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ORIGINAL ARTICLE

Clinical and molecular characterisation of metastatic papillary thyroid cancer according to radioiodine therapy outcomes

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Abstract

Purpose Radioiodine (RAI) therapy remains the gold-standard approach for distant metastatic differentiated thyroid cancer (TC). The main objective of our work was to identify the clinical and molecular markers that may help to predict RAI avidity and RAI therapy response of metastatic lesions in a cohort of papillary thyroid cancer (PTC) patients.

Methods We performed a retrospective analysis of 122 PTC patients submitted to RAI therapy due to distant metastatic disease. We also analysed, through next-generation sequencing, a custom panel of 78 genes and rearrangements, in a smaller cohort of 31 metastatic PTC, with complete follow-up, available RAI therapy data, and existing tumour sample at our centre.

Results The most frequent outcome after RAI therapy was progression of disease in 59.0% of cases ($n = 71$), with median estimate progression-free survival of 30 months. RAI avidity was associated with PTC subtype, age and stimulated thyroglobulin at first RAI therapy for metastatic disease. The most frequently altered genes in the cohort of 31 PTC patients' primary tumours were *RAS* isoforms (54.8%) and *TERT* promoter (*TERT*p) (51.6%). The presence of *BRAF* p.V600E or *RET*/PTC alterations was associated with lower avidity ($p = 0.012$). *TERT*p mutations were not associated with avidity ($p = 1.000$) but portended a tendency for a higher rate of progression ($p = 0.063$); similar results were obtained when *RAS* and *TERT*p mutations coexisted ($p = 1.000$ and $p = 0.073$, respectively).

Conclusions Early identification of molecular markers in primary tumours may help to predict RAI therapy avidity, the response of metastatic lesions and to select the patients that may benefit the most from other systemic therapies.

Keywords Papillary thyroid cancer · Radioiodine therapy · Avidity · Metastasis · *RAS* genes · *TERT* promoter

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Introduction

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer (TC), usually portending an excellent prognosis. Distant metastases occur in a minority of these patients, but represent their main cause of death [1]. The metastases from well-differentiated thyroid tumours (WDTC) have the unique property of retaining and responding to radioiodine (RAI) therapy, which remains the gold-standard approach for these patients [2]. Despite the favourable outcome of RAI-avid metastatic thyroid cancer, the 10-year survival rate drops from nearly 60% to 10% in those that are, or become, RAI-refractory [3].

Although somehow controversial, the definition of RAI therapy refractoriness [2, 4] settles in two aspects: RAI avidity and response to RAI therapy, two concepts that are not necessarily interdependent.

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The genomic landscape of TC has been broadly studied by many authors. The largest and most extensive is The Cancer Genome Atlas (TCGA) analysis of PTC [5]. However, only 4.8% and 1.0% of that cohort presented high risk and distant metastatic disease, respectively, at the time of the study. In the last years, several authors have also been reporting the molecular spectrum of more aggressive TC, namely of metastatic TC, and of poorly and anaplastic thyroid cancer (PDTC and ATC, respectively) [6–9]. Different studies analysing TC molecular alterations and their association with RAI avidity or refractoriness have also been published [10, 11]. However, some investigated a small group of TC-related genes, and others studied a non-homogeneous cohort in terms of TC histological types, with inherent distinct molecular profiles [10, 11], and different RAI therapy behaviour [12]. Furthermore, assembling the two concepts of RAI avidity, *i.e.*, sodium-iodine symporter (NIS) proper function, and RAI response, *i.e.*, cellular events that lead to tumour shrinkage, in the sole definition RAI refractory (RAIR) disease may also contribute for a heterogeneous cohort.

The main objective of our work was to identify the clinical and molecular markers that may help to predict RAI avidity and RAI therapy response of metastatic lesions in a homogenous cohort of PTC patients.

Materials and methods

Study design

Following Institutional Review Board (IRB) approval (approval number 1056), we performed a retrospective analysis of all PTC patients submitted to RAI therapy due to distant metastatic disease (M1), between 2006 and 2018, at Instituto Português de Oncologia de Lisboa Francisco Gentil (partially described in [13]). Then, we selected a smaller group of patients, whose tumours were analysed through next generation sequencing (NGS), using a custom multigene panel, after obtaining their signed consent forms. Further details are described in the Materials and Methods section of Supplementary Data.

Pathology

The Tumour-Node-Metastasis (TNM) classification of all PTC specimens was performed in accordance with criteria described in the Union for International Cancer Control (UICC)/American Joint Committee Cancer (AJCC) 8th edition [14]. Histological specimens were always reviewed by in-house expert pathologists. For the selection of primary tumour areas for NGS analysis, pathologists selected the areas with at least 90% of tumour content.

Definition of clinical outcomes

After whole-body scintigraphy (WBS), patients were classified as having RAI-avid disease, if all the known distant metastases showed RAI uptake, or as RAI-non-avid disease, if the metastases or, at least, the larger and the most clinically significant did not evidence RAI uptake. Metastases were also classified as micro (<10 mm) or macrometastases (≥10 mm).

