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BRAF V600E and risk stratification of thyroid microcarcinoma: a multicenter pathological and clinical study

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Studies from single institutions have analyzed *BRAF* in papillary microcarcinomas, sometimes with contradictory results. Most of them have provided limited integration of histological and clinical data. To obtain a comprehensive picture of *BRAF* V600E-mutated microcarcinomas and to evaluate the role of *BRAF* testing in risk stratification we performed a retrospective multicenter analysis integrating microscopical, pathological, and clinical information. Three hundred and sixty-five samples from 300 patients treated at six medical institutions covering different geographical regions of Italy were analyzed with central review of all cases. *BRAF* V600E statistical analysis was conducted on 298 microcarcinomas from 264 patients after exclusion of those that did not meet the required criteria. *BRAF* V600E was identified in 145/298 tumors (49%) including the following subtypes: 35/37 (95%, $P < 0.0001$) tall cell and 72/114 (64%, $P < 0.0001$) classic; conversely 94/129 follicular variant papillary microcarcinomas (73%, $P < 0.0001$) were *BRAF* wild type. *BRAF* V600E-mutated microcarcinomas were characterized by markedly infiltrative contours ($P < 0.0001$) with elongated strings of neoplastic cells departing from the tumor, and by intraglandular tumor spread ($P < 0.0001$), typically within 5 mm of the tumor border. Multivariate analysis correlated *BRAF* V600E with specific microscopic features (nuclear grooves, optically clear nuclei, tall cells within the tumor, and tumor fibrosis), aggressive growth pattern (infiltrative tumor border, extension into extrathyroidal tissues, and intraglandular tumor spread), higher American Thyroid Association recurrence risk group, and non-incidental tumor discovery. The following showed the strongest link to *BRAF* V600E: tall cell subtype, many neoplastic cells with nuclear grooves or with optically clear nuclei, infiltrative growth, intraglandular tumor spread, and a tumor discovery that was non-incidental. *BRAF* V600E-mutated microcarcinomas represent a distinct biological subtype. The mutation is associated with conventional clinico-pathological features considered to be adverse prognostic factors for papillary microcarcinoma, for which it could be regarded as a surrogate marker. *BRAF* analysis may be useful to identify tumors (*BRAF* wild type) that have negligible clinical risk.
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Papillary microcarcinoma is traditionally defined as a papillary thyroid carcinoma measuring 1 cm or less. Many autopsy studies over the years have shown that small papillary carcinomas are a common finding in humans, supporting the existence of a subgroup of papillary cancers that are biologically of very low malignant potential and clinically 'dormant'. A meta-analysis reviewing ~1000 such cases from 15 autopsy series from different world regions found an overall prevalence for papillary microcarcinoma of 11.5%.[1] This prevalence is compatible with the 12–13% rate for microcarcinomas diagnosed incidentally in thyroid glands surgically removed for goiter or other reasons.[2]

The advent of preoperative fine needle aspiration biopsies and the widespread utilization of imaging techniques have allowed the identification of a progressively larger number of small papillary carcinomas.[3,4] Some of these small cancers are presumably those endowed with only a very limited malignant potential found in autopsy series, and a sensible proposal to re-name papillary thyroid microcarcinoma 'papillary microtumor' (instead of 'microcarcinoma'), has been made for cases where the neoplastic focus is single, entirely contained within the thyroid gland, and found incidentally in a patient older than 18 years.[5] Other microcarcinomas, however, are simply small tumors with the potential to progress. As they are low stage, the large majority have an excellent prognosis, but ~25% of cases are at risk of persistent/recurrent disease.[6]

Conventional staging criteria do not necessarily predict an unfavorable outcome in patients with thyroid microcarcinomas. In a large meta-analysis[7] the size of the microcarcinoma, the presence of extrathyroidal extension, or the sex of the patient were not associated with tumor recurrence. The presence of distant metastases at the time of diagnosis resulted in persistent disease in some of the patients, but was not statistically associated with the development of new metastases during follow-up. On the other hand, recurrent disease was independently associated with tumors that were clinically evident, multifocal within the gland, or with lymph node involvement at the time of diagnosis.[7] Younger, as opposed to older age, was independently associated with recurrent disease.[7] Large metastatic lymph nodes harboring foci of poorly differentiated carcinoma have been consistently associated with tumor recurrence and even patient death,[8,9] but little is generally known about the histological features of the microcarcinomas that are associated with tumor recurrence or unfavorable prognosis. Not surprisingly, the management of papillary microcarcinoma is debated and there is a need to identify those microcarcinoma patients who are exposed to potential clinical risk.

The *BRAF* V600E mutation represents a very specific marker for papillary thyroid carcinoma and a remarkable body of evidence has linked the mutation to aggressive features and loss of differentiation.[10–15] Several studies have associated *BRAF* V600E with prognostic factors generally related to aggressive behavior[16–19] and with persistent disease[20] in patients with microcarcinoma. In other studies however, the mutation did not correlate with adverse prognostic features.[21–24] Some of the discrepancies, common also to studies analyzing *BRAF* mutation in tumors larger than 1 cm, may reflect the selection of cases, mutation detection procedures, geographic variation (even within the same country),[17,21,25] or the lack of integration between mutation analysis and tumor histotype/growth features.[26]

The studies that have analyzed the *BRAF* V600E mutation in thyroid microcarcinomas have been based on single institutions, and have usually provided very limited integration of *BRAF* mutation results with clinicopathological data and histological features. We report the findings of a systematic analysis of the microscopic characteristics, growth pattern, and clinical properties of *BRAF* V600E-mutated tumors < 1 cm in a multicenter study large enough to draw relevant conclusions on the biological and clinical significance of the *BRAF* V600E mutation in papillary thyroid microcarcinoma.

Materials and methods

Study Group and Inclusion Criteria

See Supplementary Materials and Methods. Microcarcinomas from six medical institutions covering different geographical areas of Italy (Bellaria and Maggiore Hospitals in Bologna-North; University Hospital-Università 'Sapienza' in Rome-Center; Casa Sollievo della Sofferenza Hospital in San Giovanni Rotondo-South, the city Hospitals of Matera and Catanzaro-South) were subjected to central review of histology slides, and of all pathological and clinical data. The study was approved by the ethics committee of the Università 'Sapienza' in Rome on behalf of all the centers.

Pathological and Clinical Data

Tumor subtype. Defined according to the World Health Organization (2004) criteria used for tumors > 1 cm. See Table 1 and Supplementary Materials and Methods.

Microscopic appearance of the tumor. See Supplementary Materials and Methods for the definition of the variables analyzed in Table 2.

Extent and type of tumor growth. See Supplementary Materials and Methods for the definition of the variables analyzed in Table 3. Intraglandular tumor spread was defined as the presence of neoplastic cell aggregates separated from a principal tumor mass by at least one layer of non-neoplastic thyroid. For the purpose of this study, tumor multicentricity was

Table 1 Tumor subtype and *BRAF* status

	BRAF		*Univariate analysis*
	V600E (%)	*WT (%)*	P-*value*
Papillary microcarcinomas	145/298 (49)	153/298 (51)	
Classic ($n=114$)	72/145 (50)	42/153 (27)	**< 0.0001**
Follicular variant ($n=129$)	35/145 (24)	94/153 (61)	**< 0.0001**
Tall cell ($n=37$)	35/145 (24)	2/153 (1)	**< 0.0001**
Solid trabecular ($n=8$)	1/145 (1)	7/153 (5)	*0.0674*
Other ($n=10$)	2/145 (1)	8/153 (5)	0.1047

Abbreviations: V600E, *BRAF* V600E-mutated tumors; WT, *BRAF* wild-type tumors.
In bold $P<0.05$; in italics $0.05<P<0.1$.
Data based on 298 papillary microcarcinomas.

defined as the presence of at least two separate tumor foci of similar dimensions, irrespective of the histological appearance of the tumor, or the presence of at least two separate tumor foci of different histological appearance, regardless of their diameter. Since, according to the above definition, multicentric thyroid microcarcinomas may represent synchronous independent tumors, multicentric microcarcinoma foci were analyzed separately.

