Mammalian Target of Rapamycin Pathway Activation Is Associated to RET Mutation Status in Medullary Thyroid Carcinoma

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Context: The genetic pathways involved in medullary thyroid carcinomas (MTC), except for RET mutations, are largely unknown, as is the detailed mapping of proteins activated as a consequence of RET tyrosine kinase phosphorylation.

Objective: The present study was designed to screen for the presence of mutations in other genes downstream to RET activation and to detect the activation patterns of a panel of intracellular regulators of cell growth.

Design: Forty-nine cases of MTC were analyzed for mutations in RET, BRAF, N-, H-, and K-RAS, and phosphatidylinositol-3 (PI3) kinase genes. Immunohistochemical analysis was performed using antibodies against several intracellular transducers. The effect of mammalian target of rapamycin (mTOR) inhibition was assessed *in vitro* onto TT cells by means of methyl thiazolyl tetrazolium and Western blot assays.

Results: BRAF, K-, H-, and N-RAS, and PI3 kinase mutations were absent in all cases examined. Germline RET mutations were detected in 20% of cases overall, whereas somatic RET mutations represented 53% of sporadic tumors. RET mutational status was associated to age, presence of multifocal tumors, and nodal status, but not disease outcome. Protein expression of markers investigated was highly heterogeneous, with a strong association between phospho-mTOR, phospho-AKT, and phospho-p70S6K, positively correlated to the presence of germline RET mutations. Moreover, selective mTOR inhibition affected cell proliferation of RET-mutant TT cells.

Conclusions: Taken together, our findings indicate that mTOR intracellular signaling pathway is functionally activated in MTC with a preferential expression in cases with germline RET mutations; genes downstream to RET tyrosine kinase such as BRAF, RAS isoforms, and PI3 kinase are not mutated in MTC. **(*J Clin Endocrinol Metab* 96: 2146–2153, 2011)**

Medullary thyroid cancer is a neuroendocrine tumor derived from thyroid C cells, and it accounts for approximately 5% of all thyroid malignancies (1). Germline RET mutations are responsible for inherited tumors (25% of the cases), whereas somatic RET mutations occur in one third of sporadic cases. However, the genetic pathways involved in RET wild-type medullary carcinomas are largely unknown.

Few data from genomic profiling studies— by means of high-resolution array comparative genomic hybridization— detected clusters of allelic imbalances (mostly losses) frequently occurring in medullary carcinomas, al-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2010-2655 Received November 10, 2010. Accepted April 8, 2011.
First Published Online May 4, 2011

Abbreviation: mTOR, Mammalian target of rapamycin; PI3, phosphatidylinositol-3.

TABLE 1. PCR and sequencing primer sequences used for RET and PI3K mutations analysis

Gene (exon)	Codons	Forward PCR primer	Reverse PCR primer
RET (11) [*NG_007489*]	630–634	5′-AGCCATGAGGCAGAGCATACG-3′	B-5′-GACAGCGGCTGCGATCAC-3′
RET (15) [*NG_007489*]	883	B-5′GGGACTTGGCAGCCAGAAA-3′	5′-GGGACAAGCCGAAATCCG-3′
RET (16) [*NG_007489*]	918	B-5′GGGCCTGGCCTTCTCCTTTA-3′	5′-CGTCGTGGCCCCACTACA-3′
PI3K (9) [*NG_012113*]	542–546	5′-GCACTTACCTGTGACTCCATAGAA3′	B-5′-CAGCTCAAAGCAATTTCTACACG3′
PI3K (20) [*NG_012113*]	1007–1031	5′-TAGCTATTCGACAGCATGCCAAT-3′	B-5′-TGAAATACTCCAAAGCCTCTTGC-3′
PI3K (20) [*NG_012113*]	1041–1060	5′-AGCAAGAGGCTTTGGAG-TATTTC-3′	B-5′-GCCTGCTGAGAGTTATTAACAGTG-3′

GenBank accession numbers are shown in *brackets*. B, Biotin; Ann T, annealing temperature; bp, base pairs.

though such genetic abnormalities were similar in cases without RET mutations as well as in those harboring somatic or germline point mutations (2). These data support the existence of several putative suppressor genes involved in medullary carcinoma tumorigenesis, although the lack of association with RET mutations seems to suggest that most, if not all, such abnormalities are acquired during cancer progression rather than associated to early initiation. Gene expression profiling on cellular models harboring different RET mutations (MEN2A and MEN2B) showed that these mutants modulated genes with similar functional ontologies (3). Moreover, microarray analysis of medullary carcinoma tumor samples identified two separate clusters of tumors, with specific genes—such as pleiotrophin, further validated by real-time PCR analysis and immunohistochemistry—associated with aggressive features, but not with specific RET mutation status (4). Taken together, all these data support the existence of peculiar molecular profiles in medullary thyroid cancer that are not directly related to the presence and type of activating mutations of the RET gene.

