for 5 seconds at 55°C, and extension for 10 seconds at 72°C with fluorescence acquisition; holding step for 20 seconds at 72°C. After completion of the cycling process, samples were subjected to melting curve analysis. Briefly, samples were slowly cooled (0.1°C/second) to 50°C for 30 seconds and then to a temperature ramp from 50 to 90C° at 0.1°C/second with continuous fluorescence monitoring. For each sample, the −dF1/dT versus T plot was displayed, and a single narrow peak was obtained, indicating specific amplification without significant by-products. Positive results were further confirmed by using the B-RAF Mutector kit (Trimgen; Tebu-Bio, Milan, Italy) and following the manufacturer's protocol. Randomly selected mutated (n = 5) and wild-type (n = 5) cases (as confirmed by Mutector assay) were further sequenced by the primers described by Davis et al.,[4] with the use of the ABI BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif) and subjected to automatic sequencing by using ABI Prism 310 Genetic Analyzer (Applied Biosystems) (data not shown).

For multifocal tumors and multiple LN metastases, $BRAF^{V600E}$ mutation was detected on all lesions; if any of the tumor foci was $BRAF^{V600E}$ positive, the case was included in the "mutated" group.

Statistical Methods

Continuous variables were analyzed as mean values plus or minus a standard deviation. Rates and proportions were calculated for categorical data. For categorical variables, differences were analyzed by means of the chi-square test and Fisher exact test when appropriate. As continuous variables were without normal distribution, we used nonparametric tests, and differences were analyzed by the Mann-Whitney *U* test. The differences between paired continuous variables were analyzed by means of the Wilcoxon test. Correlations among continuous variables were determined by using the Spearman test, the nonparametric equivalent for the Pearson test. $P < .05$ was considered statistically significant. All analyses were performed with SPSS software (2001 release 11.0; Chicago, Ill).

RESULTS

Clinicopathological characteristics of the 214 PTC ≤1 cm included in this study are shown in Table 1. An examination of the distribution of tumors without LN metastases (Group I) and with LN metastases (Group II), showed significant differences, apart from clinical signs, among intraglandular multifocality, bilaterality, sclerosis, $BRAF^{V600E}$ mutation, and $p27^{Kip1}$ protein expression.

$BRAF^{V600E}$ mutation was detected in 88 (41%) of the tumors examined (Fig. 1); sequencing confirmed the allele-specific and the Mutector analysis. An examination of the distribution of tumors with and those without $BRAF^{V600E}$ mutation showed no significant differences in clinical signs, sex, maximum diameter of the primary lesion, vascular invasion, grade of sclerosis, degree of encapsulation, multifocality, bilaterality, extrathyroidal extension, or $p27^{Kip1}$ protein expression (data not shown). Statistical anal-

TABLE 1
Comparison of Clinicopathological Parameters in Nonmetastatic (Group I) and Metastatic (Group II) Papillary Thyroid Carcinomas ≤1 cm

Parameters	Group I Node− (%)	n = 172	Group II Node+ (%)	n = 42	*P**
Clinical signs					
Nonincidental	(48)	83	(86)	36	
Incidental	(52)	89	(14)	6	<.001
Age, y					
<45	(46)	79	(55)	23	
≥45	(54)	93	(45)	19	NS
Sex					
Women	(82)	141	(71)	30	
Men	(18)	31	(29)	12	NS
Maximum diameter of primary lesion					
<5mm	(43)	74	(43)	18	
≥5mm	(57)	98	(57)	24	NS
Intraglandular multifocality					
Present	(26)	45	(76)	32	<.001
Bilaterality					
Present	(3)	6	(71)	30	<.001
Extrathyroidal extension					
Present	(15)	26	(17)	7	NS
Vascular invasion					
Present	(5)	9	(7)	3	NS
Grade of sclerosis					
Sclerosing	(1)	1	(12)	5	
Nonsclerosing	(99)	171	(88)	37	<.001
Degree of encapsulation					
Encapsulated	(52)	89	(55)	23	
Nonencapsulated	(48)	83	(45)	19	NS
BRAF^{V600E}					
Wild-type	(62)	107	(45)	19	
Mutant	(38)	65	(55)	23	.034
P27^{Kip1} protein expression					
LI ≥24.8%	(87)	150	(14)	6	
LI <24.8%	(13)	22	(86)	36	<.001

NS indicates not significant; LI, labeling index.
* Chi-square test and Fisher exact test; statistical results were considered significant for *P* values <.05.

ysis, however, demonstrated a significant association between age of patients and *BRAF*^{V600E} mutation. The mean age of the patients with *BRAF*^{V600E} mutation was significantly higher than that of patients without *BRAF*^{V600E} mutation (52.7 ± 2.9 years vs 33.4 ± 2.3 years; $P < .001$).