Clinical data. See Supplementary Materials and Methods for the definition of the variables analyzed in Table 4. Stage was defined according to current criteria (American Joint Committee on Cancer, 2010). Patients were retrospectively assigned to low or intermediate recurrence risk groups following American Thyroid Association criteria (2009). Unfavorable tumor-related events were classified as persistent disease (documented within 1 year of the diagnosis), and recurrent disease (documented 1 year after the diagnosis).

BRAF V600E Mutation Analysis

See Supplementary Materials and Methods. Mutation analysis was performed using Allele Specific Locked Nucleic Acid PCR.[27]

Immunohistochemistry

See Supplementary Materials and Methods. Immunohistochemistry for cytokeratin 19, CD31, CD34, and Popodoplanin (D2-40 antibody) was performed according to previously published protocols.[28]

Statistical Analysis

See Supplementary Materials and Methods.

Results

To obtain a comprehensive picture of *BRAF* V600E-mutated thyroid microcarcinomas we studied 365 tumor samples from 300 patients from different Italian regions with central review and meticulous histological analysis of each case. After exclusion of those that did not meet the required criteria *BRAF* V600E statistical analysis was conducted on 298 microcarcinomas from 264 patients, 191 of them with detailed follow-up.

The original selection of cases from the six medical institutions participating in the study included 365 tumor samples from 300 patients. In 28 cases the amount of residual tumor material on the paraffin block was too scant and all were excluded from DNA analysis. Therefore, a total of 337 samples from 276 patients were tested for *BRAF* V600E. Eighteen additional samples were excluded: 6 cases due to lack of amplifiable DNA, 12 cases because the diagnosis of microcarcinoma could not be confirmed after histological review. Of these 12 cases, 3 were >1 cm after measuring the tumor diameter on the H&E slide, whereas in the remaining 9 cases the diagnosis was revised after microscopic examination of the histology sections. The revised histological diagnoses were follicular foci with nuclear clearing within an adenomatous nodule (five cases), follicular foci with nuclear clearing in Hashimoto thyroiditis (one case), follicular tumor of undetermined malignant potential (two cases), and hyalinizing trabecular tumor (one case). The *BRAF* V600E mutation was identified in one of the three cases >1 cm, and in none of the nine cases where the diagnosis was revised. The remaining 319 samples were obtained from 298 microcarcinomas, from 5 foci of intraglandular spread of neoplastic cells corresponding to 5 separate microcarcinomas and from 16 microcarcinoma lymph node metastases. In the five intraglandular tumor spread samples and the 16 lymph node metastases the results of *BRAF* V600E mutation analysis were consistent with that of the primary tumor.

Seventy-five of the 264 patients had multicentric microcarcinomas. In addition to the *BRAF* status of the largest tumor, that of additional microcarcinoma foci could be determined in a total of 34 tumors from 29 of the 75 patients with multicentric microcarcinomas. Twelve of the 34 multicentric microcarcinomas had divergent *BRAF* results in the

Table 2 Microscopic appearance of papillary microcarcinoma and *BRAF* status

Variables	*BRAF*		*Univariate analysis*	*Multivariate logistic regression analysis*	
	V600E (%)	*WT (%)*	P-*value*	*OR (95% CI)*	P-*value*
Nuclei					
Pseudoinclusions					
Absent ($n=196$)	74/144 (51)	122/152 (80)	< **0.0001**	NS	NS
Few ($n=53$)	34/144 (24)	19/152 (13)			
Many ($n=47$)	36/144 (25)	11/152 (7)			
Grooves					
Few ($n=123$)	33/144 (23)	90/152 (59)	< **0.0001**	3.09 (1.76–5.42; many/widespread nuclear grooves *vs* few)	**0.0001**
Many ($n=140$)	85/144 (59)	55/152 (36)			
Widespread ($n=33$)	26/144 (18)	7/152 (5)			
Nuclear membrane irregularities					
Few ($n=82$)	29/144 (20)	53/152 (35)	**0.0002**	NS	NS
Many ($n=126$)	59/144 (41)	67/152 (44)			
Widespread ($n=88$)	56/144 (39)	32/152 (21)			
Optically clear nuclei					
Few ($n=80$)	29/144 (20)	51/152 (34)	< **0.0001**	1.99 (1.07–3.72; widespread *vs* few/many optically clear nuclei)	**0.030**
Many ($n=133$)	56/144 (39)	77/152 (51)			
Widespread ($n=83$)	59/144 (41)	24/152 (16)			
Cytoplasm					
Cells with cytoplasmic eosinophilia					
Few ($n=132$)	38/144 (26)	94/152 (62)	< **0.0001**	NS	NS
Many ($n=64$)	36/144 (25)	28/152 (18)			
Widespread ($n=100$)	70/144 (49)	30/152 (20)			
Cells with cytoplasmic clearing					
Few ($n=232$)	121/144 (84)	111/152 (73)	**0.0118**	NS	NS
Many ($n=51$)	20/144 (14)	31/152 (20)			
Widespread ($n=13$)	3/144 (2)	10/152 (7)			
Tall cells					
Absent ($n=231$)	87/144 (60)	144/152 (95)	< **0.0001**	11.86 (2.68–52.51; >50% tall cells *vs* ≤50%)	**0.001**
1–50% of the tumor ($n=28$)	22/144 (15)	6/152 (4)			
>50% of the tumor ($n=37$)	35/144 (24)	2/152 (1)			
Tall cell % within each tumor (mean ± s.e.m.)	25.8 ± 3.1 ($n=144$)	2.1 ± 0.9 ($n=152$)	< **0.0001**		
Tumor growth patterns (mean% ± s.e.m.)					
Papillary	40.5 ± 3.0	19.1 ± 2.6	< **0.0001**	NS	NS
Follicular	49.1 ± 3.0	69.1 ± 3.0	< **0.0001**		
Solid/trabecular	10.2 ± 1.4	11.9 ± 1.8	0.4751		

Table 2 (Continued)