Thus, the molecular background of tumors with wild-type RET status remains unclear. Mutations in genes frequently involved in thyroid tumorigenesis, such as RAS isoforms, BRAF, and phosphatidylinositol-3 (PI3) kinase have been investigated in medullary thyroid carcinoma only in a limited series of cases. The latter gene has been tested in 13 cases (all negative) in a single study (5), whereas BRAF gene has been found to lack mutations in two small series (6, 7) but was apparently mutated in up to 68% of cases in another series of 44 cases from Greece (8). In this same study, K-RAS mutations were also detected in about 40% of medullary thyroid cancers, a finding that has never been confirmed or called into question in the literature.

Concerning protein expression profiling, several markers related to growth control and invasion/metastatic potential have been tested in medullary carcinoma, but a single study compared the expression of one of these with RET mutation status (9); in this study, nuclear factor-κB, a molecule involved in several biological responses including proliferation, migration, and apoptosis and activated by multiple intracellular pathways, was found to be altered more frequently in RET-mutated compared with RET-wild-type cases. By contrast, proteins that are activated as a consequence of RET tyrosine kinase phosphorylation have never been extensively investigated in tumor samples of medullary thyroid carcinoma. These molecules include phospho-MAPK (10) and Stat-3 (11) that are directly activated by RET tyrosine kinase, and galectin-3, which has been shown to be expressed in medullary thyroid cancer (12) and modulated by nuclear factor-κB (13). Moreover, several examples of evidence support the activation of the AKT/mammalian target of rapamycin (mTOR) signaling cascade in medullary thyroid cancer (14, 15), and this pathway seems to be a candidate for target therapies in medullary thyroid carcinoma due to *in vitro* evidence that indirect blocking, such as the use of PI3 kinase inhibitors (16), or direct inhibition of mTOR (17) mediates induction of apoptosis and decrease in cell viability of medullary carcinoma TT cell line.

Based on such a heterogeneous background, the present study was designed in a large series of medullary thyroid carcinomas with a 3-fold aim: 1) to screen for the presence of mutations in several genes involved in thyroid carcinogenesis; 2) to detect the prevalence of expression of a panel of intracellular regulators of cell growth; and 3) to compare their activation patterns with RET mutation status.

Subjects and Methods

Case selection

Sixty-five cases of medullary thyroid carcinoma were selected from the databases of the Division of Pathology of the University of Turin (from years 1983 to 2007). Eleven cases occurred as familial tumors in the setting of MEN2 syndrome (one case as familial medullary thyroid cancer, eight cases as MEN2A, and two cases as MEN2B forms), as proved by genetic testing of RET exons 8, 10, 11, and 13–16 in peripheral blood samples performed elsewhere, determining the presence of germline RET mutations. The remaining cases were apparently sporadic. Inclusion criteria were the availability of representative hematoxylin-eosin sections for histological review and paraffin blocks for immunohistochemistry and molecular testing, and of clinical pathological information.

The study has been approved by the Institutional Review Board of San Luigi Hospital. All pathological material was de-

TABLE 1. Continued

Sequencing primer	Ann T (C)	Product size (bp)
5′-CCCACCCACAGATCC-3′	55	132
5′-TTCATCTTCCGCCCC-3′	55	72
5′-ATATGATCAAAAAGGGATTCA-3′	57	221
5′-CCTGTGACTCCATAGAAAA-3′	57	111
5′-TCTTCATAAATCTTTTCTCA-3′	55	153
5′-CAAGAGGCTTTGGAGTA-3′	57	181

identified using codes that were not available to the researchers involved in the study.

