mTOR activation in medullary thyroid carcinoma with *RAS* mutation

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Abstract

Objective: Rearranged during transfection (*RET*) mutations are well-known genetic events in sporadic and familial medullary thyroid carcinoma (FMTC). The presence of *RAS* mutations in sporadic cases, challenging the *RET* paradigm in these tumors, has been recently reported. We intend to evaluate mTOR pathway activation in *RET*- and *RAS*-mutated MTC.

Materials and methods: In this study, we analysed the presence of *RET*, *H-RAS*, and *K-RAS* mutations in a series of 87 MTCs (82 apparently sporadic and five FMTCs; five apparently sporadic MTCs were eventually found to be familial). We also evaluated mTOR activation – using the expression of its downstream effector phospho-S6 ribosomal protein (p-S6) and the expression of the mTOR inhibitor, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) – by immunohistochemistry.

Results: Our results revealed that *RET* mutations were present in 52.9% of the cases (46/87) and *RAS* mutations in 12.6% (11/87) of the whole series of MTCs and 14.3% of the 77 sporadic MTCs. The presence of *RET* and *RAS* mutations was mutually exclusive. *RAS* mutations were significantly associated with higher intensity of p-S6 expression ($P=0.007$), suggesting that the mTOR pathway is activated in such MTCs. We observed also an increased expression of p-S6 in invasive tumors ($P=0.042$) and in MTCs with lymph node metastases ($P=0.046$). Cytoplasmic PTEN expression was detected in 58.8% of the cases; cases WT for *RAS* showed a significantly lower expression of PTEN ($P=0.045$).

Conclusions: We confirmed the presence of *RAS* mutation in 14.3% of sporadic MTCs and report, for the first time, an association between such mutations and the activation of the mTOR pathway. The evaluation of the mTOR activation by pS6 expression may serve as an indicator of invasive MTC.

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Introduction

Medullary thyroid carcinoma (MTC) is a rare neuro-endocrine tumor arising from the calcitonin-producing parafollicular C cells of the thyroid. It accounts for 5–8% of all clinically evident thyroid tumors. MTCs can present as part of hereditary syndromes (20–25%) (1) known as multiple endocrine neoplasia type 2 (MEN2): MEN2A, MEN2B, and familial MTC (FMTC). More frequently, MTCs present as sporadic cases (75–80%). MTC is thought

to be responsible for up to 14% of all thyroid cancer-related deaths, distant metastases being the main cause in this setting (2). Treatment of patients with MTC is still difficult in many cases and alternative therapeutic options have been proposed (2, 3, 4, 5).

The most important genetic events contributing to MTC development are mutations in the proto-oncogene rearranged during transfection (*RET*). Germline *RET*

mutations are present in almost 100% of the hereditary cases, whereas 30–50% of sporadic MTC cases also display somatic *RET* mutations (6, 7). The most common mutations in the hereditary setting involve exon 10 (codons 609, 611, 618, and 620) or exon 11 (codon 634) in *MEN2A*; exon 16 (codon 918) – Met918Thr – in *MEN2B*; in FMTC families, mutations in exons 8, 10, 11, 13, 14, and 15 partly overlap with the mutations detected in *MEN2A* (4, 8).

According to Elisei *et al.* (9), p.Met918Thr somatic mutation was present in more than 50% of *RET*-mutated sporadic cases and it was associated with a worse prognosis (9). 40–60% of sporadic MTCs remained without a recognised genetic event (10, 11). Recently, Moura *et al.* (12) have reported the presence of *RAS* somatic mutations in 68% of *RET*-negative sporadic MTCs (12). *RAS* mutations were only detected in *H-RAS* and *K-RAS* genes (none in *N-RAS* gene), apparently representing an alternative genetic event to *RET* mutations in sporadic MTC (6, 10, 11, 12, 13, 14). These results were reproduced by others (10, 11, 13, 14) with the percentage of *RAS*-mutated cases ranging from 0% (15) to 26.2% of MTCs (11). According to a meta-analysis by Ciampi *et al.* (11), the average prevalence of *RAS* mutations in sporadic MTC is 8.8%.

It has been demonstrated that the mTOR pathway is activated in sporadic and hereditary MTC (13, 15, 16, 17) and that *RET* mutations seem to play a role in the activation of such pathway in MTC (4, 6, 15). It remains unknown whether *RAS* mutations can also lead to mTOR pathway activation in MTCs. It is also not known whether there are alterations of phospho-S6 ribosomal protein (p-S6) expression and other major components in this cascade, such as phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*), in MTCs cases with *RAS* mutation (18).