Variables	*BRAF*		*Univariate analysis*	*Multivariate logistic regression analysis*	
	V600E (%)	*WT (%)*	P-*value*	*OR (95% CI)*	P-*value*
Fibrosis associated with the tumor					
Absent ($n=154$)	55/144 (38)	99/152 (65)	**0.0001**	1.79 (1.04–3.10; fibrosis present *vs* absent)	**0.037**
Mild ($n=59$)	40/144 (28)	19/152 (13)			
Moderate/extensive ($n=83$)	49/144 (34)	34/152 (22)			
Mitoses					
No ($n=243$)	113/144 (96)	130/152 (99)	0.1136		
Yes ($n=53$)	31/144 (4)	22/152 (1)			
Tumor necrosis					
No ($n=289$)	138/144 (96)	151/152 (99)	**0.0471**	NS	NS
Yes ($n=7$)	6/144 (4)	1/152 (1)			
Psammoma bodies					
Within the microcarcinoma					
Absent ($n=226$)	102/144 (71)	124/152 (82)	*0.0906*		
Few ($n=42$)	27/144 (19)	15/152 (10)			
Many ($n=28$)	15/144 (10)	13/152 (8)			
In the non-neoplastic tissue surrounding the microcarcinoma					
Absent ($n=266$)	125/144 (87)	141/153 (92)	0.2749		
Few ($n=20$)	13/144 (9)	7/153 (5)			
Many ($n=11$)	6/144 (4)	5/153 (3)			
Lymphoid cell infiltration					
Within the microcarcinoma					
Absent/scarce ($n=235$)	104/144 (72)	131/152 (86)	**0.0033**	NS	NS
Moderate ($n=44$)	28/144 (19)	16/152 (11)			
Diffuse ($n=17$)	12/144 (8)	5/152 (3)			
At the interface between the microcarcinoma and the surrounding non-neoplastic tissue					
Absent/scarce ($n=157$)	54/143 (38)	103/153 (67)	< **0.0001**	1.73 (1.00–3.02; moderate or diffuse lymphoid infiltration *vs* absent/scarce)	*0.051*
Moderate ($n=83$)	50/143 (35)	33/153 (22)			
Diffuse ($n=56$)	39/143 (27)	17/153 (11)			

Abbreviations: NS, not significant ($P>0.1$); OR, odds ratio; s.e.m., standard error of mean; V600E, *BRAF* V600E-mutated tumors; WT, *BRAF* WT tumors.
In bold $P<0.05$; in italics $0.05<P<0.1$.
Data based on 298 papillary microcarcinomas.

Table 3 Extent and type of papillary microcarcinoma growth and *BRAF* status

	BRAF		*Univariate analysis*	*Multivariate logistic regression analysis*	
Variables	*V600E (%)*	*WT (%)*	P-*value*	*OR (95% CI)*	P-*value*
Tumor size (mm)[a]					
Tumor size < 5 mm ($n=147$)	56/145 (39)	91/153 (60)	< **0.0001**	1.58 (0.93–2.68; tumor size ≥ 5 mm *vs* < 5 mm)	*0.089*
Tumor size ≥ 5 mm ($n=151$)	89/145 (61)	62/153 (40)			
Mean ± s.e.m.	5.9 ± 0.2 ($n=145$)	4.5 ± 0.2 ($n=153$)	< **0.0001**		
Tumor growth pattern at the border with the surrounding non-neoplastic tissue					
Predominantly smooth/pushing ($n=123$)	41/144 (29)	82/153 (54)	< **0.0001**	2.24 (1.22–4.12; predominantly irregular/infiltrative *vs* predominantly smooth/pushing or both pushing and infiltrative)	**0.010**
Both pushing and infiltrative ($n=84$)	39/144 (27)	45/153 (29)			
Predominantly irregular/infiltrative ($n=90$)	64/144 (44)	26/153 (17)			
Invasion of extrathyroidal tissues					
Absent ($n=229$)	94/145 (65)	135/153 (88)	< **0.0001**	2.06 (1.06–4.02; present *vs* absent)	**0.034**
Minimal ($n=35$)	28/145 (19)	7/153 (5)			
Conspicuous ($n=34$)	23/145 (16)	11/153 (7)			
Vascular invasion					
Absent ($n=280$)	130/144 (90)	150/153 (98)	**0.0093**	NS	NS
Single focus ($n=13$)	11/144 (8)	2/153 (1)			
Multiple foci ($n=4$)	3/144 (2)	1/153 (1)			
Microcarcinoma and IGTS					
Absent (no IGTS foci; $n=184$)	65/143 (46)	119/153 (78)	< **0.0001**	2.40 (1.37–4.21; present *vs* absent)	**0.002**
Small (3 or less IGTS foci; $n=64$)	46/143 (32)	18/153 (12)			
Large (4 or more IGTS foci; $n=48$)	32/143 (22)	16/153 (10)			
Microcarcinoma multicentric					
No ($n=194$)	97/145 (67)	97/153 (63)	0.6090		
Yes ($n=104$)	48/145 (33)	56/153 (37)			
Number of multicentric tumor foci (mean ± s.e.m.)	1.5 ± 0.1 ($n=145$)	1.5 ± 0.1 ($n=153$)	0.9379		
Tumor capsule					
Absent ($n=194$)	109/144 (76)	110/153 (72)	0.2239		
Present without infitration ($n=25$)	8/144 (6)	17/153 (11)			
Present and infiltrated ($n=53$)	27/144 (18)	26/153 (17)			
Cystic component					
No ($n=277$)	132/144 (92)	145/153 (95)	0.4040		
Yes ($n=20$)	12/144 (8)	8/153 (5)			
Microcarcinoma developed within a larger hyperplastic nodule					
No ($n=283$)	143/144 (99)	140/153 (92)	**0.0040**	0.16 (0.20–1.23)	*0.078*
Yes ($n=14$)	1/144 (1)	13/153 (8)			
Surgical margins					
Negative ($n=273$)	127/143 (89)	146/153 (95)	*0.0684*		
Positive-focal infiltration ($n=15$)	11/143 (8)	4/153 (3)			
Positive-extensive infiltration ($n=8$)	5/143 (3)	3/153 (2)			

Abbreviations: IGTS, intraglandular tumor spread; NS, not significant ($P>0.1$); OR, odds ratio; s.e.m., standard error of mean; V600E, *BRAF* V600E-mutated tumors; WT, *BRAF* WT tumors.
[a]5 mm is the mean (and median) tumor size.
In bold $P<0.05$; in italics $0.05<P<0.1$.
Data based on 298 papillary microcarcinomas.

Table 4 Clinicopathological data and *BRAF* status

	BRAF		*Univariate analysis*	*Multivariate logistic regression analysis*	
Variables	*V600E (%)*	*WT (%)*	*P-value*	*OR (95% CI)*	*P-value*
Papillary microcarcinomas	132/264 (50)	132/264 (50)			
Tumor subtype					
Classic ($n=105$)	67/132 (51)	38/132 (29)	< **0.0001**		
Follicular variant ($n=108$)	30/132 (23)	78/132 (59)	< **0.0001**	0.42 (0.22–0.78)[a]	**0.006**[a]
Tall cell ($n=35$)	34/132 (25)	1/132 (1)	< **0.0001**	24.72 (3.20–192.00)[a]	**0.002**[a]
Solid trabecular ($n=7$)	0/132 (0)	7/132 (5)	**0.0215**	NS	NS
Other ($n=9$)	1/132 (1)	8/132 (6)	**0.0419**	NS	NS
Age (at diagnosis)[b]					
< 50 years old ($n=125$)	70/127 (55)	55/128 (43)	*0.0523*	NS	NS
> 50 years old ($n=130$)	57/127 (45)	73/128 (57)			
Mean ± s.e.m.	48.2 ± 1.2 ($n=127$)	51.4 ± 1.1 ($n=128$)	**0.0470**		
Sex					
Male ($n=85$)	44/132 (33)	41/132 (31)	0.7922		
Female ($n=179$)	88/132 (67)	91/132 (69)			
Microcarcinoma discovery					
Incidental ($n=195$)	84/131 (64)	111/129 (86)	< **0.0001**	2.85 (1.37–5.92)	**0.005**
Not incidental ($n=65$)	47/131 (36)	18/129 (14)			
LN metastases as the presenting sign (occult microcarcinoma)					
No ($n=252$)	124/131 (95)	128/129 (99)	*0.0761*	NS	NS
Yes ($n=8$)	7/131 (5)	1/129 (1)			
Stage					
1 ($n=214$)	98/129 (76)	116/132 (88)	**0.0179**	NS	NS[c]
3 ($n=38$)	25/129 (19)	13/132 (10)			
4a ($n=9$)	6/129 (5)	3/132 (2)			
LN status and anatomical distribution of metastatic spread for pN1 cases					
pN0 ($n=218$)	96/118 (81)	122/131 (93)	**0.0438**	NS	NS
pN1- limited to central compartment (level VI; $n=16$)	13/118 (11)	3/131 (2)			
pN1- limited to lateral cervical lymph nodes homolataeral to the largest tumor ($n=9$)	5/118 (4)	4/131 (3)			
pN1- limited to lateral cervical lymph nodes contralateral to the largest tumor ($n=0$)	0/118 (0)	0/131 (0)			
pN1- involvement of central compartment lymph nodes and lateral cervical lymph nodes homolateral to the largest tumor ($n=4$)	3/118 (3)	1/131 (1)			
pN1- involvement of central compartment lymph nodes and lateral cervical lymph nodes contralateral to the largest tumor ($n=0$)	0/118 (0)	0/131 (0)			
pN1- bilateral involvement of lateral cervical lymph nodes (regardless of whether lymph nodes of the central compartment were involved; $n=2$)	1/118 (1)	1/131 (1)			