Molecular analysis

Nucleic acid isolation

Genomic DNA was isolated from formalin-fixed, paraffin-embedded, neoplastic and corresponding normal thyroid tissues using the standard proteinase K-phenol-chloroform extraction method. RNA was isolated from paraffin-embedded material using the high pure RNA paraffin kit (Roche, Mannheim, Germany) following manufacturer's instructions. The quantity of isolated DNA and RNA was assessed using a Biophotometer (Eppendorf, Hamburg, Germany).

Point mutation analysis

The presence of *BRAF* V600E and K601E, *NRAS* codon 61, *HRAS* codon 61, and *KRAS* codons 12 and 13 point mutations was analyzed using pyrosequencing method and PCR primers following previously published protocols (18). RET exons 11, 15, and 16 and PI3 kinase exons 9 and 20 were analyzed in both tumor and normal peritumoral thyroid tissue by means of pyrosequencing method. PCR and sequencing primers were designed using the PSQ Assay Design Software version 1.0.6 (Biotage AB, Uppsala, Sweden). One primer of each pair has a 5′-biotin label necessary for post-PCR processing. The corresponding primer sequences, amplicon sizes, and annealing temperatures are shown in Table 1. PCR amplification for the pyrosequencing assay was performed according to standard protocols. The amplicons were mixed with sequencing primers, and sequencing was performed using a PyroGold reagent Kit (Biotage AB) according to the manufacturer's protocol. Results were analyzed using the PSQ-96 MA 2.0.2 software.

Immunohistochemistry

Eleven tissue microarrays were prepared for immunohistochemical analysis using the Quick-RAY tissue arrayer (Bio-Optica, Milan, Italy). For each case, two different samples of tumor tissue were selected from the more representative hematoxylin-eosin-stained slide, and tissue cylinders with a diameter of 3 mm were punched under the stereomicroscope from the specific areas of the "donor" block and brought into the "recipient" paraffin block.

The cases were analyzed by means of immunohistochemistry using the following antibodies: phospho-mTOR (rabbit monoclonal, 49F9, Ser2448, diluted 1:100; Cell Signaling Tech, Beverly, MA), phospho-p70S6K (mouse monoclonal, 1A5, Thr389, diluted 1:400; Cell Signaling), phospho-AMPK (rabbit monoclonal, 40H9, Thr172, diluted 1:100; Cell Signaling), phospho-AKT (rabbit monoclonal, 736E11, Ser473, diluted 1:1000; Cell Signaling), Stat-3 (mouse monoclonal, F-2, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-MAPK (p44–42) (polyclonal, Thr202/Tyr204, diluted 1:250; Cell Signaling), and galectin-3 (rat monoclonal, diluted 1:500; Mabtech, Nacka, Sweden).

Immunohistochemical reactions were performed according to standard automated immunohistochemical procedure (Dako Autostainer, Glostrup, Denmark), and immunoreactions were revealed by means of a biotin-free, dextran-chain detection system (Envision; DakoCytomation, Glostrup, Denmark) and developed using diaminobenzidine as the chromogen. For all antibodies, immunohistochemical staining was scored as a sum of the two scores as follows: score 0, no immunoreactivity; score 1, no more than 25% of positive tumor cells; score 2, 25–50% of positive tumor cells; and score 3, more than 50% of positive tumor cells. For statistical purposes, scores were considered individually for Spearman correlation test and were grouped as "negative" (score 0 and 1) and "positive" (scores 2 and 3) for clinical pathological and molecular correlations.

Cell culture, proliferation assay, and Western blot

The RET germline mutant MTC cell line (TT) was purchased from ATCC (LGC Standards s.r.l., Sesto San Giovanni, Milan, Italy); a pancreatic neuroendocrine tumor cell line (BON-1) was kindly provided by Dr. Bertram Wiedenmann (University of Berlin) and served as a control. Cell lines were maintained in Ham's F12 (TT) and in 1:1 DMEM/F12 mixture (BON-1) supplemented with 10% fetal calf serum, 2 nM glutamine, penicillin (25 U/ml), and streptomycin (25 μg/ml) (all from Sigma-Aldrich, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37 C. BON-1 and TT cell lines were plated into 96-well plates in triplicate and treated with different doses of RAD001 (from 0.1 nmol/liter to 1 μmol/liter; Novartis Pharma AG, Basel, Switzerland) for 48 and 72 h. Cell viability was evaluated by adding 0.5 mg/ml methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich) solution, incubating for 4 h, then adding 100 μm dimethyl sulfoxide. The 570-nm absorbance was measured using a microplate reader (model 540; Bio-Rad, Hercules, CA), and differences were evaluated by means of Student's *t* test.

