**CLINICAL STUDY**

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

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**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 $^{18}$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 χ²-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.**

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

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 131I-negative recurrences, and 11 had 131I-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 131I-positive cases than in 131I-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 131I-positive cases than in 131I-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 (T2N0M0) 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 131I-negative, 18F-FDG-positive, and BRAF V600E-mutated cases (Fig. 2).

BRAF status and 131I and 18F-FDG uptake

In the 131I-negative group, 79% (31/39) carried a BRAF V600E mutation in their recurrence, while this was true of only 18% (2/11) in the 131I-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 131I-negative in 94% (31/33) of the cases.

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

In non-iodine avid recurrence group, 59% (23/39) underwent 18F-FDG-PET–CT; 83% (19/23) showed positive foci of 18F-FDG uptake. In the group of patients undergoing 18F-FDG-PET–CT, there were 20/23 BRAF V600E cases, of which 80% were (16/20) 18F-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 131I is a challenge because 131I is of no benefit in such cases for diagnostic or therapeutic purposes, making surgery the only curative strategy.
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.

Figure 3 Relationship between BRAF status and 131I uptake in thyroid recurrences.
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 131I uptake; at the same time, they correlate with the ability to concentrate 18F-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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