Table 4 (Continued)

	BRAF		*Univariate analysis*	*Multivariate logistic regression analysis*	
Variables	*V600E (%)*	*WT (%)*	*P-value*	*OR (95% CI)*	*P-value*
ATA (2009) recurrence risk groups					
Low ($n=184$)	75/132 (57)	109/132 (83)	**< 0.0001**	2.22 (1.17–4.22)	**0.014**[d]
Intermediate ($n=80$)	57/132 (43)	23/132 (17)			
Administration of RAI					
No ($n=160$)	83/129 (64)	77/132 (58)	0.3847		
Yes ($n=101$)	46/129 (36)	55/132 (42)			
Unfavorable disease-related patient events (persistent or recurrent disease)					
No ($n=184$)	87/92 (95)	97/99 (98)	0.2095		
Yes ($n=7$)	5/92 (5)	2/99 (2)			
Non neoplastic thyroid disease co-existing with the microcarcinoma[e]					
Lymphocytic thyroiditis					
Absent or focal ($n=262$)	133/144 (92)	129/153 (84)	**0.0489**	NS	NS
Diffuse ($n=35$)	11/144 (8)	24/153 (16)			
Nodular hyperplasia					
Absent, few hyperplastic nodules ($n=171$)	94/144 (65)	77/153 (50)	**0.0128**	NS	NS
Numerous hyperplastic nodules, some > cm 1 ($n=126$)	50/144 (35)	76/153 (50)			

Abbreviations: LN, lymph node; NS, not significant ($P>0.1$); OR, odds ratio; RAI, radioactive iodine; s.e.m., standard error of mean; V600E, *BRAF* V600E-mutated tumors; WT, *BRAF* wild-type tumors.

[a]For the multivariate analysis of tumor subtype categories OR and *P*-values have been calculated using the Classic subtype as the reference category.

[b]50 is the mean (and median) age of the patients.

[c]In the multivariate analysis model for stage the following variables were included in addition to the stage variable: histologic subtypes, preoperative microcarcinoma diagnosis, coexisting thyroid pathology (lymphocytic thyroiditis and nodular hyperplasia); not considered for the model because of their inclusion in the definition of stage were the following variables: age, tumor size, extrathyroidal extension, and lymph node metastases.

[d]In the multivariate analysis model for the American Thyroid Association (2009) recurrence risk groups the following variables were included in addition to the American Thyroid Association recurrence risk variable: all the histologic subtypes but the Tall Cell category, age, preoperative microcarcinoma diagnosis, tumor size, and coexisting thyroid pathology; not considered for the model because of their inclusion in the definition of American Thyroid Association recurrence risk groups were the following variables: Tall Cell category, extrathyroidal extension, and lymph node metastases.

[e]The presence of non-neoplastic thyroid disease was evaluated histologically.

$P>0.1$. In bold $P<0.05$; in italics $0.05<P<0.1$.

Data based on 264 patients with papillary microcarcinoma; when more than one microcarcinoma was present in the same thyroid gland, the statistical analysis refers to the features of the largest tumor. The statistical analysis for non neoplastic thyroid disease (thyroiditis, nodular hyperplasia) was based on the review of 297 microcarcinomas.

different samples analyzed from the same thyroid gland, supporting the assumption that multicentric microcarcinomas, as defined in this study, represent indeed synchronous tumors that are most likely of independent origin ($P=0.043$, McNemar's test).

Overall, *BRAF* V600E was identified in 145/298 tumors (49%). The statistical relationship of *BRAF* V600E with the histologic subtypes of the 298 microcarcinomas subjected to screening for *BRAF* mutation is summarized in Table 1, with their microscopic features in Table 2, and with the characteristics of the papillary thyroid microcarcinoma growth in Table 3.

Figure 1 illustrates the relationship of *BRAF* V600E with the microcarcinoma subtype (Figure 1a), the proportion of different growth patterns within the microcarcinoma (Figure 1b), the degree of intraglandular tumor spread (Figure 1c), and the geographical origin of the microcarcinomas (Figure 1d). Although there were differences in the *BRAF* V600E mutation prevalence in microcarcinomas from patients of different Italian regions these differences were not statistically significant ($P=0.1687$, χ^2 test). Figure 2 illustrates histopathological features that were associated with *BRAF* V600E.

The statistical relationship after both univariate and multivariate analyses of *BRAF* V600E with the clinicopathological characteristics of the microcarcinoma in the 264 patients whose tumors were subjected to screening for *BRAF* mutation is summarized in Table 4. If more than one papillary thyroid microcarcinoma was present in the same thyroid gland (multicentric cases) the largest tumor was considered for statistical analysis.

Comprehensive multivariate study of all histopathological and clinical parameters with $P<0.1$ after multivariate analyses in Tables 1–4, revealed the following features to be independently associated with *BRAF* V600E: tall cell subtype ($P=0.004$), many neoplastic cells ($>20\%$) with nuclear grooves ($P=0.0001$) or with optically clear nuclei ($>60\%$; $P=0.015$), infiltrative tumor borders ($P=0.034$), intraglandular tumor spread ($P=0.032$), and a non-incidental discovery of the tumor ($P=0.010$; Table 5).

Table 6 summarizes tumor features, *BRAF* status and clinical data of the seven patients with unfavorable tumor-related events (persistent disease at 1year or recurrent disease). The *BRAF* status of tumors in patients with follow-up information is summarized in Table 7. Figure 3 shows the Kaplan–Meier curve of unfavorable tumor-related events among patients with a *BRAF* V600E mutated or with a *BRAF* wild-type microcarcinoma.

Papillary Microcarcinoma Subtype and *BRAF* V600E

The tall cell subtype showed the strongest link with *BRAF* V600E (Table 1; Figures 1a and 2a), and the association was independent of all other pathological and clinical variables tested (Tables 4 and 5). The classic subtype was also strongly associated with *BRAF* V600E (Figure 1a). The follicular variant subtype was inversely associated with *BRAF* V600E (Figure 1a).