Western blot experiments were performed on protein extracts at basal or after 24 h of treatment with different doses of RAD001 (from 0.1 to 10 nmol/liter). All samples were homogenized and lysated in Tris-NaCP-EDTA lysis buffer supplemented with 1% protease inhibitor cocktail (Complete; Roche Diagnostic Corporation, Indianapolis, IN). The protein concentration was evaluated using BCA protein assay kit (Pierce, Milwaukee, WI), and 50 μg of protein were resolved in 8% SDS-PAGE and transferred to nitrocellulose membranes for each experiment. The membrane blots were blocked for 1 h with 5% BSA in Tris-buffered saline-Tween 0.1% and incubated over-

TABLE 2. Clinical pathological features of 49 cases of medullary carcinoma according to RET mutational status

RET mutational status	F/M ratio	Mean age ± SEM (yr)	Multifocal (rate)	Mean size ± SEM (mm)	pT ≥3 stage (rate)	pN+ stage (rate)	AWD/DOD status (rate)
Absence of RET mutations (18)	1.2	60.3 ± 2.2	2/17	30.92 ± 5.3	5/17	4/12	4/13
Sporadic RET mutations (20)	3	55.3 ± 3.1	3/20	26.7 ± 3.4	8/20	11/13	7/13
Germline RET mutations (11)	2.6	40.4 ± 6.2	6/10	21.6 ± 3.5	4/10	7/9	5/10
P all groups	*P* = 0.32	***P* = 0.003**	***P* = 0.008**	*P* = 0.45	*P* = 0.77	***P* = 0.017**	*P* = 0.45
P RET− *vs.* RET+	*P* = 0.24	***P* = 0.03**	*P* = 0.29	*P* = 0.32	*P* = 0.68	***P* = 0.014**	*P* = 0.37
P sporadic *vs.* germline RET+	*P* = 0.77	***P* = 0.02**	***P* = 0.03**	*P* = 0.35	*P* = 0.69	*P* = 0.88	*P* = 0.81
IM RET mutations (13)	3.3	45.1 ± 4.7	33.3%	22.7 ± 3.2	3/12	6/9	3/12
TK RET mutations (18)	2.6	53.6 ± 4.2	27.7%	26.6 ± 3.7	9/18	12/13	9/15
	P = 0.90	*P* = 0.19	*P* = 0.93	*P* = 0.46	*P* = 0.32	*P* = 0.33	*P* = 0.21

IM, Mutations in the iuxta-membrane domain; TK, mutations in the tyrosine kinase domain; F, female; M, male; AWD, alive with disease status; DOD, died of disease status; pT, pathological tumor stage; pN, pathological node stage. *Bold* indicates statistical significance. Numbers in parentheses indicate number of cases.

night at 4 C with the same phospho-mTOR and phospho-p70S6K antibodies used for immunohistochemistry and β-actin (Santa Cruz Biotechnology), all diluted 1:1000. Immunoreactive proteins were visualized using horseradish peroxidase-conjugated antimouse or antirabbit antibody (1:3000 and 1:1000, respectively) and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) as the substrate. The OD of the appropriately sized bands was measured using the ImageJ free software (http://rsbweb.nih.gov/ij/).

Statistical analysis

The correlation between genetic alterations, known clinical pathological parameters, and immunohistochemical findings was assessed by χ^2 test or nonparametric *t* test. Univariate survival analysis was based on the Kaplan-Meier product limit estimate of overall survival distribution. Unadjusted differences between survival curves were tested using the log rank test. The Spearman test was used to analyze the correlation index among markers expression. The level of significance was set at $P = 0.05$. Statistical analysis was performed using the GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) and SPSS (SPSS Inc., Chicago, IL) software.

Results

Molecular profile

Sixteen cases were excluded from further molecular analysis due to very low amount or poor quality of DNA extracted.