Definition of target lesions, and distant tumour response to RAI therapy – progressive disease (PD), stable disease (SD), complete response (CR) and partial response (PR) – were established according to RECIST 1.1 criteria [15] (these criteria are described in the Materials and Methods section of Supplementary Data). We considered biochemical progression when non-stimulated thyroglobulin (Tg) or Tg-antibody (Ab-Tg) after RAI therapy increased at least 20%; stable biochemical response was defined as a variation of ±20%; partial biochemical response as a decrease of 20–100%; and complete response when the decrease reached 100% [10].

Progression free survival (PFS) was defined as the period from the day of first RAI therapy with distant metastatic disease to the day of biochemical or structural progression, and disease-specific survival (DSS) as the period from the day of first RAI therapy for distant metastatic disease and PTC-specific death.

For the study of somatic molecular alterations, patients were classified in three groups: RAI-avid and RAI therapy response for at least 24 months; RAI-avid and progression after RAI therapy (if it occurred in less than 24 months); and RAI-non-avid and progression after RAI therapy. The corresponding radiological exams and WBS were reviewed by a radiologist and a nuclear medicine specialist in a blinded manner. The nuclear medicine physician analysed, in terms of avidity, the post-RAI therapy WBS performed in the presence of metastatic disease. The radiologist analysed, in terms of structural response, the CT scans performed after each RAI therapy for metastatic disease and also during the entire follow-up. Both physicians did not know the name or the outcomes (structural and/or biochemical) of each patient. These physicians are dedicated to thyroid cancer field, and they also integrate the multidisciplinary team of thyroid oncology of our centre. Further details are described in the Materials and Methods section of Supplementary Data.

DNA extraction from normal and tumour tissues

The details of DNA extraction from formalin-fixed paraffin-embedded (FFPE) normal and tumour tissues are detailed in the Materials and Methods section of Supplementary Data.

Next-generation sequencing (NGS) analysis of a custom multigene panel in PTC samples

NGS was performed with a custom panel (78 genes and 2 rearrangements) (Supplementary Table 1). Among the 122 patients from the whole cohort, a smaller group of 31 patients could be selected, based in the following requirements: i) ready availability of patient's primary tumour FFPE sample in our centre; ii) patient with complete follow-up; and iii) patient liable to be classified in terms of avidity and response to RAI therapy. Three patients' matched normal samples were also included. These patients were grouped according to RAI treatment outcomes. The details of NGS and bioinformatics analysis are presented in the Materials and Methods section of Supplementary Data.

Statistics

Categorical variables are presented as absolute number and percentage. Gaussian distribution of continuous variables was determined with Shapiro-Wilk test; non-normally variables are presented as median [interquartile range (IQR); minimum-maximum]. Categorical variables were compared using Chi-Square test or Fisher's Exact test. Comparison of continuous variables between groups was made with Mann–Whitney and Kruskal–Wallis tests.

Independent factors associated with RAI avidity and response were identified with binary logistic regression. Estimate median and mean DSS and PFS were determined by the Kaplan–Meier method. For the multivariable models, the continuous variables were converted into categorical ones with the cut-off point being the median of each variable, except for age at diagnosis, in which we used the cut-off <55 and ≥55 years as recommended by UICC/AJCC for thyroid tumours. A p-value <0.05 was considered statistically significant. Missing data were handled by complete-case analysis. The main reasons for missing data were absence of information in the clinical files or loss to follow-up. Statistical analysis was performed with IBM SPSS Statistics version 25 (IBM Corp. New York. USA).

Results

Clinical data

Patients' clinicopathological features and therapeutic strategies

We included 122 PTC patients with distant metastatic disease. Fifty-seven (46.7%) patients presented with distant metastasis at PTC diagnosis, and the remaining patients were diagnosed with distant disease throughout the follow-

