CLINICAL STUDY

***BRAF* in primary and recurrent papillary thyroid cancers: the relationship with ^{131}I and 2- ^{18}F]fluoro-2-deoxy-D-glucose uptake ability**

Susi Barollo¹, Gianmaria Pennelli², Federica Vianello^{1,3}, Sara Watutantrige Fernando⁴, Isabella Negro⁴, Isabella Merante Boschini⁵, Maria Rosa Pelizzo⁵, Massimo Rugge², Franco Mantero⁴, Davide Nacamulli⁴, Maria Elisa Girelli⁴, Benedetto Busnardo⁴ and Caterina Mian⁴

¹Istituto Oncologico Veneto – IRCCS, 35128 Padova, Italy, ²II Pathology Unit, Department of Medical and Diagnostic Sciences, 35121 Padova, Italy, ³Radiotherapy Department, Istituto Oncologico Veneto – IRCCS, 35128 Padova, Italy, ⁴Endocrinology Unit and ⁵Special Surgery Unit, Department of Medical and Surgical Sciences, University of Padua, Via Ospedale n.105, 35128 Padova, Italy

(Correspondence should be addressed to C Mian; Email: caterina.mian@unipd.it)

Abstract

Objective: *BRAF* V600E is a potential marker of poor prognosis in papillary thyroid cancers (PTC). In a previous report, we showed that recurrent PTC with no radioiodine (^{131}I) uptake are frequently associated with *BRAF* mutations, a low expression of thyroid-related genes and a high expression of glucose type-1 transporter gene.

Aim: The aim of the present study was to assess *BRAF* status in a large series of recurrent PTC patients, considering paired primary and recurrent cancers. The *BRAF* genotype was correlated with the ability to concentrate ^{131}I and/or 2- ^{18}F]fluoro-2-deoxy-D-glucose (^{18}F -FDG) in the recurrent cancers, serum markers of recurrence, and patient outcome.

Design and methods: We studied 50 PTC patients with recurrent cervical disease submitted to a re-intervention, followed up in median for 9 years. *BRAF* analysis was conducted by direct sequencing and mutant allele-specific PCR amplification. In 18 cases, molecular analysis was also assessed in the primary cancer. Out of 50 patients, 30 underwent ^{18}F -FDG-positron emission tomography–computed tomography.

Results: *BRAF* V600E-positive recurrent patients were found ^{131}I -negative in 94% of cases ($P < 0.001$); 73% of the cancers carrying *BRAF* V600E were both ^{131}I -negative and ^{18}F -FDG positive. In paired primary and recurrent PTC, *BRAF* V600E was observed in 79% of the primary cancers and 84% of their recurrences. Three patients with ^{131}I -negative and *BRAF* V600E-positive recurrent cancers deceased during follow-up.

Conclusions: *BRAF* mutations are more common in thyroid recurrences with no ^{131}I uptake than in ^{131}I -positive cases. They are correlated with the ability to concentrate ^{18}F -FDG, and they can appear, albeit rarely, as a *de novo* event in the course of PTC recurrences.

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Introduction

Well-differentiated papillary thyroid cancers (PTC) typically have a favorable prognosis, particularly in low-risk patients, 80% of whom are cured by primary surgery followed by radioiodine (^{131}I) ablation (1). Approximately, 15–20% of patients experience recurrences; however, two in three of which occur in the first decade. One in three of these recurrences loses the ability to trap ^{131}I , thus making the most important tool available for treating the tumor ineffectual (2, 3). When the loss of ^{131}I uptake tends to coincide with the ability to actively concentrate glucose (the so-called ‘flip-flop’ phenomenon), the patient’s prognosis becomes particularly unfavorable, associated with an early poor

outcome (4–6). Conventional approaches are of marginal benefit in such cases, hence the need to develop novel medical strategies. In this scenario, mapping the particular genetic alterations in recurrent PTC enables the use of novel ‘targeted’ molecular therapies (7, 8).

Since it was first described in thyroid cancer, the *BRAF* V600E mutation has proved to be the most common genetic event in the onset of PTC, responsible for around 45% of cases. Several studies have also shown that primary PTC harboring *BRAF* mutations are more aggressive and more prone to recur and lose the capacity for iodine uptake (9, 10).

The molecular profile of paired primary and recurrent PTC and its relationship to ^{131}I and

2-¹⁸F]fluoro-2-deoxy-D-glucose (¹⁸F-FDG) uptake have been little characterized in the literature. In a previous report on a small series of recurrent PTC, we found a high percentage of *BRAF* mutations in ¹³¹I-negative recurrences, and this genetic event has a key role in silencing the expression of the whole iodine metabolism enzyme chain and in increasing the expression of glucose transporter gene (11).