BRAF; K-, H-, and N-RAS; and PI3 kinase mutations were absent in all cases examined. RET germline mutations in MEN2 patients, detected elsewhere, included Val804Met (one case), Cys618Arg (two cases), Cys634Tyr (five cases), Cys634Arg (one case), and Met918Thr (two cases) substitutions. The remaining 38 clinically sporadic cases showed no mutations in exons 11, 15, and 16 in nontumoral DNA, whereas tumor DNA harbored the Met918Thr mutation in 16 cases, the Cys630Ala mutation in two cases, the Cys634Arg mutation in one case, and the delta6 mutation in exon 11 (nucleotides 1894–1899 of the corresponding RET cDNA) (19) in one case.

RET mutational status was associated to age, presence of multifocal tumors, and nodal status (Table 2): mean age was lower in RET-positive cases ($P = 0.03$) and, among them, in patients with germline mutations ($P = 0.023$); multifocality was present in a minority of sporadic tumors but associated to the presence of germline RET mutations

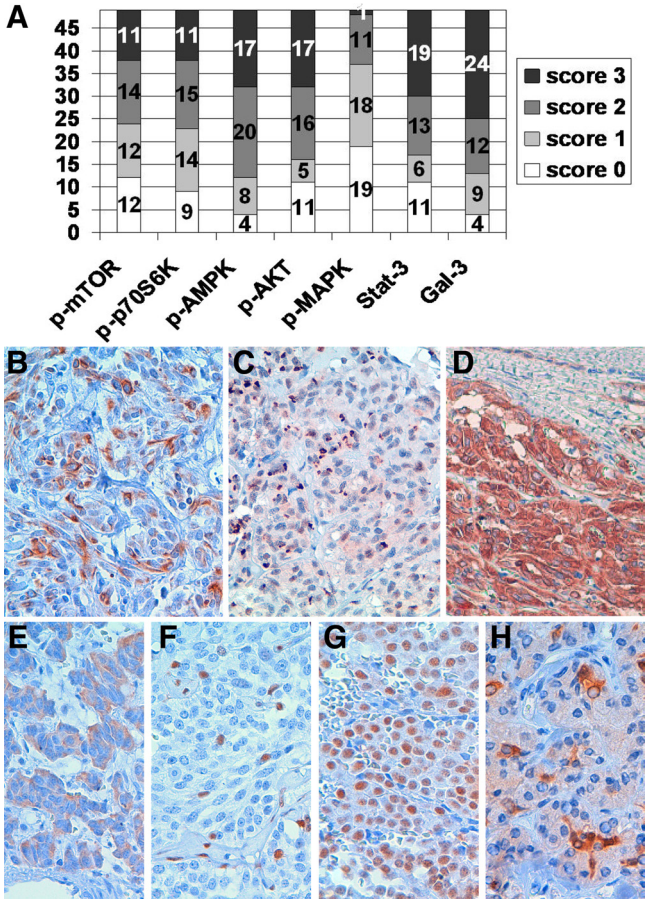

FIG. 1. A, Graph distribution of individual immunohistochemical markers in medullary carcinomas, divided according to immunohistochemical score. B–H, Representative illustrations of phospho-mTOR, phospho-p70S6K, phospho-AMPK, phospho-AKT, phospho-MAPK, Stat-3, and galectin-3, respectively (immunoperoxidase; original magnification, ×400 for all panels).

TABLE 3. Correlation among the immunohistochemical expression of investigated molecules according to the Spearman method

	p-p70S6K	p-AMPK	p-AKT	p-MAPK	STAT-3	GAL-3
p-mTOR	**R = 0.4636**	R = −0.037	**R = 0.304**	R = 0.1581	R = 0.2721	R = 0.2176
P	**0.0012**	0.8047	**0.024**	0.2884	0.0643	0.1417
p-p70S6K		R = 0.2183	**R = 0.2716**	**R = 0.3382**	R = 0.2404	**R = 0.3420**
P		0.1450	**0.0419**	**0.0215**	0.1036	**0.0215**
p-AMPK			R = 0.0539	R = 0.2541	R = 0.1513	R = 0.0872
P			0.7216	0.0884	0.3156	0.5688
p-AKT				R = 0.1665	R = 0.1301	R = 0.1368
P				0.2742	0.3942	0.3757
p-MAPK					0.1313	0.1525
P					0.3789	0.3115
STAT-3						−0.2223
P						0.1377

Bold indicates statistical significance.