Table 1 Main clinical and pathological characteristics of the cohort

Variable	Total ($n = 122$)
Median follow-up since PTC diagnosis (years)	6.0 (IQR 7.0; 0.0–27.0)
Median age at PTC diagnosis (years)	61.0 (IQR 21.0; 19.0–85.0)
Female	81 (66.4%)
Primary surgical approach	
• TT	67 (54.9%)
• TT + LND	49 (40.2%)
• *En bloc* resection	6 (4.9%)
PTC subtype	
• Classical	41 (33.6%)
• Follicular	46 (37.7%)
• Classical + follicular	12 (9.8%)
• Aggressive subtypes/mixed, including aggressive subtypes	13 (10.7%)
• Others	4 (3.3%)
• Unknown	6 (4.9%)
Median tumour size (mm)	36.5 (IQR 35.0; 3.0–120.0)
Multifocal	52 (42.6%)
Extrathyroidal extension	72 (59.0%)
Bilateral	34 (27.9%)
Poorly-differentiated areas	10 (8.2%)
LN metastasis	58 (47.5%)
Resection status of primary surgery	
• R0	74 (60.3%)
• R1	29 (24.0%)
• R2	8 (6.6%)
• Rx	11 (9.1%)
T at PTC diagnosis	
• T1	24 (19.7%)
• T2	20 (16.4%)
• T3	48 (39.3%)
• T4	17 (13.9%)
• Tx	13 (10.7%)
N at PTC diagnosis	
• N0	7 (5.7%)
• N1a	8 (6.6%)
• N1b	50 (41.0%)
• Nx	57 (46.7%)
M at PTC diagnosis	
• M0	23 (18.9%)
• M1	57 (46.7%)
• Mx	42 (34.4%)
Status at last follow-up	
• Alive with NED	6 (4.9%)
• Alive with BED	3 (2.5%)
• Alive with locoregional disease	3 (2.5%)

Table 1 (continued)

Variable	Total ($n = 122$)
• Alive with distant metastatic disease	66 (54.1%)
• Lost to follow-up	3 (2.5%)
• Dead due to PTC	37 (30.3%)
• Dead due to other reason	4 (3.3%)

The PTC subtypes were solid/trabecular and cribiform. TNM classification was performed in accordance UICC/AJCC 8th edition

BED biochemical evidence of disease, *IQR* Interquartile range, *LN* lymph-node, *LND* lymph-node dissection, *NED* no evidence of disease, *PTC* papillary thyroid cancer, *TT* total thyroidectomy

up. Table 1 presents the main clinical and pathological characteristics of the cohort.

Radioiodine therapy outcomes

The RAI therapy features are provided in Table 2, including RAI avidity and RAI therapy response. Median estimate PFS after RAI therapy for distant metastatic disease was 2.5 years, which is close to the cut-off of 24 months, that we had established to consider at least a transitory stable disease after RAI therapy. Then, we investigated the independent variables associated with a partial or complete response or stable disease that lasted at least 24 months [$n = 78$ (63.9%)] (Table 3) and with avidity (Table 4).

Tumour molecular data

Frequently altered genes in PTC with distant metastases

Figure 1 shows the molecular alterations found in the 31 primary tumours of PTC patients that presented distant metastatic disease (detailed data in Supplementary Tables 2 and 3). Distant metastases were present at primary diagnosis in 27 (87.1%) of these patients. Follicular variant (fvPTC) represented the majority of these cases (45.2%). More than 90% of the genes had >80% of the exons with a coverage >20×. The median variant read depth was 336.0 (IQR 659.0, 25.0–2537.0); median number of variants was 4.0 (IQR 3.0; 1.0–10.0). The observed variants were present in oncogenes in 46.3% of all variants, and in tumour-suppressor genes in 21.5%. The most frequently altered genes were *RAS* isoforms [$n = 17$ (54.8%)] and *TERT*p [$n = 16$ (51.6%)], followed by *USH2A* and *FAT4* [$n = 5$ (16.1% each)]. *BRAF* p.V600E mutation (c.1799T>A) was found in two (6.5%) tumours; a *BRAF* non-p.V600E mutation (c.2156G>A; p.Arg719His) was seen in a tumour with *NRAS* mutation (c.182A>G; p.Gln61Arg), and according to TCGA study [5], these mutations have a *RAS*-like behaviour. *EIF1AX* mutations [$n = 3$ (9.7%)] always coexisted with *NRAS* mutations. *RET*/PTC fusion was observed in one tumour without any other molecular alterations. The most frequent *TERT*p altered variant was located at −124 bp (Chr 5: 1295228 C>T; −124C>T; C228T) ($n = 14$, 87.5%). Variants in MAPK genes were more frequent in males than in females ($p = 0.026$). We also observed a tendency for the association of *BRAF* p.V600E and *RET*/PTC alterations with classic PTC (cPTC) ($p = 0.060$).

These primary tumours did not harbour *TP53* nor *CDKI* genes mutations; the prevalence of alterations in mismatch repair (MMR) genes was low [$n = 1$ (3.2%)]. Only one tumour (3.2%) presented a hotspot mutation in *PIK3CA*.

Table 2 RAI therapy characteristics, avidity, structural response, progression-free survival and disease-specific survival