In this mono-institutional study, we analyzed *BRAF* status in a relatively large series of recurrent PTC patients followed up at our Operative Units, comparing samples of paired primary and recurrent PTC from the same patient, when available. *BRAF* status was correlated with the classical markers of cancer recurrence and the ability to trap ¹³¹I and/or ¹⁸F-FDG.

Materials and methods

From 2006 to 2009, we collected a series of consecutive recurrent cancers surgically removed from 50 patients (16 males and 34 females) with well-differentiated recurrent PTC, followed up at our operative units; they all came from north-eastern Italy, a borderline iodine-sufficient area according to WHO criteria (12). At diagnosis, all the patients underwent total thyroidectomy, 16 underwent central, 26 underwent central and lateral, and 1 underwent central and bilateral compartment lymph nodes dissection. ¹³¹I ablation was performed in all the patients after surgery.

Tumors were staged according to the 6th TNM classification (13). Pathological diagnosis showed a well-differentiated papillary thyroid carcinoma in all but one case that was a tall cell variant.

After primary treatment, serum thyroglobulin (Tg) under hormonal suppressive therapy, anti-Tg autoantibodies (anti-Tg abs), and neck ultrasonography (US) were evaluated every 6 months. At the first follow-up, ten patients had a suspect of persistence. When the recurrent disease was proved by US-FNAB and/or neck positive ¹³¹I whole body scan (WBS) and/or positive ¹⁸F-FDG-positron emission tomography (PET)–computed tomography (CT), the patients underwent re-intervention.

At the time of recurrence, WBS was performed after a therapeutic dose of ¹³¹I (range 3.7–7.4 GBq), following thyroid hormone withdrawal in all the patients. Thirty patients also underwent ¹⁸F-FDG-PET–CT. In cases who were found positive at ¹⁸F-FDG-PET–CT, the surgeon was careful to isolate the cervical lesion observed in the scan. The latest stimulated Tg serum level measured prior to surgery was retained for statistical analysis in all but eight patients, who had persistent anti-Tg abs.

For 18 patients, paired primary and recurrent PTC were analyzed simultaneously. In three cases, we studied different thyroid recurrences from the same patient.

DNA extraction and BRAF status detection

After examining the section stained with hematoxylin and eosin, cancer tissue specimens containing >60–70% tumor cells were chosen for molecular analysis. In cases of multifocal primary PTC, different microdissected cancer specimens were considered. In all, we analyzed 71 thyroid cancer tissues, i.e. 46 neck lymph node recurrences, 6 neck soft tissue recurrences, 1 distant metastasis (skin), and 18 primary PTC. Genomic DNA was extracted from 5-μm sections of archived paraffin-embedded tissues using the DNeasy blood and tissues kit (Qiagen) according to the manufacturer’s protocol. The *BRAF* status of exon 15 was assessed by both direct sequencing and mutant allele-specific PCR amplification for the T to A substitution at nucleotide 1799 (V600E), using the procedure described elsewhere (14).

Statistical analysis

The Kaplan–Meier method and standard log-rank test were used to evaluate the effect of iodine uptake in recurrences on the probability of disease detection, and of the *BRAF* status on patient survival. The *t*-test was used to assess the relationship between *BRAF* status and Tg serum level, after logarithmic transformation. The χ^2 -test was used to analyze the relationship between *BRAF* status and iodine uptake in recurrences. A *P* < 0.05 was considered statistically significant.

Table 1 Demographic and histological characteristics of patients according to *BRAF* status at recurrence level.

Clinical features	<i>BRAF</i> V600E (<i>n</i> = 33)	<i>BRAF</i> wild type (<i>n</i> = 17)
Gender	F 23 (70%) M 10 (30%)	F 11 (65%) M 6 (35%)
Age	Median 51 years (17–73) Mean 46 years ± 17	Median 35 years (16–74) Mean 37 years ± 15
T1	11 (33%)	6 (35%)
T2	5 (15%)	1 (6%)
T3	13 (40%)	7 (41%)
T4	4 (12%)	3 (18%)
N0	2 (6%)	1 (6%)
N1	25 (76%)	12 (70%)
Nx	6 (18%)	4 (24%)
Stage		
I	15 (46%)	12 (70%)
II	0	1 (6%)
III	9 (27%)	0
IV a	7 (21%)	1 (6%)
b	0	0
c	0	1 (6%)
NA	2 (6%)	2 (12%)
Multifocality	14 (42%)	6 (35%)
Site of recurrence		
Thyroid bed	11 (33%)	2 (12%)
Neck lymph nodes	22 (67%)	15 (88%)
Distant metastasis	5 (15%)	3 (18%)

NA, not available.