($P = 0.008$); moreover, the presence of lymph node metastases was associated to RET point mutations ($P = 0.014$), irrespective of somatic or germline. By contrast, the presence or type of RET mutations was not correlated to sex, tumor size, stage, disease status, and survival.

Immunohistochemistry

Immunohistochemical evaluations were performed in the 49 cases suitable for molecular analysis. The distribution of individual scores for each marker is reported in Fig. 1. Positivity (scores 2 and 3) ranged from 24% for phospho-MAPK to 76% for phospho-AMPK. Correlation analysis (Table 3) demonstrated a strong association between phospho-mTOR, phospho-p70S6K, and phospho-AKT, suggesting an activated status of the AKT/mTOR pathway in these cases; phospho-p70S6K was also slightly associated with phospho-MAPK (R = 0.33) and galectin-3 (R = 0.34), but phospho-AKT and phospho-mTOR were not. With respect to clinical pathological correlation, none of the markers investigated was directly associated to clinical (sex, age, disease status) or pathological (size, multifocality, pathological stage) features. The presence of positive phospho-mTOR immunoreactivity was more prevalent in node-negative (69% of cases) compared with node-positive cases (36% of cases), although not reaching statistical significance ($P = 0.102$). By means of univariate analysis, all markers investigated failed to demonstrate a prognostic impact.

When checking the expression of these markers in correlation with RET mutation status (Table 4), phospho-mTOR and phospho-p70S6K showed the same profile, being expressed predominantly in cases harboring germline RET mutations, irrespective of whether the mutations were in codons coding amino acids in the iuxta-membrane or tyrosine kinase portion of the receptor. Phospho-MAPK was positive in cases with RET mutations only, both sporadic or germline, except for one sporadic case lacking somatic RET mutation.

In vitro mTOR pathway inhibition

TT and BON-1 cell lines showed a differential sensitivity to mTOR inhibition (Fig. 2) with a significant reduction of cell viability at high doses. Western blot analysis showed that in both cell lines RAD001 strongly inhibited phosphorylation status of phospho-mTOR and phospho-p70S6K.

TABLE 4. Immunoprofiling of 49 cases of medullary carcinoma according to RET mutational status

	Rate immunohistochemistry +ve cases						
RET mutational status	**p-mTOR**	**p-p70S6K**	**p-AMPK**	**p-AKT**	**p-MAPK**	**Stat-3**	**Gal-3**
Absence of RET mutations (18)	11/18	7/18	11/18	10/18	1/18	11/18	13/18
Sporadic RET mutations (20)	5/20	9/20	16/20	16/20	6/20	12/20	15/20
Germline RET mutations (11)	9/11	10/11	10/11	7/11	5/11	9/11	8/11
P all groups	***P* = 0.006**	***P* = 0.016**	*P* = 0.16	*P* = 0.27	***P* = 0.04**	*P* = 0.42	*P* = 0.98
P RET− *vs.* RET+	*P* = 0.43	*P* = 0.22	*P* = 0.15	*P* = 0.23	***P* = 0.045**	*P* = 0.87	*P* = 0.85
P sporadic *vs.* germline RET+	***P* = 0.008**	***P* = 0.034**	*P* = 0.78	*P* = 0.88	*P* = 0.64	*P* = 0.40	*P* = 0.77
IM RET mutations (13)	6/13	9/13	11/13	7/13	5/13	10/13	9/13
TK RET mutations (18)	8/18	10/18	15/18	16/18	6/18	11/18	14/18
	P = 0.78	*P* = 0.69	*P* = 0.69	*P* = 0.07	*P* = 0.93	*P* = 0.59	*P* = 0.90

IM, Mutations in the iuxta-membrane domain; TK, mutations in the tyrosine kinase domain; IHC, immunohistochemistry; Gal-3, galectin-3.