Variable	Total ($n = 122$)
Median follow-up since first RAIT with M1 (months)	47.0 (IQR 57.5; 3.0–157.0)
Median age at first RAIT with M1 (years)	66.5 (IQR 22.5; 21.0–85.0)
Macrometastases	47 (38.5%)
Median total RAI activity (mCi) for M1	300 (IQR 300; 100–886)
Median number of RAIT with M1	2.0 (IQR 2.0; 1.0–6.0)
Median number of RAIT showing M1 avidity	1.0 (IQR 2.0; 0.0–6.0)
Number of patients showing avid M1	63 (51.6%)
RAIT preparation	
• Levothyroxine withdrawal	32 (26.2%)
• Recombinant TSH	83 (68.0%)
• Both	7 (5.7%)
Median stimulated Tg at first RAIT with M1 (ng/mL)	457.0 (IQR 7877.0; 0.0–300000.0)
Structural response to RAIT with M1 during follow-up[a]	
• PD	71 (59.0%)
• SD	31 (25.8%)
• PR	12 (10.0%)
• CR	6 (5.0%)
Other therapeutic approaches that may have influenced RAI avidity	8 (6.6%)
Other therapeutic approaches that may have influenced RAIT response	14 (11.5%)
Median estimate PFS after RAIT for M1 [95%CI] (months)[a]	30.0 [22.1–37.9]
Median estimate DSS after RAIT for M1 [95%CI] (months)[a]	121 [95% CI not provided]

CI confidence interval, *CR* complete response, *IQR* Interquartile range, *M1* distant metastasis, *PFS* progression-free survival, *PD* progressive disease, *PR* partial response, *RAI* radioiodine, *RAIT* radioiodine therapy

[a]In these parameters, 120 patients were included because two were loss to follow-up. "Not provided" means that the statistics software did not calculate it

Table 3 Factors associated with disease stability or response that lasted at least 24 months after radioiodine therapy for distant metastatic disease

Variable	Outcome: stable disease <24mo ($n = 44$) *vs.* ≥24mo ($n = 78$)	Univariate	Multivariate	Odds ratio (95% CI)
PTC subtypes		$P = 0.071$	–	–
• Classical	17 (41.5%) *vs.* 24 (58.5%)			
• Follicular	10 (21.7%) *vs.* 36 (78.3%)			
• Classical + follicular	6 (54.5%) *vs.* 5 (45.5%)			
• Mixed, including aggressive subtypes	6 (46.2%) *vs.* 7 (53.8%)			
Age		$P = 0.001$	$P = 0.010$	0.2 [0.1–0.7]
• <55 years[a]	5 (13.5%) *vs.* 32 (86.5%)			
• ≥55 years	38 (45.2%) *vs.* 46 (54.8%)			
Sex		$p = 0.006$	$P = 0.011$	3.4 [1.3–8.7]
• Female	22 (27.2%) *vs.* 59 (72.8%)			
• Male[a]	21 (52.5%) *vs.* 19 (47.5%)			
Poorly-differentiated areas		$P = 0.031$	$P = 0.062$	–
• No[a]	31 (31.6%) *vs.* 67 (68.4%)			
• Yes	7 (70.0%) *vs.* 3 (30.0%)			
Stimulated Tg at first RAIT with M1		$P = 1.000$	–	–
• ≤450 ng/mL	20 (34.5%) *vs.* 38 (65.5%)			
• >450 ng/mL	20 (33.9%) *vs.* 39 (66.1%)			
Metastases dimension		$P = 0.562$	–	–
• <10 mm	24 (32.9%) *vs.* 49 (67.1%)			
• ≥10 mm	18 (38.3%) *vs.* 29 (61.7%)			
Metastases' sites		$P = 0.562$	–	–
• Lungs	32 (35.2%) *vs.* 59 (64.8%)			
• Bones	8 (32.0%) *vs.* 17 (68.0%)			
• Lungs + bones	2 (50.0%) *vs.* 2 (50.0%)			
• Others	1 (100.0%) *vs.* 0 (0.0%)			
Avidity		$P = 0.022$	$P = 0.082$	–
• No[a]	27 (46.6%) *vs.* 31 (53.4%)			
• Yes	16 (25.4%) *vs.* 47 (74.6%)			
Total RAI activity for M1		$P = 1.000$	–	–
• <600 mCi	37 (34.9%) *vs.* 69 (65.1%)			
• ≥600 mCi	5 (35.7%) *vs.* 9 (64.3%)			
Other therapeutic approaches that may have influenced response		$P = 0.374$	–	–
No	40 (37.4%) *vs.* 67 (62.6%)			
Yes	3 (21.4%) *vs.* 11 (78.6%)			

M1 distant metastasis, *RAIT* radioiodine therapy, *PTC* papillary thyroid cancer, *Tg* thyroglobulin, *TSH* thyrotropin

[a]Reference category in multivariate analysis

Alterations in non-MAPK/PI3K/AKT pathways genes

In our dataset there were alterations in three genes that belong to the WNT pathway: *TCF7L2* gene, mutated in 9.7% ($n = 3$) of the cases, a likely pathogenic variant in *RFN43* gene in one tumour (3.2%), and a variant of unknown significance in the *AXIN1* gene, always coexisting with alterations in MAPK pathway genes. Other genes, such as *USH2A* (that encodes usherin, found in the basement membrane, which interacts with collagen IV and fibronectin via its laminin EGF-like domains) and *FAT4* (involved in cell adhesion) were also mutated, both in 16.1% of the cases, as referred above.