In addition to LN metastasis, low p27^{Kip1} protein expression was significantly associated with multifocality, bilaterality, and extrathyroidal extension (Table 2).

BRAF^{V600E} Mutation and p27^{Kip1} Expression in LN Metastases

Of the 42 cases in Group II, 22 were classified as pN1a and 20 as pN1b; 22 cases showed metastatic lesions with an extracapsular extension of the lymph node (ECE-LN).

Twenty-three of 42 harbored the *BRAF*^{V600E} mutation in primary PTC ≤1 cm, and 17 of 23 (74%) also presented the same mutation in their respective LNs. All the remaining 6 cases with *BRAF*^{V600E} mutation in primary tumors with no mutation in LNs had multiple foci of PTC ≤1 cm in the thyroid gland with *BRAF*^{V600E}-positive and *BRAF*^{V600E}-negative tumors coexisting in the same patient. Nineteen cases did not harbor the *BRAF*^{V600E} in the primary PTC, and of these, 13 cases did not show the *BRAF*^{V600E} mutation in their respective LNs, whereas the other 6 showed the *BRAF*^{V600E} mutation in the LN metastasized tumor tissue.

We observed that metastasized LNs harboring the *BRAF*^{V600E} mutation presented a wider diameter of the largest metastatic area, a higher number of involved metastatic LNs, and a higher percentage of ECE-LN in comparison with LNs without mutations. No difference was found in *BRAF*^{V600E} with regard to compartment involvement (N1a or N1b) (Table 3).

An evaluation of p27^{Kip1} expression in LN metastasized tumor cells showed a significantly lower mean value than that observed in corresponding primary PTC (5.77 ± 6.14% vs 14.27 ± 18.24%; $P = .006$). Furthermore, whereas 36 (86%) tumors showed low p27^{Kip1} protein expression (labeling index <24.8%) in primary PTC, in all cases of LN metastases, the p27^{Kip1} labeling index of cancer cells was <24.8%. The lowest mean value of p27^{Kip1} labeling index was detected in ECE-LNs when compared with LNs without ECE (1.78 ± 2.19% vs 10.16 ± 6.11%; $P < .001$). No difference was found in p27^{Kip1} protein expression in relation to compartment involvement (N1a or N1b [7.73 ± 7.17% vs 3.61 ± 3.89%; $P = .068$]), whereas an inverse correlation was found between the p27^{Kip1} labeling index and the number of metastatic LNs (Rho −0.659; $P < .001$), as well as between the p27^{Kip1} labeling index and the diameter of largest metastatic area in LN (Rho −0.732; $P < .001$) (Fig. 2).

With regard to p27^{Kip1} protein expression and *BRAF*^{V600E}, we found a significant lower mean value of p27^{Kip1} labeling index in LNs harboring the *BRAF*^{V600E} mutation in comparison with those without the mutation (1.87 ± 2.44% vs 10.49 ± 5.97%; $P < .001$) (Fig. 3).

By applying an arbitrary score analysis on the basis of the number of metastasized LNs >3, the presence of ECE-LNs, and the diameter of the largest metastatic area >3 cm (score 0 = none of the selected evaluation criteria present; 1 = 1 single cri-