Microscopic Papillary Microcarcinoma Features and *BRAF* V600E

The microscopic appearance of *BRAF* V600E mutated cases was distinctive (Table 2, Figure 1b, Figures 2a to 2k).

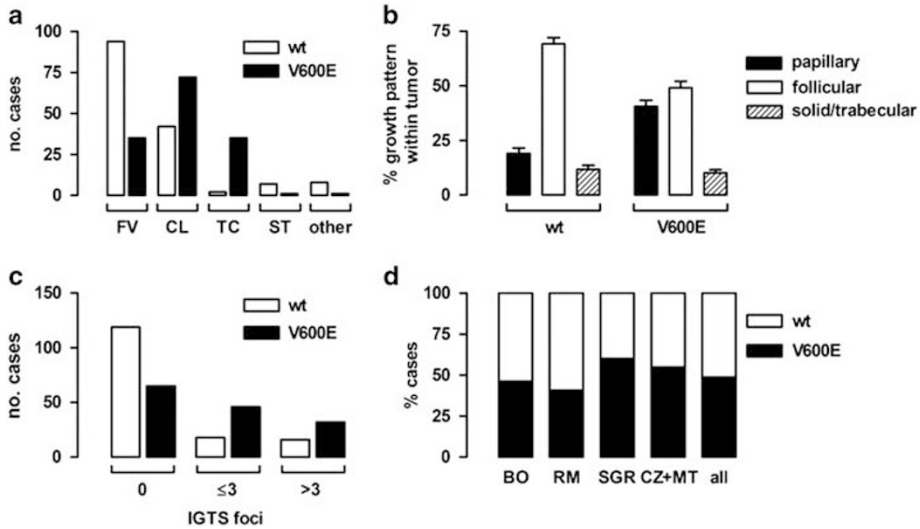

Figure 1 (**a**) Thyroid microcarcinoma subtype and *BRAF* status ($n=298$): CL, classic; FV, follicular variant; ST, solid trabecular; TC, tall cell. (**b**) Proportion of different growth patterns within the microcarcinoma and *BRAF* status ($n=298$). (**c**) Intraglandular tumor spread (IGTS) and *BRAF* status ($n=298$). (**d**) *BRAF* status of the microcarcinomas originating from different Italian regions ($n=298$): BO, Bologna (northern Italy); CZ+MT, Catanzaro and Matera (southern Italy); RM, Rome (central Italy); SGR, San Giovanni Rotondo (southern Italy).

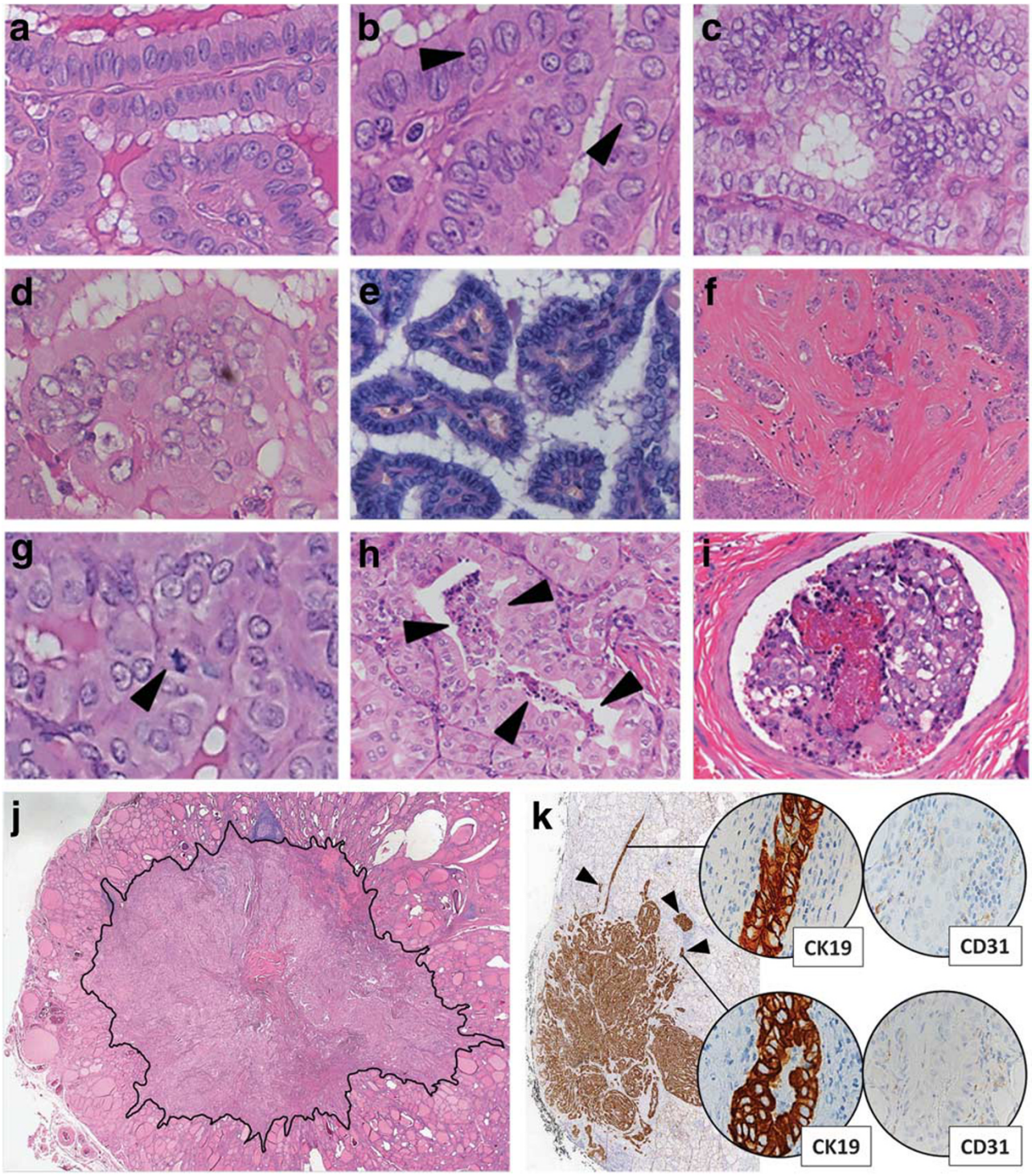

Figure 2 Histopathological features of microcarcinomas associated with the *BRAF* V600E mutation (Tables 2 and 3): (**a**) tall cells (×400). (**b**) Nuclear grooves and intranuclear inclusions (arrowheads; × 400). (**c**) Optically clear nuclei (×400). (**d**) Eosinophilic cytoplasm (×400). (**e**) Papillary growth (×100). (**f**) Stromal fibrosis with desmoplasia (×40). (**g**) Mitoses (arrowhead; ×600). (**h**) Necrotic microfoci (arrowheads; × 200). (**i**) Vascular invasion (×200). (**j**) Infiltrative growth pattern featuring a markedly irregular interface with non-neoplastic thyroid (×20). (**k**) Intraglandular peritumoral spread (arrowheads) and elongated digitiform projections of neoplastic cells into non-neoplastic thyroid (×20); immunohistochemical stain with cytokeratin 19 (CK19) highlights neoplastic cells (×400); immunohistochemistry for the endothelial cell marker CD31 (CD31) does not demonstrate positive immunoreactivity around neoplastic cells (×400), indicating that they are not growing into blood or lymphatic vascular channels.