Molecular profiling of PTC according to RAI therapy outcomes

Tumours were clustered according to their RAI therapy outcomes in terms of avidity and response: group 1) RAI-avid and RAI therapy response; group 2) RAI-avid and

Table 4 Factors associated with metastases' radioiodine avidity in post-therapy whole body scintigraphy

Variable	Outcome: Avidity Yes ($n = 63$) *vs.* No ($n = 59$)	Univariate	Multivariate	Odds ratio (95% CI)
PTC subtypes		$P < 0.001$	$P = 0.005$	
• Classical[a]	8 (19.5%) *vs.* 33 (80.5%)			
• Follicular	35 (76.1%) *vs.* 11 (23.9%)			7.4 [2.3–23.7]
• Classical + follicular	5 (41.7%) *vs.* 7 (58.3%)			2.1 [0.4–10.1]
• Mixed, including aggressive subtypes	9 (69.2%) *vs.* 4 (38.2%)			5.3 [1.1–25.2]
Age		$P = 0.020$	$P = 0.009$	0.2 [0.1–0.7]
• <55 years[a]	25 (67.6%) *vs.* 12 (32.4%)			
• ≥55 years	38 (44.7%) *vs.* 47 (55.3%)			
Sex		$P = 0.653$	—	
• Female	43 (53.1%) *vs.* 38 (46.9%)			
• Male[a]	20 (48.8%) *vs.* 21 (51.2%)			
Poorly-differentiated areas		$P = 1.000$	—	—
• No	50 (51.0%) *vs.* 48 (49.0%)			
• Yes	5 (50.0%) *vs.* 5 (50.0%)			
Stimulated Tg at first RAI therapy with M1		$P < 0.001$	$P = 0.011$	4.5 [1.4–14.2]
• ≤450 ng/mL[a]	20 (33.9%) *vs.* 39 (66.1%)			
• >450 ng/mL	43 (72.9%) *vs.* 16 (27.1%)			
Metastases dimension		$P = 0.003$	$P = 0.920$	
• <10 mm[a]	30 (40.5%) *vs.* 44 (59.5%)			
• ≥10 mm	32 (68.1%) *vs.* 15 (31.9%)			
Metastases' sites		$P = 0.001$	$P = 0.481$	
• Lungs[a]	38 (41.3%) *vs.* 54 (58.7%)			
• Bones	21 (84.0%) *vs.* 4 (16.0%)			
• Lungs + bones	3 (75.0%) *vs.* 1 (25.0%)			
• Others	1 (100.0%) *vs.* 0 (0.0%)			
Other therapeutic approaches that may have influenced avidity		$P = 0.718$	–	
No	58 (50.9%) *vs.* 56 (49.1%)			
Yes	5 (62.5%) *vs.* 11 (37.5%)			

M1 distant metastasis, *RAIT* radioiodine therapy, *PTC* papillary thyroid cancer, *Tg* thyroglobulin, *TSH* thyrotropin

[a]Reference category in multivariate analysis

progression; and group 3) RAI-non-avid and progression (Fig. 1). The median number of somatic alterations was similar between groups ($p = 0.186$). The presence of *BRAF* p.V600E or *RET*/PTC alterations was associated with lower avidity ($p = 0.012$). Isolated *RAS* mutations were not associated with avidity ($p = 0.412$) or with progression ($p = 0.576$). *TERT*p mutations were not associated with avidity ($p = 1.000$) but portended a tendency for a higher rate of progression ($p = 0.063$); similar results were obtained when *RAS* and *TERT*p mutations coexisted ($p = 1.000$ and $p = 0.073$, respectively). The proportion of altered genes in MAPK and WNT pathways was similar between the three groups. Likely pathogenic variants in *TSHR* and *DICER1* genes were only observed in progressive tumours; on the other hand, alterations in *FAT4* gene were only detected in group 1, and variants in *USH2A* gene were only detected in RAI-avid tumours (groups 1 and 2).

Regarding the therapeutic options for patients refractory to RAI, the mutated targetable genes detected in our cohort, and the corresponding drugs, are represented in Fig. 2.

Discussion

Herein, we present the clinical and molecular analysis of markers of RAI avidity and RAI therapy response, in a homogeneous cohort of PTC patients with distant metastatic disease, who were submitted to RAI therapy.