Results

Patients and BRAF mutation status

Patients' clinical characteristics at diagnosis and *BRAF* status at recurrence level were shown in Table 1. Patients were followed up for a mean 9 ± 6 years (median 7, min 1, max 28 years).

In our series, 66% of patients (33/50) harbored a *BRAF V600E* mutation in their recurrent cancer.

Thirty-nine patients had ^{131}I -negative recurrences, and 11 had ^{131}I -positive recurrences. Calculating the time elapsing between the end of initial treatment and the first surgery for recurrence, we found the interval to be lower in ^{131}I -positive cases than in ^{131}I -negative cases: the median interval before re-intervention was 1 year in the former group, and 5 years in the latter group. In statistical terms, the likelihood of detecting the recurrence was higher in ^{131}I -positive cases than in ^{131}I -negative cases ($P=0.01$), who were followed up for longer before the site of recurrent disease could be identified (Fig. 1).

On average, *BRAF*-mutated patients had lower stimulated Tg serum levels than those carrying a wild-type *BRAF* (19 ng/ml, 95% confidence interval (CI) 7–54 vs 49 ng/ml, 95% CI 9–250), but the difference was not statistically significant.

Three patients died due to disease progression: one female 68 years of age with PTC (T2N1M0) recurred 3 years later in thyroid bed and lung, and died 2 years later from lung disease; one female 73 years of age with PTC (T2NxM0) recurred 6 years later in thyroid bed and lung, and died 2 years later from cerebral metastases; one male 61 years of age with PTC (T3mN1bM0) recurred 1 year later in lateral neck, and died 7 months later for local and lung disease. They were all ^{131}I -negative, ^{18}F -FDG-positive, and *BRAF V600E*-mutated cases (Fig. 2).

Figure 1 Probability of detecting recurrence over time in patients with or without ^{131}I uptake in the recurrence.

Figure 2 Survival curves for recurrent PTC patients with and without *BRAF V600E* mutations. All patients who died during the follow-up belonged to ^{131}I negative and *BRAF V600E* positive cases.

BRAF status and ^{131}I and ^{18}F -FDG uptake

In the ^{131}I -negative group, 79% (31/39) carried a *BRAF V600E* mutation in their recurrence, while this was true of only 18% (2/11) in the ^{131}I -positive group, in which most patients harbored a wild-type gene ($P<0.001$; Fig. 3). On a whole, *BRAF V600E*-positive recurrent patients were ^{131}I -negative in 94% (31/33) of the cases.

In iodine-avid recurrence group, 63% (7/11) underwent ^{18}F -FDG-PET-CT: 86% (6/7) showed positive foci of ^{18}F -FDG uptake. In the group of patients undergoing ^{18}F -FDG-PET-CT, there were 2/7 *BRAF V600E* cases, who were all ^{18}F -FDG PET positive.

In non-iodine avid recurrence group, 59% (23/39) underwent ^{18}F -FDG-PET-CT: 83% (19/23) showed positive foci of ^{18}F -FDG uptake. In the group of patients undergoing ^{18}F -FDG-PET-CT, there were 20/23 *BRAF V600E* cases, of which 80% were (16/20) ^{18}F -FDG PET positive.

BRAF status in paired primary and recurrent PTC

In 18 cases, paired primary and recurrent cancers were simultaneously analyzed for *BRAF* status: all but one showed a concordant genotype, 13 carried a *BRAF V600E* mutation in both the primary and the recurrence, and 4 had wild-type *BRAF* in both, while in one case the primary harbored a wild-type *BRAF*, and the recurrence harbored a *BRAF V600E* mutation. In the three cases where different recurrences were studied, the genotype was always concordant, i.e. mutated in two cases and wild type in one.

Discussion

The clinical management of recurrent PTC failing to trap ^{131}I is a challenge because ^{131}I is of no benefit in such cases for diagnostic or therapeutic purposes, making surgery the only curative strategy.

Figure 3 Relationship between *BRAF* status and ¹³¹I uptake in thyroid recurrences.

Chemotherapy and external radiation are often only palliative measures, and clinical trials with compounds for blocking the effectors of the MAPK pathway are currently underway.