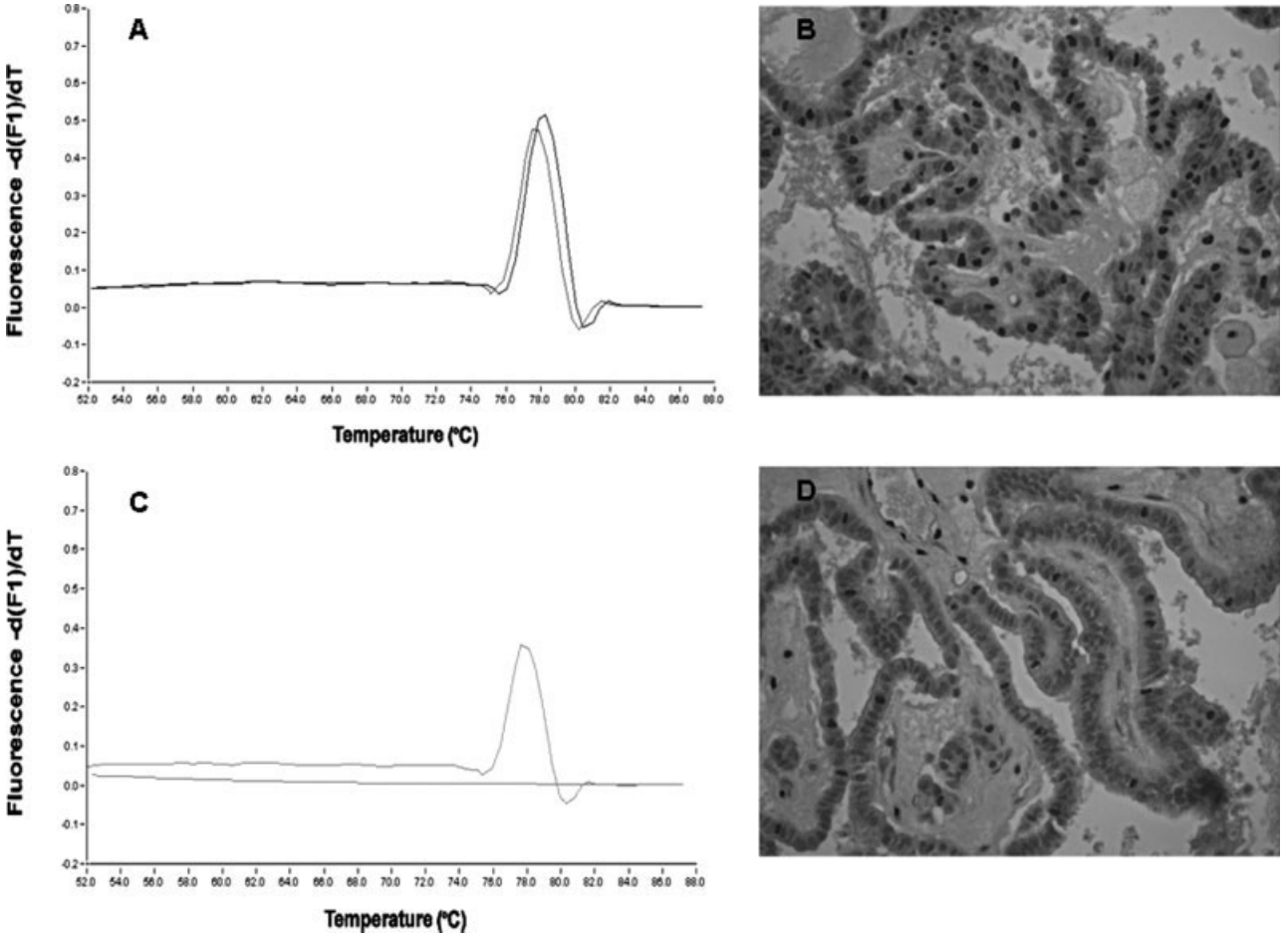

FIGURE 1. Representative cases of PTC with (A) wild-type or (C) *BRAF*^{V600E} mutation and with (B) high expression or (D) low expression of p27^{Kip1}.

TABLE 2
Low p27^{Kip1} Protein Expression (LI <24.8%) and Lymph Node Status, Intraglandular Multifocality, Bilaterality, Extrathyroidal Invasion in 214 Patients With PTC ≤1 cm

Parameters	Total	Low p27^{Kip1} protein expression, no. (%)	*P**
Node			
Node−	172	22 (13)	
Node+	42	36 (86)	<.001
Intraglandular multifocality			
Absent	137	18 (14)	
Present	77	40 (52)	<.001
Bilaterality			
Absent	178	25 (14)	
Present	36	33 (92)	<.001
Extrathyroidal invasion			
Absent	181	27 (15)	
Present	33	31 (94)	<.001

LI indicates labeling index.
* Chi-square test; statistical results were considered significant for *P* values <.05.

terion; 2 = 2 criteria present; 3 = all 3 selected criteria present) and then subdividing the 42 patients with LN metastases, we found that *BRAF*^{V600E} mutation was found in 11 of 12 (92%) patients belonging to score 3, in 50% of patients belonging to score 1 and to score 2, and in 1 of 8 (12.5%) patients with score 0; these differences proved to be significant ($P < .001$). When we analyzed p27^{Kip1} protein expression, we found that the progressive reduction of p27^{Kip1} labeling index was significantly associated with the worst score (Fig. 4).