The most frequent outcome in the present series was progression of disease, that occurred in 59.0% of cases ($n = 71$), with median estimate PFS of 30 months,

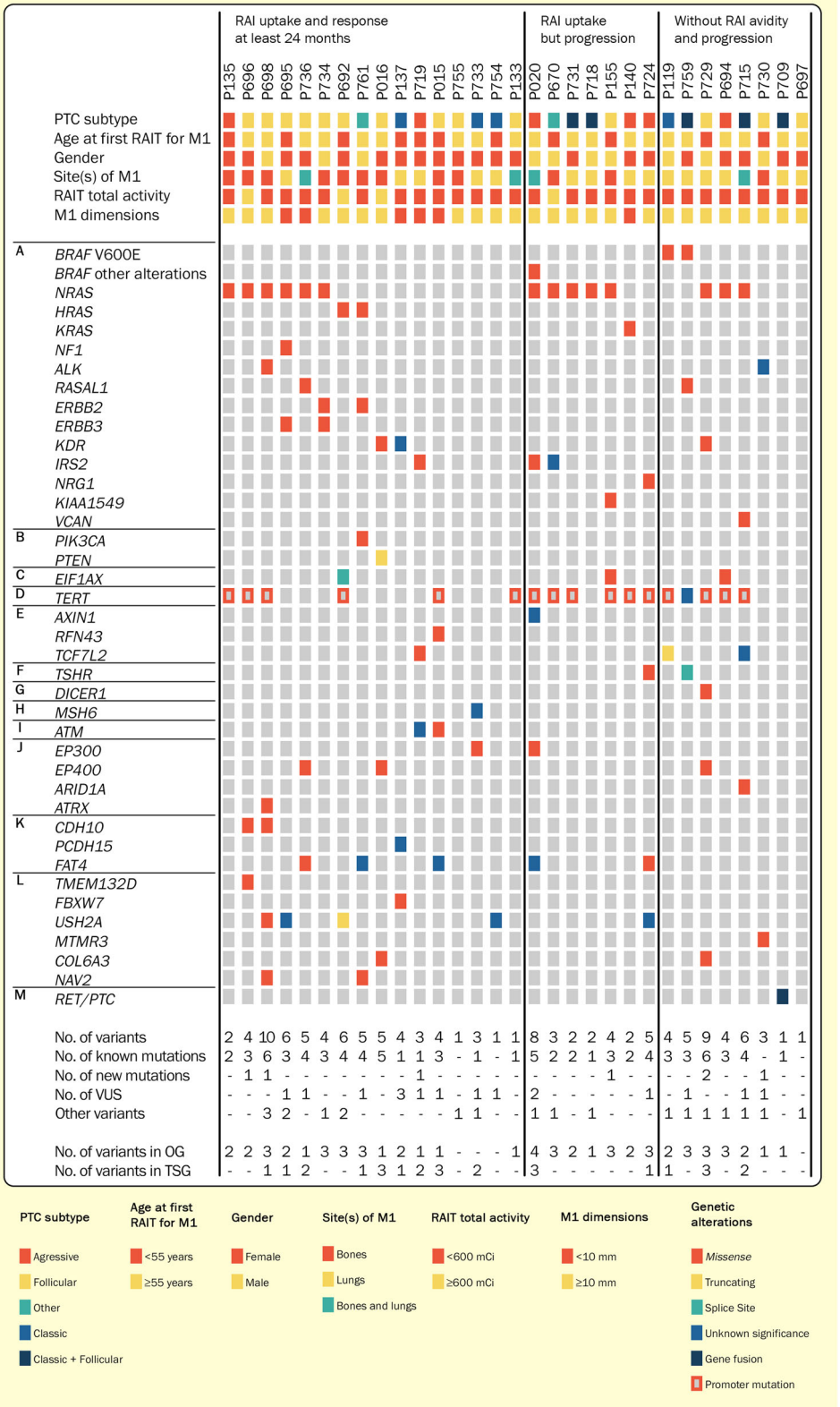

Fig. 1 Molecular alterations of metastatic papillary thyroid cancers. A, MAPK pathway; B, PI3K/AKT pathway; C, Eukaryotic translation initiation; D, Telomere maintenance; E, WNT pathway; F, Thyroid metabolism; G, Gene silencing by RNA; H, Mismatch repair; I, DNA damage repair; J, Chromatin modifiers; K, Cell adhesion; L, other pathways; M, gene fusions. M1 metastatic disease, OG oncogene, PTC papillary thyroid carcinoma, RAI radioiodine, RAIT radioiodine therapy, TSG tumour suppressor gene, VUS variant of unknown significance