Table 5 Histopathological and clinical parameters associated to *BRAF* V600E ($P < 0.1$) after multivariate analyses in Tables 1–4

	Multivariate logistic regression analysis	
Variables	*OR (95% CI)*	P-*value*
Tumor subtype (Table 1)		
Tall cell (*vs* other subtypes)	20.89 (2.66–163.66)	**0.004**
Microscopic appearance of the tumor (Table 2)		
Nuclear grooves	4.18 (2.17–8.04; many/widespread *vs* few)	**0.0001**
Optically clear nuclei	2.41 (1.18–4.91; widespread *vs* few/many)	**0.015**
Extent and type of tumor growth (Table 3)		
Tumor growth pattern at the border with the surrounding non-neoplastic tissue	2.19 (1.06–4.54; predominantly irregular/infiltrative *vs* smooth or partially infiltrative)	**0.034**
Intraglandular tumor spread	2.11 (1.07–4.18; present *vs* absent)	**0.032**
Clinicopathological data (Table 4)		
Microcarcinoma discovery	2.68 (1.27–5.68; incidental *vs* not incidental)	**0.010**

Data based on 264 patients. Variables that were significantly correlated to each other after the Pearson test were excluded from the multivariate model.

Mutated tumors had a high proportion of tall cells, irrespective of the microcarcinoma subtype. The average proportion of tall cells (Figure 2a) in *BRAF* V600E mutated cases was 26% *versus* 2% in *BRAF* wild-type cases (Table 2, $P < 0.0001$).

Other variables statistically associated with *BRAF* V600E by univariate analysis were: nuclear features linked to classic papillary carcinoma morphology (chromatin clearing, nuclear membrane irregularities, grooves, and nuclear pseudoinclusions; Figures 2a to 2c); eosinophilic cytoplasm (Figure 2d), whereas cytoplasmic clearing was inversely related to the BRAF mutation; papillary pattern of growth (the average proportion of papillary growth in *BRAF* V600E-mutated microcarcinomas was 41% *versus* 19% in *BRAF* wild-type cases, $P < 0.0001$, Table 2, Figures 1b and 2e), whereas follicular growth was inversely related to the *BRAF* mutation; tumor fibrosis with stromal desmoplasia (positive association; Figure 2f); lymphoid cell infiltration within the tumor and at the interface between the microcarcinoma and the surrounding non-neoplastic parenchyma (positive association; Table 2). Mitotic activity was present in 53 cases and 31 of them were *BRAF* V600E mutated (Table 2, $P = 0.1136$; Figure 2g). Microscopic foci of necrosis were identified in seven microcarcinomas and six of the cases with necrosis were *BRAF* V600E mutated (Table 2, $P = 0.0471$; Figure 2h).

Multivariate analysis showed that among the microscopic appearance variables considered in this study (Table 2) the presence within the microcarcinoma of many cells with nuclear grooves (>20%) or optically clear nuclei (>60%), the presence of >50% tall cells, and of tumor-associated fibrosis were independently associated with *BRAF* V600E. The independence from other microscopic variables of lymphoid cell infiltration at the interface between the microcarcinoma and the surrounding non-neoplastic parenchyma was statistically borderline (Table 2, $P = 0.051$).

Extent and Type of Tumor Growth and *BRAF* V600E

BRAF V600E-mutated microcarcinomas were larger (5.9 *versus* 4.5 mm, $P < 0.0001$, Table 3) and were characterized by invasive features. Histological signs of blood vessel invasion were observed in 17 microcarcinomas and 14 of them were *BRAF* V600E mutated (Table 3, $P = 0.0093$; Figure 2i). *BRAF* V600E-mutated microcarcinomas had a predominantly infiltrative (as opposed to smooth and pushing) tumor border (Table 3, $P < 0.0001$; Figure 2j). Typically, the interface between the microcarcinoma and the surrounding parenchyma was markedly irregular (Figure 2j), showing in some cases thin strings and elongated digitiform projections of neoplastic cells departing from the tumor and growing deeply into the surrounding tissue (Figure 2k). *BRAF* V600E-mutated microcarcinomas commonly infiltrated extrathyroidal tissue (Table 3, $P < 0.0001$).

Mutated tumors were associated with intraglandular tumor spread (Table 3, $P < 0.0001$; Figure 2k). Most of the foci of intraglandular neoplastic cell spread were microscopic and located within a 5 mm radius of the outer border of the microcarcinoma (in 92 of 112 microcarcinomas with intraglandular tumor spread, 82%, Figure 2k). Immunohistochemical analysis of 14 *BRAF* V600E-mutated microcarcinomas with intraglandular tumor spread for the papillary carcinoma marker cytokeratin 19, endothelial markers (CD31 and CD34) and lymphatic endothelial cells (Podoplanin, D2-40 antibody) did not demonstrate positive immunoreactivity around the foci of intraglandular neoplastic cell dissemination, suggesting that they are not the result of permeation of blood vessels or lymphatic vascular channels (Figure 2k).

Table 6 Clinicopathological features and *BRAF* status in the seven patients with unfavorable tumor-related events

Features	*Case 1*	*Case 2*	*Case 3*	*Case 4*	*Case 5*	*Case 6*	*Case 7*
Age	59	40	46	16	29	65	48
Sex	F	M	F	F	F	M	F
Microcarcinoma discovered incidentally	Yes	Yes	Yes	No (LN metastases at presentation)	No (LN metastases at presentation)	No (LN metastases at presentation)	No (LN metastases at presentation)
Microcarcinoma subtype	TC	ST	CL	CL	CL	TC	TC
Tumor size (mm)	3.6	8.5	10.0	10.0	7.3	8.0	9.0
Intraglandular tumor spread	Yes (≤3 foci)	Yes (≤3 foci)	Yes (≤3 foci)	Yes (>3 foci)	Yes (>3 foci)	Yes (>3 foci)	Yes (>3 foci)
Multicentricity	No	Yes	No	No	No	No	No
Extrathyroidal extension	No	No	Yes (C)	Yes (C)	Yes (M)	Yes (C)	Yes (C)
Stage at diagnosis (pTNM)	1 (pT1a N0 Mx)	1 (pT1am Nx Mx)	3 (pT3 Nx Mx)	1 (pT3 N1b Mx)	1 (pT3 N1b Mx)	4a (pT3 N1b Mx)	4a (pT3 N1b Mx)
Surgery (type)	TT	TT	TT	TT; central, lateral LND	TT; central, lateral LND	TT; central, lateral LND	TT; central, lateral LND
Surgical margin	Negative	Negative	Positive	Negative	Positive	Negative	Negative
American Thyroid Association recurrence risk group	0	0	1	1	1	1	1
1st year FU visit	BIR	SIR	SIR	SIR	SIR	SIR	ER
Persistent disease site	NA	Lateral neck	Lateral neck	Lung	Lateral neck	Mediastinum	NA
Recurrent disease	Yes	UNK	NA	NA	NA	NA	Yes
Site of recurrence	Mediastinum	UNK	NA	NA	NA	NA	Lateral neck
Recurrence time (months from initial diagnosis)	57	UNK	NA	NA	NA	NA	16
Additional therapy	RAI	UNK	Surgery, RAI	RAI	No	Surgery, RAI	No
Status of last FU	ER	SIR	ER	ER	SIR	ER	SIR
FU total (months)	156	12	68	90	95	44	19
BRAF status	V600E	WT	V600E	WT	V600E	V600E	V600E

Abbreviations: BIR, biochemical incomplete response (abnormal thyroglobulin values); C, conspicuous extrathyroidal extension: >1 submillimetric focus of tumor or a single focus ≥1 millimeter in the extrathyroidal tissues; CL, classic microcarcinoma; ER, excellent response (no biochemical, structural or clinical evidence of disease); FU, follow up; LN, lymph node; LND, lymph node dissection; M, minimal extrathyroidal extension: one submillimetric focus of tumor in the extrathyroidal tissues; NA, not applicable; RAI, radioactive iodine ablation; SIR, structural incomplete response (positive imaging studies); ST, solid/trabecular microcarcinoma; TC, tall cell microcarcinoma; TT, total thyroidectomy; UNK, unknown; V600E, *BRAF* V600E-mutated tumor; WT, *BRAF* wild-type tumor.
Data from 191 patients with follow-up information.