In this study, we searched for mutations of *RET*, *H-RAS*, and *K-RAS* in a series of 87 MTCs. We evaluated the mTOR activation in *RET*- and *RAS*-mutated MTCs using immunohistochemistry (IHC) for p-S6 (a downstream effector) and *PTEN* (a mTOR pathway inhibitor).

Material and methods

Human cancer samples

A total of 87 MTC samples from patients with apparently sporadic (AS-MTC, $n=82$) and familial ($n=5$) MTCs were analysed by molecular and IHC methods. Formalin-fixed, paraffin-embedded tissues were retrieved from the files of Centro Hospitalar S. João (HSJ)/Medical Faculty of Porto (FMUP)/Ipatimup (63 cases) and from the

Portuguese Institute of Oncology, Coimbra (IPO-C; 24 cases). The diagnosis of MTC was revised by M Sobrinho-Simões and C Eloy and confirmed by calcitonin staining. Clinicopathological and follow-up data were retrieved from the files of the Department of Pathology and Oncology of HSJ and from IPO databases (Table 1). Ipatimup cases included many consultation cases from which only limited demographic and clinical information was available. The study was approved by the Local Ethical Committee. National Ethical rules were followed in every procedure. In the cases suspected to be familial, peripheral blood samples were obtained with informed consent and genetic counselling was available for participants.

DNA extraction, PCR, and Sanger sequencing

DNA was obtained from formalin-fixed, paraffin-embedded tissues (10-micron sections) after careful microdissection. DNA extraction was performed using the Ultrapep Tissue DNA Kit (AHN Biothecnologie, Nordhausen, Germany) following the manufacturer's instructions.

Sanger's direct sequencing analysis of exons 10, 11, 13, 14, 15, and 16 of *RET* oncogene and exons 1 and 2 of both *H-RAS* and *K-RAS* was performed after specific amplification by PCR. Sequences of oligonucleotide primers to screen for *RET* hotspot mutations in the exons are mentioned above, and *H-RAS* and *K-RAS* hotspots can be found in Supplementary Table 1, see section on supplementary data given at the end of this article. Amplification

Table 1 Clinical and pathological data of MTC.

	MTC
Gender ($n=62$) ^a	
Female	36 (58.1%)
Male	26 (41.9%)
Age ($n=62$; mean $\pm$ s.d.) ^a	53.2 ($\pm$ 14.3)
Tumor size (cm) ($n=43$; mean $\pm$ s.d.) ^a	2.61 ($\pm$ 1.74)
Nodular metastasis ($n=31$) ^a	
Absent	10 (32.3%)
Present	21 (67.7%)
Invasion ($n=30$) ^a	
Absent	5 (16.7%)
Present	25 (83.3%)
Amyloid stroma ($n=46$) ^a	
Absent	16 (34.8%)
Present	30 (65.2%)
<i>RET</i> mutations ($n=87$)	46 (52.9%)
<i>RAS</i> mutations ($n=87$)	11 (12.6%)
<i>RET</i> and <i>RAS</i> WT ($n=87$)	30 (34.5%)

^aIt was not possible to obtain complete clinicopathologic data from all the cases included in the series.

of genomic DNA (25–100 ng) for *RET* was performed by PCR using the Qiagen Multiplex PCR Kit (Qiagen) according to the manufacturer's instructions. For *H-RAS* and *K-RAS*, we used PromegaGoTaq G2 Flexi DNA Polymerase (Promega). The sequencing reaction was performed with the ABI Prism BigDye Terminator Kit (Perkin-Elmer, Foster City, CA, USA), and the fragments were run in an ABI prism 3100 Genetic Analyser (Perkin-Elmer). The sequencing reactions were performed in a forward direction for all the exons, except for exon 2 of *H-RAS*, which was done in reverse direction. An independent PCR amplification/sequencing, in both directions, was performed in positive samples and in samples that were dubious. The genetic analysis was done first in tumoral tissue, and whenever a mutation was found, the germinative line was analysed in blood DNA or adjacent tissue DNA. We did not screen exon 16 for germline *RET* mutations because there was no clinical indication of *MEN2B* phenotype in the cases harbouring somatic mutation in this exon.