Moreover, we also investigated possible associations between the clinicopathological features of the primary tumors with LN metastasis (Group II) and our arbitrary score; the only parameter significantly associated ($P = .044$) was the mean value of p27^{Kip1} labeling index of primary tumors with LN metastasis in score 0 (27.68% ± 16.24%) versus mean value of p27^{Kip1} labeling index of primary tumors with LN metastasis in score 3 (11.32% ± 9.44%).

TABLE 3
Features of 42 Metastasized Lymph Nodes in Relation to *BRAF*

Features	*BRAF* Wild-type (n = 19)	*BRAF*^{V600E} Mutant (n = 23)	*P*
Largest metastatic area diameter, cm, Mean ± SD	1.79 ± 0.78	3.06 ± 0.47	<.001*
No. metastatic lymph nodes, Mean ± SD	2.47 ± 1.3	4.13 ± 1.18	<.001*
Extracapsular extension of lymph nodes, No. (%)	5 (26)	17 (74)	.003†
Node pN1a, No. (%)	13 (68)	9 (39)	
pN1b, No. (%)	6 (32)	14 (61)	.059†

SD indicates standard deviation (standard error of the mean).
* Mann-Whitney test; significant values when $P < .05$.
† Chi-square test; significant values when $P < .05$.

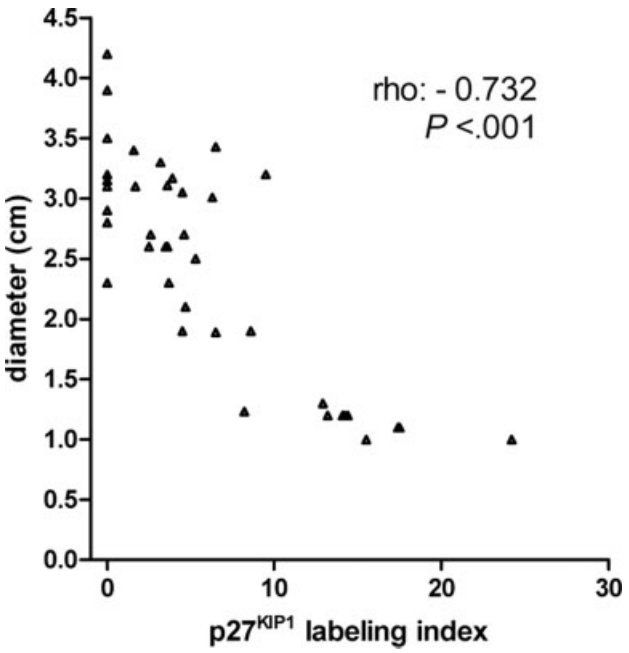

FIGURE 2. Bivariate scatter plot of p27^{Kip1} labeling index and diameter (cm) of largest metastatic area in lymph nodes. Correlations among continuous variables and *P* values were determined by using the Spearman test; statistical results were considered significant for *P* values <.05.