BRAF V600E has proved to be the most frequent genetic event in the onset of PTC in adults, being found in 36–69% of cases (15). Several retrospective studies have also shown that primary PTC with *BRAF* mutations are more aggressive and more likely to recur and progress toward dedifferentiation processes, making *BRAF V600E* a new predictor of poor outcome, whatever the status of the other, classical prognostic factors (10, 16). In a previous study on a small series of thyroid recurrences, we demonstrated that such cancers (with no ¹³¹I uptake) carried a high frequency – around 77% – of *BRAF* mutations, and that this genetic event was accompanied by a decreased expression of TPO, Tg, and Pendred's syndrome genes, and an increased expression of the *GLUT1 (SLC2A1)* glucose transporter gene. In accordance with earlier findings in primary PTC, we demonstrated that *BRAF* mutations may promote iodine uptake impairment in recurrences, and we gave a biological basis for the use of scintigraphy with ¹⁸F-FDG (11). Our preliminary data were subsequently confirmed by Ricarte-Filho *et al.*, (17) who analyzed a large series of metastatic radioactive iodine-refractory recurrent cancers, 19 of which belonging to the papillary phenotype. These authors found that *BRAF V600E* was the main genetic event in these cancers, responsible for around 95% of cases and 100% of the ¹⁸F-FDG-PET-positive ones. Similar results were also reported by Henderson *et al.*, who demonstrated that recurrent PTC were significantly associated with *BRAF V600E* mutations in 77% of cases, though they did not examine the ¹³¹I uptake status in the recurrences (18).

To our knowledge, the present study concerns the largest reported series of recurrent PTC with a relatively lengthy follow-up (around 9 years) evaluated in terms of their capacity to concentrate ¹³¹I and ¹⁸F-FDG.

As far as the follow-up is concerned, we observed ~4-year delay in cancer coming to site diagnosis and re-intervention, in patients with ¹³¹I-negative recurrences. US scan was routinely assessed to demonstrate persistent or recurrent disease during follow-up in all the patients; however if it was not clearly negative, it was not sufficient by itself to perform the surgery, and US-FNAB was needed to confirm the suspicion. So, in patients with serum Tg rising or anti-Tg abs persistence, with a negative ¹³¹I WBS in the presence of recurrent tiny or not accessible lymph nodes being unable to uptake ¹³¹I, a more prolonged follow-up was needed to confirm the site of recurrence. During this lapse of time, we cannot rule out the risk of the disease spreading beyond the neck, such event being particularly unfavorable in those cases which cannot be treated by ¹³¹I. Actually, the only three deaths – all ¹³¹I-negative patients – were due to distant metastases. As a consequence, an effort should be made for early detection of persistent or recurrent disease, mainly in ¹³¹I-negative patients.

As regards molecular data, we validated our previous findings demonstrating that *BRAF V600E*-positive recurrent patients were ¹³¹I-negative in 94% of cases. Such observations are consistent with previous studies by Riesco-Eizaguirre *et al.*, who demonstrated *in vitro* that the *BRAF V600E* transfection had several effects on NIS regulation: first, the transcriptional activity of the NIS promoter decreased, then NIS targeting to the membrane was impaired, and finally protein expression progressively diminished (19).

The present study also confirms our preliminary findings that recurrent PTC carrying a mutated *BRAF* gene are more likely to be identified using ¹⁸F-FDG (which was positive in 80% of our cases). As a consequence, our data suggest the search for *BRAF* mutations – possibly even before surgery as proposed by some authors (20, 21), but still not proven – could help to stratify PTC patients, identifying persistent or recurrent cancers, that should be followed up not only with ¹³¹I WBS, but also with ¹⁸F-FDG-PET.

We also found that *BRAF*-mutated cancers were also associated with a trend of low Tg serum levels in comparison with cases harboring a wild-type *BRAF*: such variation was not statistically different and should be validated by future studies. The present study also analyzed *BRAF* status in the largest reported series of paired primary and recurrent PTC in an attempt to ascertain whether the molecular pattern of the cancer in a given patient could change during the follow-up. We confirmed the findings reported by Ficarte-Filho *et al.*, i.e. most primary and recurrent cancers had a concordant *BRAF* genotype. Only one of our patients acquired a *BRAF* mutation as a *de novo* event in the PTC

recurrence: so that *BRAF* testing at the time of primary PTC diagnosis could identify nearly all mutated cases. Indeed, a relatively earlier study on paired primary PTC and metastatic lymph nodes had found that *BRAF* mutations could be a *de novo* event at lymph node level, due to the favorable influence of the local milieu (22).

In conclusion, our study showed that *BRAF* mutations are very frequent in thyroid recurrences with no ^{131}I uptake; at the same time, they correlate with the ability to concentrate ^{18}F -FDG, and they can appear, albeit rarely, as a *de novo* event in the cancer recurrence.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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