Immunohistochemistry

Immunohistochemistry for p-S6 and PTEN was performed in representative tumor tissue sections of 85 cases for PTEN and 84 cases for p-S6 (the remaining two and three cases, respectively, for PTEN and pS6, were excluded due to technical reasons). Briefly, deparaffinised and rehydrated sections were subjected to one of the antigen retrieval treatment methods, in a pressure cooker in 10 mM sodium citrate buffer of pH 6.0 (PTEN) or EDTA buffer of pH 9 (p-S6) for antigen retrieval. The sections were incubated overnight at 4 °C in a humidified chamber with the primary antibody for PTEN (polyclonal, rabbit, 1:75) and p-S6 (polyclonal, mouse, 1:75), both from Cell Signaling Technology, Inc. (Danvers, MA, USA). The detection was obtained with a labelled streptavidin–biotin immunoperoxidase detection system (Thermo Scientific/Lab Vision, Fremont, CA, USA), and the immunohistochemical staining was developed with 3,3'-diaminobenzidine substrate. Omission of the primary antibody incubation was used as negative control. Previously tested samples of thyroid and pancreas were used as positive control. IHC evaluation was performed by two observers (C Eloy and J Lyra), independently. An IHC score was defined using the intensity score for expression (absent=0, faint=1, moderate=2, and strong=3) and the extension score of the expression (0–5%=0, 6–25%=1, 26–50%=2, 51–75%=3, and 76–100%=4). We considered as 'low expression' when

the p-S6 intensity score was 0 or 1, and as 'high expression' when p-S6 intensity score was 2 or 3. For PTEN it was also taken into account the cellular localisation of the staining (nuclear or cytoplasmic) and for IHC evaluation the same score as the one used for p-S6 was applied.

Statistical analyses

Statistical analysis was performed using 21.0 SPSS Statistical Package (SPSS, Inc., 2003). χ^2 , Fisher's exact test, and independent-samples *t*-test were performed in an attempt to correlate *RAS* and *RET* genotype results and clinicopathological data. The same statistical analysis was performed to evaluate immunohistochemical results (presented as intensity expression scores) and *RET* genotype, *RAS* genotype, and clinicopathological data. Values of $P \leq 0.05$ were considered statistically significant.

Results

Genetic analysis

In the 87 MTC cases screened for *RET* oncogene, 52.9% (46/87) were mutated: 9.2% (8/87) were mutated in exon 10, 20.7% (18/87) in exon 11, 1.1% (1/87) in exon 13, 2.3% (2/87) in exon 15, and 20.7% (18/87) in exon 16. We confirmed the germline nature of *RET* mutations in the five familial cases suspicious to be *MEN2A* and we detected five additional germline mutations in five apparently sporadic MTC. In the ten cases with germline mutations, there were seven (70.0%) with mutations in exon 11, one in exon 10 (10.0%), one in exon 13 (10.0%), and one in exon 15 (10.0%).

RAS mutations were detected in 12.6% (11/87) of the tumors: eight in exon 2 of *HRAS* – 9.2% (8/87) – and three in exon 1 of *KRAS* – 3.4%. A mutually exclusive pattern was observed between *RET* and *RAS* mutations; *RAS* mutations were only present in *RET*-negative cases (10, 11, 12). *RAS* mutations were found in 26.8% (11/41) of WT *RET* MTCs.

Patients with MTCs displaying germline *RET* mutations were younger than those with MTCs with a somatic *RET* mutation or no *RET* mutation at all (41.8 years vs 55.2 years, $P=0.008$). We did not find any other significant association between *RET* mutations and gender, tumor size, nodal metastasis, amyloid stroma, or invasive features. A similar analysis for *RAS*-mutated cases did not disclose any associations with any clinicopathological feature (Table 2).

Clinical Study	J Lyra and others	p-S6 and PTEN expression in MTC	171:5	636
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Table 2 Clinicopathologic data, p-S6 expression, and *RET* and *RAS* mutational status.