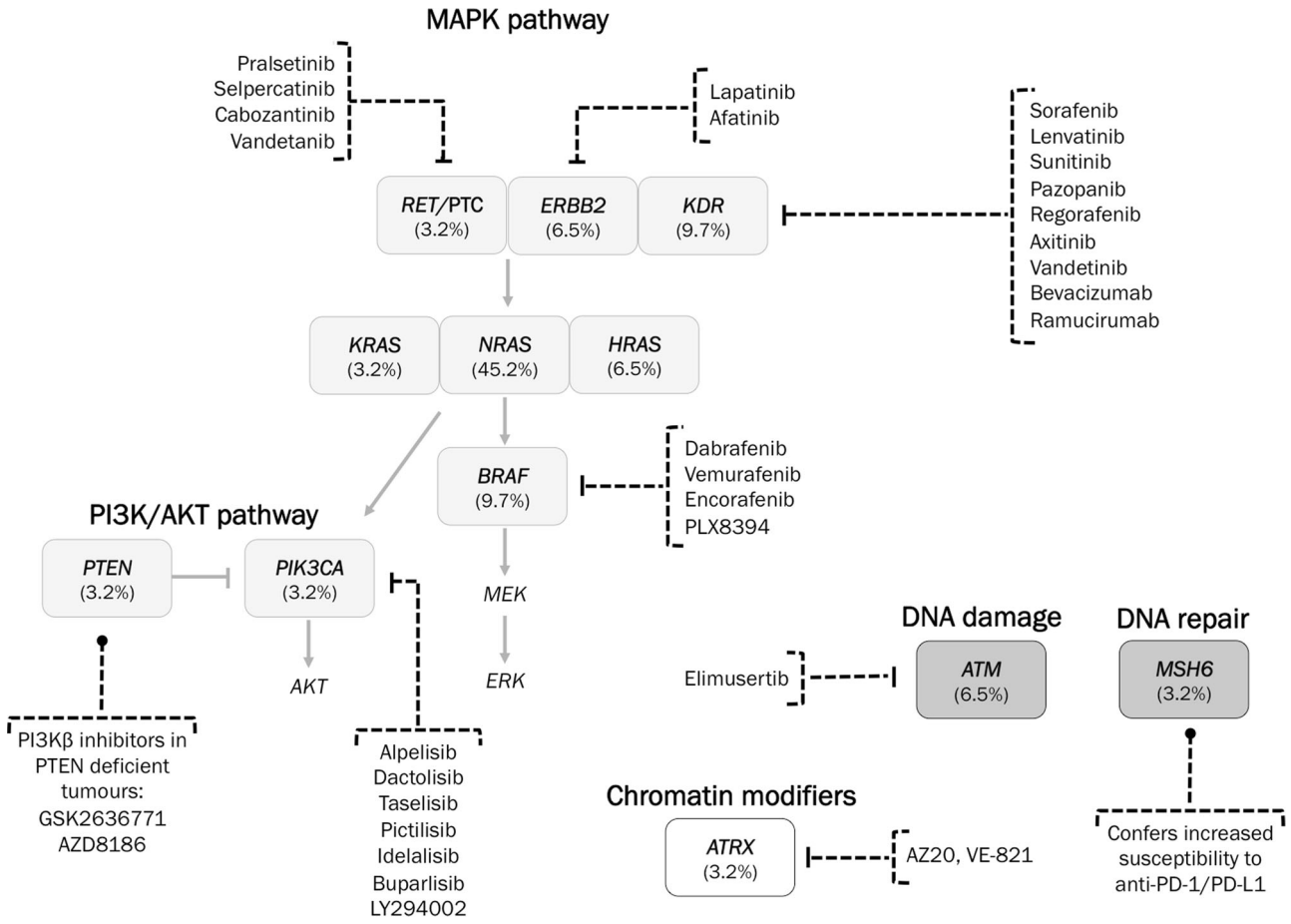

Fig. 2 Frequency of target genes and the corresponding available directed drugs. The dashed lines indicate studied target drugs directed to each mutated gene. Information regarding studied target drugs is based on OncoKB (https://www.oncokb.org/cancerGenes), CIVic (https://civicdb.org/home) and PubChem (https://pubchem.ncbi.nlm.nih.gov/)

demonstrating the characteristic indolent course of this disease [16]. More than half [$n = 78$ (63.9%)] of our cohort exhibited stable disease at least for 24 months.

One of the major gaps in the definition of RAIR is which metrics is most appropriate to evaluate the response to RAI therapy and the duration of that response. Slow or no progression, for an acceptable period of time, may be considered as RAI therapy response and constitute an indication for repeating RAI therapy, rather than considering the disease as RAIR. As advocated by other authors [17], documenting any degree of tumour growth at any time point during follow-up may be too imprecise to classify the disease as RAIR. Thus, based on our data, we propose that a patient may be considered RAIR when biochemical and/or structural progression are documented in less than 24 months after RAI therapy. Another controversial point in RAIR definition is "≥600 mCi of cumulated RAI therapy" [4, 17]. For this classification, the evidence is very scarce. Durante et al. [3] observed that almost half of the patients (48%) achieved negative WBS after a cumulative activity of less than 200 mCi. A negative WBS does not always represent a favourable response, and, beyond that, in our study, total activity of <600 *vs*. ≥600 mCi did not impact on RAI therapy response. Thus, we consider that cumulative RAI therapy activity may be higher than 600 mCi if there is any benefit (i.e., at least stable disease), throughout the follow-up, in the absence of significant adverse effects.

The variables significantly associated with RAI avidity were PTC subtype, age and stimulated Tg at first RAI therapy for metastatic disease. Our group recently reported the influence of histotype in RAI therapy outcomes: besides avidity, histotype was also an independent variable associated with PFS, but not with disease-specific survival [13].