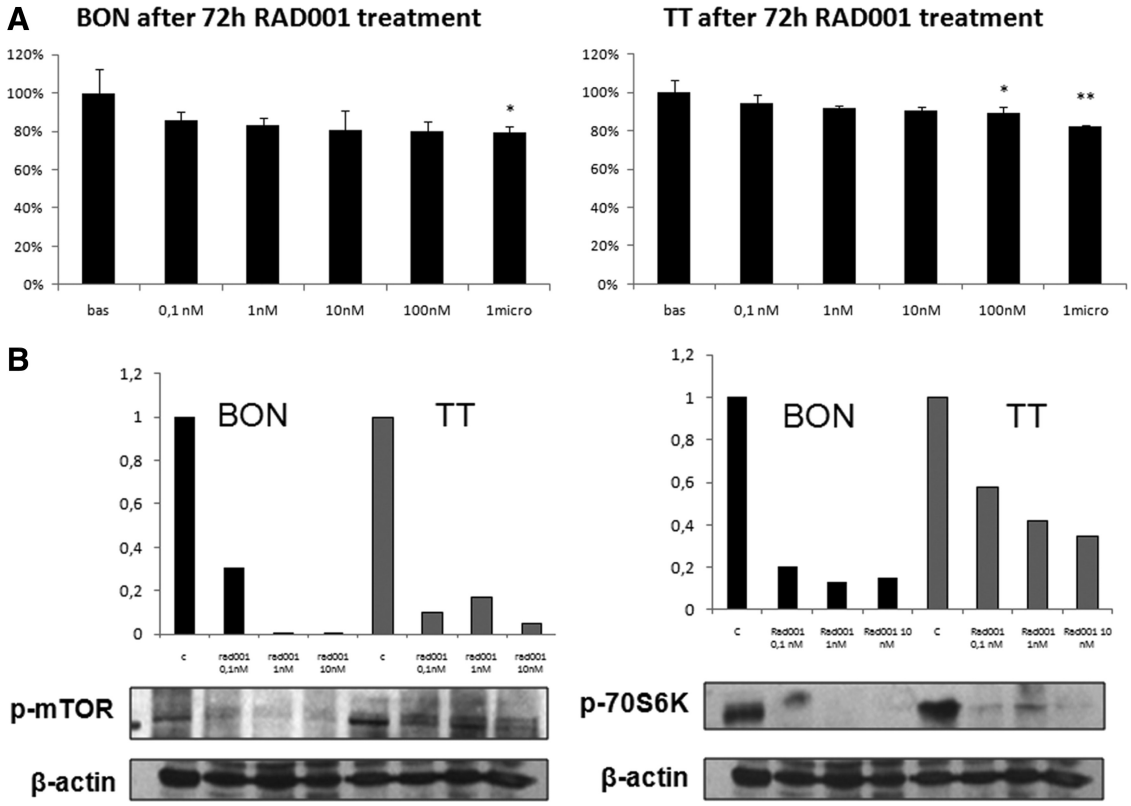

FIG. 2. Methyl thiazolyl tetrazolium assay (A) and Western blot analysis (B), including densitometric graph and corresponding blot of BON-1 and TT cells under RAD001 treatment. *, $P < 0.05$; and **, $P < 0{,}001$, compared with untreated cells. bas, Basal condition.

Discussion

Seventeen years passed since the discovery that RET protooncogene mutations are responsible for inherited (20) and sporadic (21) medullary thyroid carcinoma. However, RET mutations remain the only well-recognized genetic alteration occurring in this tumor type, bearing also prognostic significance (22), whereas the molecular pathogenesis of up to two thirds of sporadic cases remains unclear. Point mutations of genes involved in thyroid tumorigenesis and linked to RET pathway have been screened in limited case series. Therefore, a first aim of the present study was to determine in RET wild type as well as in RET mutated medullary carcinomas the occurrence of point mutations in genes involved in thyroid tumorigenesis and acting downstream to RET tyrosine kinase activation. Our study results confirm the data already published in a small series of cases (5) showing that the PI3 kinase gene is not mutated in medullary thyroid cancer, and are in line with the majority of the authors that failed to demonstrate the presence of BRAF mutations in this tumor type, whereas they contrast with the very high prevalence encountered in a Greek study (8); although ethnical differences are to be considered, the significance of such a discrepancy is unclear. Moreover, we could not confirm the high prevalence of K-RAS mutations described in this same study (8), and we could not detect any mutation in N- and H-RAS isoforms, frequently altered in several thyroid tumor histotypes. So far, we could not detect any molecular alteration in the genes investigated, either associated to RET mutations or in RET wild-type tumors in our series of medullary carcinomas.