	<i>RET</i>			<i>RAS</i>			p-S6 expression ^a		
	WT	Mutation ^b	P value	WT	Mutation	P value	Low expression, S≤1	High expression, S≥2	P value
Gender									
Female	15 (55.6%)	21 (60.0%)	0.798	31 (55.4%)	5 (83.3%)	0.387	15 (55.6%)	19 (59.4%)	0.797
Male	12 (44.4%)	14 (40.0%)		25 (44.6%)	1 (16.7%)		12 (44.4%)	13 (40.6%)	
Age	57.1 (±13.9)	50.2 (±13.9)	0.059	52.7 (±13.3)	58.5 (±22.4)	0.345	55.8 (±13.4)	51.8 (±15.1)	0.286
Tumor size (cm)	2.3 (±1.7)	2.8 (±1.8)	0.358	2.6 (±1.8)	3.3 (±1.1)	0.603	2.6 (±1.6)	2.9 (±1.8)	0.561
Nodular metastasis									
Absent	3 (30.0%)	7 (33.3%)	1.000	10 (33.3%)	0 (0%)	1.000	9 (50.0%)	1 (7.7%)	0.046
Present	7 (70.0%)	14 (66.7%)		20 (66.7%)	1 (100.0%)		9 (50.0%)	12 (92.3%)	
Invasion									
Absent	2 (20.0%)	3 (15.0%)	1.000	5 (20.0%)	0 (0%)	–	5 (33.3%)	0 (0%)	0.042
Present	8 (80.0%)	17 (85.0%)		25 (80.0%)	0 (0%)		10 (66.7%)	15 (100.0%)	
Amyloid stroma									
Absent	6 (35.3%)	10 (34.5%)	1.000	15 (34.9%)	1 (33.3%)	1.000	11 (44.0%)	4 (20.0%)	0.118
Present	11 (64.7%)	19 (65.5%)		28 (65.1%)	2 (66.7%)		14 (56.0%)	16 (80.0%)	

P values obtained from χ^2 test realized for gender, nodular metastasis, invasion, and amyloid stroma and Independent t-test for age and tumor size.

^aThese tests were carried out by stratifying p-S6 intensity score in two groups – low expression (0 or 1) and high expression (2 or 3).

^bSomatic or germline *RET* mutations.

p-S6 expression in MTC

Ribosomal protein S6 is part of the mTOR cascade and when phosphorylated it indicates upstream mTOR pathway activation. We assessed mTOR cascade activity by IHC using an antibody for p-S6. Out of the 84 cases evaluated for p-S6 expression, 53.6% (45/84) had high intensity ($S \geq 2$) vs 46.4% (39/84) presenting low intensity ($S \leq 1$) of p-S6 expression (Tables 2 and 3).

We did not observe any significant difference in the intensity score of p-S6 expression between tumors with and without *RET* mutation (germinative or somatic).

Yet, a significant difference in the intensity score of p-S6 was observed in tumors harbouring *RAS* mutation in comparison with WT *RAS* cases ($P=0.009$; Table 3). Furthermore, a Pearson's χ^2 test comparing p-S6 expression groups was applied within the series of *RAS*-mutated cases and showed a statistically significant over-representation of p-S6 high expression cases (90.9% of *RAS*-mutated cases vs 9.1% in WT *RAS*; $P=0.007$).

The intensity of p-S6 expression was significantly associated with the presence of lymph node metastases ($P=0.046$; Table 2). The intensity of p-S6 expression was significantly higher in invasive than in non-invasive

Table 3 p-S6 and *PTEN* intensity expression according to the mutational status of MTC.

	p-S6 expression ^a (n=84)			PTEN cytoplasmic expression ^a (n=85)			PTEN nuclear expression ^a (n=85)		
	Low expression, S≤1	High expression, S≥2	P value	Low expression, S≤1	High expression, S≥2	P value	Low expression, S≤1	High expression, S≥2	P value
MTC (all cases)	39 (46.4%)	45 (53.6%)	–	50 (58.8%)	35 (41.2%)	–	64 (75.3%)	21 (24.7%)	–
<i>RET</i> WT ^b	18 (46.2%)	21 (53.8%)	1.000	24 (58.5%)	17 (41.5%)	1.000	29 (70.7%)	12 (29.3%)	0.452
<i>RET</i> mutation	21 (46.7%)	24 (53.3%)		26 (59.1%)	18 (40.9%)		35 (79.5%)	9 (20.5%)	
<i>RAS</i> WT ^b	38 (52.1%)	35 (47.9%)	0.009	47 (63.5%)	27 (36.5%)	0.045	57 (77.0%)	17 (23.0%)	0.453
<i>RAS</i> mutation	1 (9.1%)	10 (90.9%)		3 (27.3%)	8 (72.7%)		7 (63.6%)	4 (36.4%)	

$P \leq 0.05$ where considered significant.

^ap-S6 and *PTEN* intensity expression was stratified in two groups, according to its intensity score – low expression when intensity score was 0 or 1 and high expression for scores of 2 or 3. χ^2 tests were performed to analyse the differences in proteins expression in mutated vs wild type (WT) cases for *RET* and *RAS*.