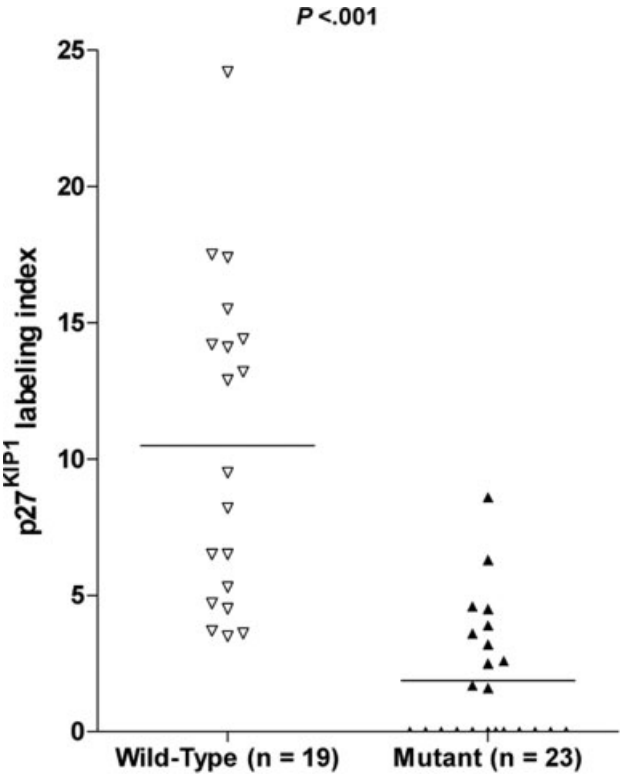

FIGURE 3. p27^{Kip1} labeling index in comparison with *BRAF*^{V600E} in lymph node metastases. A lower p27^{Kip1} protein expression was observed in LNs harboring the *BRAF*^{V600E} mutation in comparison with those without the mutation (1.87 ± 2.44% vs 10.49 ± 5.97%; $P < .001$). Differences were analyzed by the Mann-Whitney test; statistical results were considered significant for *P* values <.05.

DISCUSSION

Several studies have investigated the role of the *BRAF*^{V600E} mutation in PTC[24–30], and several of them,[17,18] though not all,[16,20] have reported a significant association of this mutation with metastasis and local invasion. These differences may be explained by the low number of cases composing the cohort[16,20] and the different histotype composition of the PTC,[20] which may obscure the association between some poor clinicopathological outcomes and *BRAF*^{V600E} mutation; several studies, however, have reported *BRAF*^{V600E} mutation at a higher rate in PTC with invasive and metastatic potential than in noninvasive tumors, although without statistical significance.[16]

In our own study, the *BRAF*^{V600E} mutation in primary tumors was associated with LN metastases. In concordance with other authors,[16,18,21] we also observed that patients with PTC lesions harboring the *BRAF*^{V600E} mutation were older in age than those presenting PTC lesions with wild-type *BRAF*. The higher age of patients with conventional PTC displaying *BRAF*^{V600E} may indicate either that tumors with the mutation start their neoplastic development (initiation and/or early phase of promotion) in older individuals or, having started in a similar age period, progress more slowly than PTC without the mutation.