Table 7 Disease-related events and *BRAF* status

	BRAF		*Univariate analysis*
Follow-up information	*V600E (%)*	*WT (%)*	P-*value*
Persistent Disease			
No ($n=186$)	89/92 (97)	97/99 (98)	0.5916
Yes ($n=5$)	3/92 (3)	2/99 (2)	
Recurrent Disease			
No ($n=188$)	90/92 (98)	98/98 (100)	0.1423
Yes ($n=2$)	2/92 (2)	0/98 (0)	
Disease at last follow-up			
No ($n=188$)	90/92 (98)	98/99 (99)	0.5180
Yes ($n=3$)	2/92 (2)	1/99 (1)	

Abbreviations: V600E, *BRAF* V600E-mutated tumors; WT, *BRAF* wild-type tumors. Data from 191 patients with follow-up information.

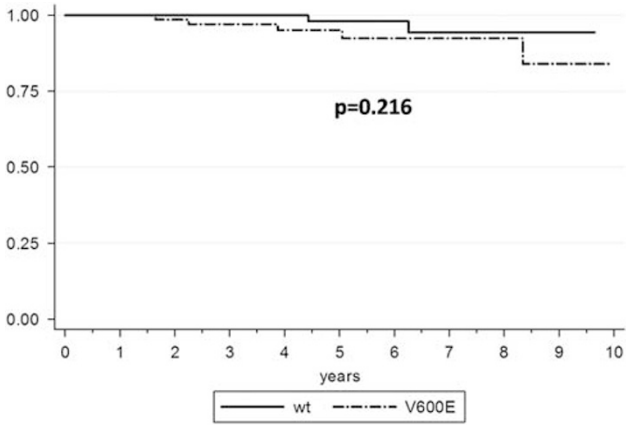

Figure 3 Kaplan-Meier curve of patients with unfavorable tumor-related events (persistent disease at 1 year, recurrent disease, alive with disease at last follow-up; $n=7$, 5 *BRAF* V600E mutated tumors, 2 *BRAF* wild type tumors). Data from 191 patients with follow-up information.

Although *BRAF* V600E was linked to mutifocality resulting from intraglandular tumor spread, in this series *BRAF* V600E did not correlate with tumor multicentricity (Table 3, $P=0.6090$). Microcarcinomas that developed within a larger hyperplastic nodule were infrequently *BRAF* V600E mutated (Table 3, $P=0.004$).

Among the variables considered in Table 3, multivariate analysis showed that intraglandular tumor spread, infiltrative tumor borders and extension into extrathyroidal tissues were independently associated with the *BRAF* V600E mutation. The independence from the other Table 3 variables of the lack of *BRAF* V600E mutation for papillary microcarcinomas that developed within a hyperplastic nodule was statistically borderline (Table 3, $P=0.078$).

Clinicopathological Data, Follow-up Status and *BRAF* V600E

Univariate analysis showed that patients with *BRAF* V600E-mutated tumors were younger than those with tumors lacking the mutation (48 *versus* 51 years of age, Table 4, $P=0.0470$). Microcarcinomas with the *BRAF* V600E mutation were less frequently diagnosed as an incidental finding (Table 4, $P<0.0001$), were more commonly of higher stage (Table 4, $P=0.0179$), more often had metastases to cervical nodes (Table 4, $P=0.0333$), and these lymph node metastases tended to more frequently involve both central compartment as well as lateral cervical nodes (Table 4, $P=0.0438$). Patients with *BRAF* V600E-mutated tumors more commonly belonged to an intermediate as opposed to a low American Thyroid Association recurrence risk group (Table 4, $P<0.0001$). Not only *BRAF* V600E-mutated microcarcinomas rarely developed within hyperplastic nodules (see above, Extent and Type of Tumor Growth and *BRAF* V600E), they were also less common in glands affected by multinodular hyperplasia (Table 4, $P=0.0128$). We observed an inverse relationship between *BRAF* V600E-mutated microcarcinomas and the occurrence of diffuse lymphocytic thyroiditis (Table 4, $P=0.0489$).

Among the variables considered in Table 4, multivariate analysis showed that non-incidental tumor discovery and intermediate (as opposed to low) American Thyroid Association recurrence risk group were independently associated with the *BRAF* V600E mutation.

One hundred and ninety-one patients had adequate follow-up information. No patient died of or with disease. Seven experienced unfavorable events related to the microcarcinoma at follow-up: five had persistent disease and two recurrent disease (Tables 6 and 7). Five of the seven patients with unfavorable tumor-related events had *BRAF* V600E-mutated microcarcinomas (Table 4, $P=0.2095$; Figure 3). Of the five patients with persistent disease three carried *BRAF* V600E-mutated tumors (3% of the 92 patients with *BRAF* V600E-mutated microcarcinomas; Table 7). Both patients with recurrent disease carried *BRAF* V600E-mutated tumors (2% of the 92 patients with *BRAF* V600E-mutated microcarcinomas; Table 7).

Discussion

A major finding of the study is the close correlation between *BRAF* V600E and specific microscopic features of the tumor. The strongest association is with microcarcinomas subtyped as tall cell variant, confirming that the link noted for larger tumors is also true for microcarcinomas.[10,29] In our series nearly 95% of microcarcinomas subtyped as tall cell are *BRAF* V600E mutated, and the correlation is independent of all other parameters tested after multivariate analysis. Interestingly, *BRAF* V600E is

also associated with specific microscopic neoplastic cell features, irrespective of microcarcinoma subtype, to the point that after the study it became easy for the pathologists who reviewed the cases to correctly guess the presence of the mutation in a given microcarcinoma. Being 'miniature' lesions, microcarcinomas sometimes display alterations of nuclear morphology that are less developed when compared with those found in larger tumors and tend to have a relative predominance of follicular growth patterns.[30] In our series, *BRAF* V600E positive microcarcinomas show full development of alterations of nuclear morphology that typically define papillary carcinoma (eg chromatin clearing, nuclear grooves etc), and have significantly higher proportions of papillary growth, compared with *BRAF* wild-type cases. We measured the proportion of tall cells within each microcarcinoma and found it to be >10 times higher in *BRAF* V600E mutated cases than in *BRAF* wild-type tumors (26% *versus* 2%). Remarkably, this proportion of tall cells is close to the 30% tall cell cut-off that has been associated with the prognostic features of tall cell variant papillary thyroid carcinoma in larger tumors.[31]

The strong similarity of microscopic features between *BRAF* V600E-mutated microcarcinomas and *BRAF* V600E-mutated carcinomas >1 cm supports the hypothesis that they belong biologically to the same class, and that the mutation induces the same set of morphologic alterations, independent of tumor size. A corollary to this hypothesis is that *BRAF* V600E-mutated microcarcinomas represent indeed low stage lesions, with the potential to evolve into larger clinically evident tumors. In our series we find mitotic activity in a small minority of microcarcinomas, and in rare cases microscopic foci of necrosis, both distinctive features of high-grade neoplasms that are usually large and poorly differentiated. Both mitotic activity and necrosis are more common in *BRAF* V600E-mutated tumors, further supporting the contention that this mutation confers to the tumor a potential to progress.