^b*RET* WT group includes the cases mutated for *RAS*. In the same way, *RAS* WT group includes cases mutated for *RET*.

tumors ($P=0.042$). No significant association was observed between p-S6 expression and age, tumor size and presence of amyloid stroma (Table 2).

PTEN immunohistochemical expression in MTC

After observing that p-S6 is highly expressed in MTC, we studied *PTEN* expression by IHC assays in an attempt to clarify whether the expression of this mTOR pathway inhibitor could be associated with the aforementioned findings. We observed total absence of *PTEN* expression in three cases (3.5%). High *PTEN* cytoplasmic expression ($S \geq 2$) was present in 41.2% (35/85) of the tumors, whereas 24.7% (21/85) of the MTC disclosed high *PTEN* nuclear intensity. In 41.2% (35/85) of the cases, no nuclear *PTEN* expression was detected ($S=0$) (Tables 2 and 3).

No significant correlation was found between *RET* mutational status and *PTEN* expression intensity or localisation. At variance with this, we observed a significantly lower cytoplasmic *PTEN* expression in WT *RAS* cases than in cases with *RAS* mutation ($P=0.045$; Table 3 and Fig.1).

No significant associations were found regarding clinicopathological features and *PTEN* expression or localisation (Table 2).

Discussion

The recent description of *HRAS* and *KRAS* mutations in sporadic MTC by Moura *et al.* has shaken the *RET* paradigm in MTCs and contributed to relaunch the interest in the molecular study of sporadic MTCs. In this study, we identified the presence of *RAS* mutations in 12.6% of the whole series of MTC (14.3% of the sporadic cases and 28.6% of the *RET*-negative cases), and confirmed that *RAS* mutations occur in a mutually exclusive pattern with *RET* mutation. Besides, this, *RET* mutations were present in 52.9% of the MTC, thus reinforcing the prominent role of such mutations in the development of hereditary and sporadic MTC. We confirmed the hereditary nature of the five suspected cases and found *RET* germinative mutations in five apparently sporadic MTCs. These figures are in accordance with previous studies in MTC (11).

We think that the most interesting results of our study concern the mTOR pathway activation in MTC (13, 15, 17). In a previous study, mTOR pathway expression was found to be frequently detected in MTC, namely in MTC metastatic lesions (13). In our series,

Figure 1

p-S6 and *PTEN* expression in tumours with and without *RAS* mutation. (A) Low p-S6 expression in a WT *RAS* tumour (intensity score ≤ 1); (B) high p-S6 expression in a tumour with *RAS* mutation (intensity score ≥ 2); (C) low *PTEN* expression in a WT *RAS* tumour (intensity score ≤ 1); and (D) high *PTEN* expression in a *RAS* mutated tumour (intensity score ≥ 2). A full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-14-0389>.

we observed mTOR activation in the majority of the tumors, 54.8% of the cases showing high p-S6 expression. mTOR activation in MTC is not surprising since *RET* oncogene is known to promote the activation of RAS/MAPKK/extracellular signal-related kinase (RAS/MEK/ERK) pathway and the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway, controlling cell proliferation, invasion and survival (13).

Like Tamburrino *et al.* (13) we did not find any association between germinative or sporadic *RET* mutations and p-S6 expression. In contrast to this, Rapa *et al.* (15) reported that phospho-mTOR, phospho-AKT, and phospho-p70S6K expression correlated positively with the presence of germline *RET* mutations. The discrepancies between the results obtained in our and Tamburrino *et al.* (13) studies and those reported by Rapa *et al.* (15) may be due to the different elements of mTOR cascade used to evaluate its activity in the three studies. Rapa *et al.* (15) did not evaluate p-S6 protein as we and

Figure 2

Schematic representation of mTOR pathway activation in MTC according to *RET* and *RAS* mutational status. mTOR activation seems to be a common feature of MTC. High expression levels of p-S6 can result from *RAS* or *RET* mutation, as well as from low levels of cytoplasmic *PTEN* in WT *RAS* tumours. A full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-14-0389>.

Tamburrino *et al.* (13) did; in contrast they focused their study on upstream elements of the mTOR pathway (15). Also, the size of the series and the relative composition in terms of sporadic and hereditary *RET*-mutated cases can underlie those differences. Larger series are needed to fully clarify these aspects. Despite these differences, in all the three studies, the prominent expression of mTOR pathway proteins is a recurrent finding in MTC.