The further step of this study was therefore to analyze the protein expression of a panel of signal transducers that act downstream to RET tyrosine kinase activation (23) and have never been investigated in medullary carcinoma compared with the RET molecular profile. This approach had the dual scope of defining protein profiles of both pathogenetic and therapeutic interest because several intracellular transducers, including those analyzed in this study, are relevant for tumor initiation and progression but are also potential candidates for targeted therapies (24). Although the analysis of phosphorylated proteins in formalin-fixed, paraffin-embedded tissue samples using specific antibodies is potentially affected by artifacts related to tissue preservation, the immunohistochemical approach allows the evaluation of retrospective series with long follow-up data and the cellular/subcellular localization of target proteins, as well. Our data at the tissue level showed that the AKT/mTOR pathway is highly activated in medullary carcinoma due the strong reciprocal association between the molecules involved in this intracellular cascade. Moreover, phospho-mTOR and its downstream

activator phospho-p70S6K were correlated to RET mutation status because the vast majority of cases with germline RET mutations were positive for both markers. Interestingly, the association was not depending on the type (codons of the iuxta-membrane or of the tyrosine kinase portion of the receptors) but on the hereditary nature of the mutation. By contrast, medullary carcinomas with sporadic RET mutations or with RET wild type showed heterogeneous expression of AKT/mTOR pathway molecules, thus suggesting alternative activation mechanisms that still need to be further elucidated. A major limitation of this study is represented by the lack of *in vitro* MTC models harboring different (germline *vs.* somatic) or lacking RET mutations that might be employed to speculate on the molecular mechanisms subtending the apparently selective activation of mTOR pathway by specific RET mutations, and our *in vitro* data on TT cells could only support the functional activation of mTOR pathway but not prove a mechanistic link with the presence of a germline RET mutation. It is of clinical relevance, however, irrespective of the pathogenetic interest of these observations, that about 50% of cases with medullary carcinoma showed expression of phospho-mTOR because, at least in *in vitro* lung carcinoid models, endogenous levels of this molecule have been shown to be indicative of response to mTOR inhibitors (25). Moreover, we could confirm previously published data *in vitro* (17) that the selective inhibition of mTOR pathway in a germline-RET mutated MTC cell line is effective to decrease cell viability and block the phosphorylated status of mTOR signaling molecules. As for the other molecules investigated, a heterogeneous distribution was observed in our case series, with the exception of phospho-MAPK that was expressed almost exclusively in cases harboring somatic or germline RET mutations, a finding that suggests a potential role of MAPK inhibition as a therapeutic strategy for RET-mutated MTC that needs to be validated in *in vitro* and *in vivo* studies. However, none of the markers investigated was inversely correlated with any of the other molecules, thus suggesting the absence of mutually exclusive intracellular signaling activation patterns in medullary carcinoma that would have supported unique, although undiscovered, molecular alterations with special reference to RET wild-type cases. Clinical pathological correlations failed to show any association between the markers investigated and any parameter considered (including survival), demonstrating that none of these markers is associated to prognosis, which conversely still remains largely unpredictable, with stage still being the most relevant prognostic indicator (26).

Taken together, our findings indicate that intracellular signaling pathways are heterogeneously activated in medullary thyroid carcinoma; RET mutation profile is associated to the preferential expression of AKT/mTOR and, to a lower extent, MAPK signaling, but in the absence of mutations in genes downstream to RET tyrosine kinase such as BRAF, RAS isoforms, and PI3 kinase, the mechanisms responsible for the functional activation of intracellular signaling cascades promoting cell growth are still unknown in medullary thyroid cancer.

Acknowledgments

The authors thank Professor Gianni Bussolati, University of Turin, for providing part of the cases. The paper is also dedicated to him in the year of his retirement.

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This work was supported by grants of the Italian Ministry of University, Rome (ex-60% to M.V. and M.P.), Regione Piemonte (RSF, DD n. 12 dated 14.01.2008, to M.V.), and unrestricted grants from Novartis Pharma (FT75/08, to M.V.; FT81/07, to M.P.).

Disclosure Summary: M.P. and M.V. received lecture fees from Novartis Pharma, and the paper is partially supported from unrestricted grants from Novartis Pharma to both of them. All other authors have nothing to disclose.

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