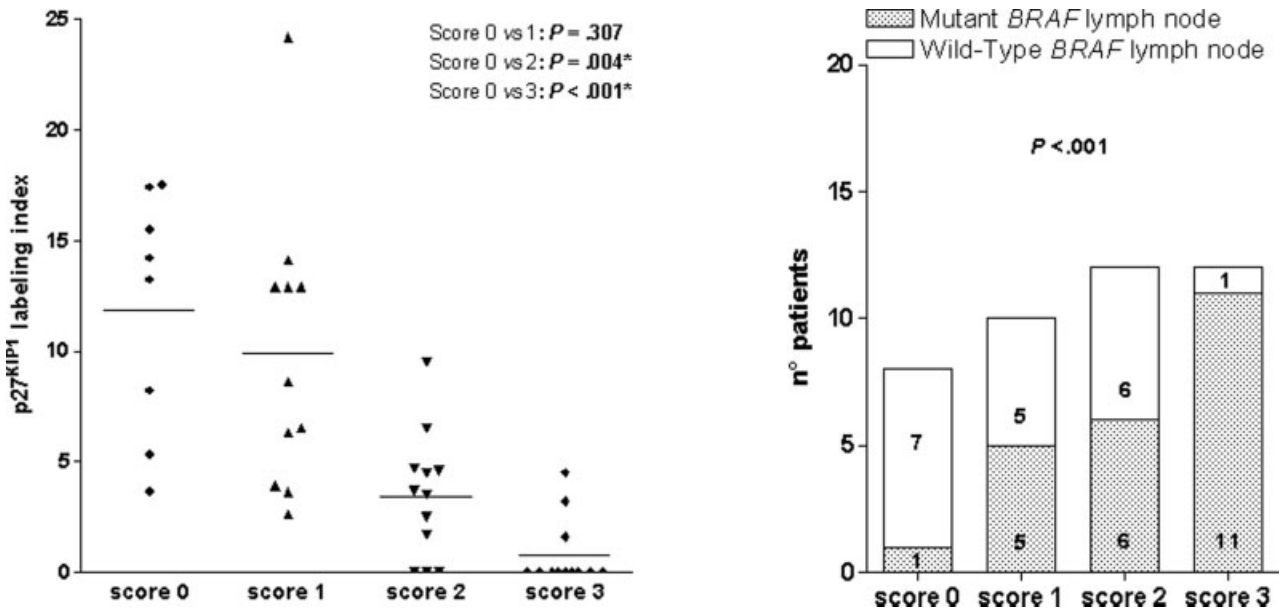

FIGURE 4. $p27^{Kip1}$ labeling index and $BRAF^{V600E}$ mutation in 42 cases of lymph node metastases were subdivided according to our scores. The progressive reduction of $p27^{Kip1}$ protein expression in metastatic lymph node tumor cells was significantly associated with the worst score (score 0 vs score 2, $P = .004$; score 0 vs score 3 $P < .001$; P values were determined by using Kruskal Wallis test.) $BRAF^{V600E}$ mutation in metastatic lymph node tumor cells was found in 92% (11 of 12) of cases belonging to score 3, in 50% of cases belonging to score 1 and to score 2, and in 12.5% cases with score 0; these differences proved to be significant ($P < .001$); P values were calculated by using the chi-square and Fisher exact tests. Statistical results were considered significant for P values $< .05$.

In our series, 6 cases with multiple foci of tumors with $BRAF^{V600E}$-positive and $BRAF^{V600E}$-negative tumors coexisted in the same patient not presenting the $BRAF^{V600E}$ mutation in their respective LN metastases, and 6 out of 19 cases did not harbor the $BRAF^{V600E}$ in the primary PTC harboring the $BRAF^{V600E}$ mutation in their respective LNs. It is, therefore, possible that the *BRAF* mutation is not absolutely essential for the metastasis of PTC $\leq$1cm to LNs; this would be consistent with other clinicopathological studies reporting the association of both *BRAF* mutation-positive and mutation-negative primary PTC tumors with LN metastases.[16–18,20,22] Moreover, recent studies have reported novel *BRAF* mutations only in LN-metastasized PTC and not in the primary tumors.[23,24] The high prevalence of the *BRAF* mutation in LN-metastasized thyroid tumors in cases showing this mutation in their primary tumors supports the notion that the *BRAF* mutation may facilitate the seeding and progression of PTC cells in LNs, consistent with the finding that the mutation is associated with a higher prevalence of LN metastasis of PTC.[25]

In the present study, the low $p27^{Kip1}$ protein expression was strongly associated with LN metastases; we also found a significant association between low $p27^{Kip1}$ protein expression and multifocality, bilaterality, and extrathyroidal extension. These findings are in line with previous studies on $p27^{Kip1}$ protein expression in thyroid papillary microcarcinomas.[26,27]

Previous studies have not, however, included an examination of $p27^{Kip1}$ expression in LN metastases and paired PTCs. It should be noted that we ourselves have evaluated the potential role of $p27^{Kip1}$ in the process of LN metastasis. The levels of $p27^{Kip1}$ expression in primary PTCs were found to be higher than those of compared LN metastases. Recent studies have revealed a significant reduction in the expression of $p27^{Kip1}$ in the metastatic site compared with that detected in the corresponding primary tumors; p27 has also been shown to have a function in adhesion-dependent cell growth.[5] In our own study, the lowest mean value of $p27^{Kip1}$ labeling index was detected in ECE-LNs when compared with LNs without ECE, and, in our arbitrary score, the progressive reduction of $p27^{Kip1}$ labeling index was significantly associated with the worst score.

With regard to the pathological characteristics of LN-metastasized tumor cells, we found a signifi-

cant lower mean value of p27^{Kip1} labeling index in LNs harboring the *BRAF*^{V600E} mutation in comparison with those without the mutation. The level of the p27^{Kip1} protein is mainly regulated by post-translational mechanisms through degradation by ubiquitin-dependent proteolysis.[28] A recent study on melanoma cells shows that mutation of *BRAF* subverts adhesion control of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway leading to deregulation of cyclin D1 and p27^{Kip1} levels.[29]

In conclusion, as far as we know, the present study is the first analysis of both *BRAF*^{V600E} mutation and p27^{Kip1} protein expression in primary PTC $\leq$1 cm and their paired LN metastases, suggesting that *BRAF*^{V600E} mutation and down-regulation of p27^{Kip1} in cancer cells of PTC $\leq$1 cm may be considered as factors facilitating tumor-cell growth and progression, once these are seeded in the LNs. Although they do not provide direct experimental evidence that, as recently demonstrated in other tumors,[29,30] *BRAF*^{V600E} mutation down-regulates p27^{Kip1} expression in thyroid cancer cells, our findings suggest an interplay between *BRAF*^{V600E} mutation and p27^{Kip1} underexpression. Further in vitro experiments are needed to clarify these possible molecular interactions.

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