Interestingly, we observed that *RAS* mutation was significantly associated with higher intensity of p-S6 expression ($P=0.007$). To our knowledge, this is the first study reporting p-S6 evaluation in *RAS*-mutated MTCs. *RAS* itself stimulates numerous intracellular transducers, including PI3K, which is part of mTOR cascade and, as so, could mediate the activation we observed in MTCs with *RAS* mutation (18, 19; Fig.2).

In our series, p-S6 expression was significantly higher in invasive tumors and in tumors presenting lymph node metastases, a finding that supports the results reported by Tamburrino *et al.* (13) who described an increased p-S6 expression in metastases from MTC in comparison with autologous matched primary MTCs. Altogether, these results suggest that p-S6 expression can be an indicator of increased metastatic capacity of MTC.

Another interesting result of this study concerns the possible dissociation between *RAS* mutation and pS6 expression with regard to the invasive capacity of MTCs. It seems that the overexpression of pS6, regardless of the presence of *RAS* mutation or its absence, is associated with a significantly higher invasive capacity of MTCs. The study of a larger series with more cases with and without *RAS* mutation and detailed clinical information is necessary to confirm the aforementioned putative role of p-S6 as a biomarker of MTC aggressiveness.

The contribution of *PTEN*, an inhibitor of mTOR pathway, has not been addressed before, in MTC, to the best of our knowledge. The analysis of *PTEN* in our series revealed that *PTEN* expression was present in almost every tumor (only three cases were negative for this protein). We observed that a significant lower cytoplasmic expression of *PTEN* was present in WT *RAS* cases ($P=0.045$). Taking into consideration that approximately half (35/73 – 47.9%) of WT *RAS* cases showed high p-S6 expression, the aforementioned decrease in *PTEN* expression may serve as an alternative mechanism for mTOR pathway activation. Indeed, when we consider only the tumors with high p-S6 expression and compare WT *RAS*- and *RAS*-mutated tumors, there is a significant association of low *PTEN* expression with WT *RAS* cases (62.9% of WT *RAS* cases vs 20.0% in *RAS* mutated; $P=0.029$). This result suggests that low *PTEN* expression is not a central mechanism of mTOR pathway activation in *RAS*-mutated cases, but it may be crucial to drive mTOR activation in WT *RAS* tumors.

Loss of *PTEN* function is not only related to mTOR pathway activation. It has been proposed that *PTEN* may also play a role inside the nucleus, where it regulates chromosomal integrity, cell cycle progression, p53 acetylation, and DNA-damage response through the interaction with RAD51 as well as in apoptosis induction (20). Putting our results together it seems that the mechanisms of action of *PTEN* may be twofold: on one hand the decrease in *PTEN* cytoplasmic expression can lead to a higher mTOR activity, and on the other hand, the high nuclear *PTEN* expression observed in some of our cases can also influence other cellular processes and putatively contribute to

tumorigenesis. Further studies are necessary to evaluate whether or not PTEN plays a major role in this setting.

It has been demonstrated that MTC cancer cell lines are sensitive to mTOR inhibitors (3, 4, 13, 21). It was also observed, in melanoma (22, 23), that sensitivity to an mTOR inhibitor was correlated with pre-treatment p-S6 levels, but not with other downstream effectors of this pathway (PI3K and p-AKT) (22, 23). Similarly, in a phase II study of everolimus in gastric cancer refractory to chemotherapy, higher p-S6^{Ser240/4} expression was significantly associated with a higher rate of disease control and achievement of clinical response or stable disease. Therefore, p-S6 expression can be a potential predictive biomarker for everolimus response (24). Further studies are necessary to verify whether p-S6 expression in MTC may represent a predictive sensitivity biomarker to mTOR inhibitors.

Summing up, we report, for the first time, an association between the activation of the mTOR pathway and the presence of RAS mutations in MTCs. Further studies are needed to confirm the usefulness of p-S6 expression as an indicator of invasive capacity of MTC and as a predictive biomarker for the treatment of MTC patients with mTOR inhibitors.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-14-0389>.

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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Author contribution statement

J Lyra, V Pinto, R Batista, and J Vinagre performed the experimental work (mutational and IHC analysis). M Sobrinho-Simões, C Eloy, and J Lyra performed the histological review of the cases and immunohistochemical evaluation. H Prazeres and F Rodrigues collect cases and clinical information. P Soares supervised the study, analysed the data, and wrote the first draft together with J Lyra. M Sobrinho-Simões performed a critical review of the MS. All the authors contributed to the writing of the final version.

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