In the small cohort of metastatic PTC, that could be selected for NGS analysis with a multigene panel, molecular profiling showed that the most commonly mutated genes were *RAS* isoforms (54.8%) and *TERTp* (51.6%), whereas *BRAF* mutations and *RET/PTC* fusions were detected in approximately 10% and 3% of these primary tumours, respectively. Our findings contrasted with the PTC TCGA study [5], in which a predominance of *BRAF* mutations was observed. Noteworthy, the TCGA cohort was mainly represented by classic PTC

(cPTC) (65.0%), and 20.0% were fvPTC, whilst in our cohort the predominant subtype was fvPTC (45.2%), followed by classic+follicular (16.1%) and cPTC (12.9%). Sabra et al. [10] studied 43 RAI-avid TC, including PDTC (33%), cPTC (28%), fvPTC (19%), and other subtypes (20%), and found mutations in *RAS* (42%), *BRAF* (23%), *PIK3CA* (2%), and *RET/*PTC fusions (10%). Shobab et al. [11] studied 24 RAIR thyroid cancers, also including PDTC and oncocytic TC, and reported activating alterations in *BRAF* (27%), *RAS* (23%) and *TP53* (23%). However, *TERT*p was not addressed in these studies. Thus, cohorts with preponderance of RAI-avid tumours, as in the present study, may be enriched with *RAS* mutations.

In our cohort, *BRAF* mutations were only detected in patients with progressive disease (RAI-avid and RAIR). In addition, the observed association of *BRAF* or *RET/*PTC alterations with lower RAI avidity, confirms that MAPK pathway output is associated with dedifferentiation, and, in particular, with NIS downregulation [5]. Interestingly, we found that isolated mutations of *RAS* or *TERT*p were not associated with lower avidity but when these two alterations coexisted, they showed a tendency for progression. Progressive tumours (groups 2 and 3) were clearly enriched in *TERT*p mutations, as this genetic alteration was present in 2/3 of the cases. In contrast, Sabra et al. [10] observed that RAI-avid patients with stable disease or partial response compared to those with progressive disease had similar *RAS*, *BRAF*, *RET/PTC* proportions, except for the presence of *PIK3CA* mutations, detected only in the later.

Regarding the genes less frequently reported in thyroid cancer, we detected mutations in two WNT pathway genes, *TCF7L2* and *RNF43*, which may be associated with thyroid cancer metastasis, as they coexisted with known driver genes, such as *BRAF* or *RAS* genes. Alterations in *USH2A* gene, were observed in 16.1% of the tumours, similarly to the frequency reported in 22 ATC (18.0%) [9], indicating that this gene may be important in thyroid cancer progression. Variants in *FAT4* gene, which plays a critical role in the epithelial-to-mesenchymal-transition [18], that are rare in thyroid tumours, were observed in five tumours (16.1%) of our cohort.

Regarding the identification of targetable alterations, we observed that 1/4 tumours with progression (with/without RAI avidity), that were *RAS* or *BRAF* negative, had a *RET*/PTC rearrangement. van der Tuin *et al*. [19] found targetable gene fusions in 7/60 (12%) of the RAI refractory tumours without *RAS* or *BRAF* mutations. A low prevalence of mutations in MMR genes was observed in our cohort, which indicates that treatment with immune checkpoint inhibitors may not be useful in these patients. Accordingly, the genomic analysis of 55 metastatic thyroid cancers requiring systemic therapy [20] revealed absence of microsatellite instability.

The present approach of analysing primary tumours as a proxy for the prediction of distant metastases behaviour is supported by two recent studies, which included matching primary tumours and distant metastases (including PTC), and found that the overall mutational status was similar [21, 22].

Our study has some limitations, namely its retrospective nature, and the number of patients that could be selected for the genetic analysis. However, this smaller group was only composed of PTC histotype, which represents a strength in this study.

In summary, although most of PTC patients with distant metastasis progress after RAI therapy during follow-up, this therapy may provide disease stability for at least two years. Early identification of molecular markers in primary tumours may help to predict RAI therapy avidity and response of metastatic lesions and select the patients that may benefit the most from other systemic therapies.

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Author contributions J.S.-P. designed the work, acquired the data (clinical and genetic), designed the custom panel, analysed the data, and wrote the manuscript; A.S., R.R. and C.P. performed the genetic analysis; M.P. designed the custom panel and acquired the FFPE material; M.H. reviewed the images to assess the structural response of metastases; D.L.-P., M.R. and R.C. reviewed the pathological slides and selected the tumour and normal counterparts; T.C.F. reviewed the post-RAI therapy WBS to assess avidity; B.M.C. planned the project, designed the custom panel, provided substantial contribution to the interpretation of the genetic data, and revised critically the manuscript; Valeriano Leite provided substantial contribution for the clinical data and revised critically the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Consent to participate The collection of biological samples from all subjects involved in this study was performed after written informed consent.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethical Committee of IPOLFG (approval number 1056).

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