A second set of important findings is the correlation between *BRAF* V600E and aggressive growth features. In our series, mutated cases are statistically associated with larger tumor size. Such a correlation has been demonstrated in a few other microcarcinoma studies.[17,32] The most striking observation is that *BRAF* V600E-mutated microcarcinomas have distinctively invasive features. Their growth is almost 'weed-like', with a very infiltrative tumor border, sometimes characterized by thin strings and digitiform projections of neoplastic cells penetrating into the parenchyma surrounding the tumor. *BRAF* V600E-mutated microcarcinomas also show a strong tendency to spread. We defined intraglandular tumor spread as satellite neoplastic cell aggregates separated from the microcarcinoma by non-neoplastic thyroid and showing its same morphologic appearance. The large majority of these aggregates are of microscopic size and located within a 5 mm radius of the tumor. By immunohistochemical analysis of intraglandular tumor spread foci using vascular and lymphatic endothelial cell markers we find no evidence that they represent invasion of vascular spaces. It is likely that at least some of these microscopic peritumoral neoplastic foci are cross sections of the digitiform projections that emanate from the tumor. Although *BRAF* V600E mutation is linked to mutifocality resulting from tumor dissemination (intraglandular tumor spread), we do not observe a correlation with tumor multicentricity. Lack of correlation between *BRAF* V600E and microcarcinoma multicentricity has been reported in other studies,[16,19,32] supporting the hypothesis that a significant proportion of multicentric microcarcinomas likely represent synchronous lesions of independent origin.[33–35] Blood vessel invasion is very uncommon in microcarcinomas and has not been previously associated with *BRAF* V600E. Other studies have indicated that *BRAF* V600E-mutated microcarcinomas often extend into extrathyroidal tissue.[16,17,19] A few have also shown that they tend to have infiltrative borders,[19,36] and to spread within the gland.[36] In our series infiltrative growth and intraglandular tumor spread show a strong and independent link to *BRAF* V600E, and emerge among the most distinctive features associated with the mutation.

We find a statistical association between *BRAF* V600E and lymph node metastases at presentation, confirming what has been reported in other series from single institutions.[16,32] In addition we demonstrate that *BRAF* V600E is also statistically linked to more extensive nodal disease, that in *BRAF*-mutated microcarcinomas tends to involve not only central compartment but also lateral cervical lymph nodes.[19]

In our series the mean age of the patients with *BRAF* V600E-mutated tumors is statistically lower compared with that of patients whose tumors are wild type. *BRAF* V600E has been linked to older age in tumors >1 cm,[10] but in some microcarcinoma series the same correlation between young age and *BRAF* V600E has emerged.[16] At variance with what happens for papillary carcinomas >1 cm, young, as opposed to older age is associated with tumor recurrence,[7] and one observational study from Japan has identified young age as an independent factor predicting microcarcinoma progression.[37]

In this study the large majority of microcarcinomas discovered incidentally lack *BRAF* V600E. We are aware of only one study[18] that has specifically addressed this issue, reporting a similar inverse relationship between the incidental discovery of the tumor and the mutation. We not only confirm the observation, but also demonstrate that the link between incidental microcarcinoma discovery and a lack of *BRAF* V600E is very firm and independent of all other factors tested after multivariate analysis. Latent papillary microcarcinomas discovered incidentally likely include that subset of tumors of very

low malignant potential found in autopsy series.[1] A large meta-analysis of thyroid microcarcinomas has shown that patients with incidentally discovered tumors experience fewer recurrences inspite of receiving more limited treatment, independently of other clinicopathological factors, suggesting that non-incidental thyroid microcarcinomas are biologically different, with more aggressive, therapy-resistant features.[38]

We show that *BRAF* V600E-mutated microcarcinomas are statistically associated with a higher risk of recurrence according to the 2009 American Thyroid Association criteria, and that the mutation correlates with those variables (multifocality consistent with intraglandular tumor dissemination, lymph node metastases, younger age of the patient, and a non-incidental discovery of the tumor) specifically linked to tumor recurrence in microcarcinoma.[7] Few patients in this series had recurrent or persistent disease, but the majority of them harbored *BRAF* V600E positive tumors. The proportion of our patients with *BRAF* V600E-mutated papillary microcarcinomas that experienced disease persistence (3%) or recurrence (2%) is in the same range of the rate for persistent or recurrent disease (7% and 1%, respectively) recently reported in a large series of clinically evident papillary carcinomas.[39] Furthermore, a meta-analysis has recently shown that the mutation is associated with papillary carcinoma recurrence even in low stage tumors and microcarcinomas.[12]

Remarkably, the correlation between *BRAF* V600E and unfavorable microcarcinoma characteristics is confirmed after excluding from the statistical analysis cases subtyped as follicular variant, a subtype that is generally regarded as particularly indolent among papillary carcinomas >1 cm: tall cell subtype, tumor size >5 mm, infiltrative growth, extrathyroidal extension, higher risk of recurrence (American Thyroid Association, 2009), and a non-incidental discovery of the microcarcinoma were still strongly linked to *BRAF* V600E ($P<0.05$; data not shown).

Observational studies carried out in Japan, where selected patients with microcarcinomas are managed conservatively and followed rather than treated up front,[40,41] are showing that only ~15% of microcarcinomas increase in size after an average follow-up of 10 years. New lymph node metastases were found in 3% of 340 patients after 10 years of observation.[40] If we assume that the prevalence of *BRAF* V600E in microcarcinoma in Japan is ~30%[22] one would have expected a larger proportion of tumors showing signs of progression. The discrepancy between the findings of this and other series showing that *BRAF* V600E-mutated microcarcinomas are endowed with worrisome features and the results of these observational studies is puzzling. It may be explained if we consider *BRAF* V600E as a 'sensitive', but by no means 'specific' marker to identify those microcarcinomas that, if untreated, will eventually evolve. Additional molecular alterations must be involved in this progression. This is supported by recent data emerging from the work of The Cancer Genome Atlas project, indicating that *BRAF* V600E-mutated papillary carcinoma is indeed a rather heterogeneous group of tumors with several molecular subtypes.[42]

To the best of our knowledge this is the first multicenter study to address in detail the microscopic, pathologic, and clinical aspects of *BRAF* V600E-mutated microcarcinomas. On the whole, our data confirm that thyroid microcarcinomas are heterogeneous and that it is inaccurate and misleading to consider them as a single category harboring the same hazard for the patient. *BRAF* V600E is consistently associated with those conventional clinico-pathological features known to be adverse prognostic factors for microcarcinomas, and may therefore be rightfully considered a surrogate marker for increased clinical risk. A major contribution of *BRAF* testing to risk stratification would be to identify those tumors (*BRAF* wild type) that can be regarded as indolent, with a very low chance of recurrence, to be managed conservatively. As the large majority of patients with mutated tumors do not experience significant disease-related events additional factors that promote progression of *BRAF* V600E-mutated microcarcinomas should be investigated.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol).