Activation of the mTOR Pathway in Primary Medullary Thyroid Carcinoma and Lymph Node Metastases

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Abstract

Purpose: Understanding the molecular pathogenesis of medullary thyroid carcinoma (MTC) is prerequisite to the design of targeted therapies for patients with advanced disease.

Experimental Design: We studied by immunohistochemistry the phosphorylation status of proteins of the RAS/MEK/ERK and PI3K/AKT/mTOR pathways in 53 MTC tissues (18 hereditary, 35 sporadic), including 51 primary MTCs and 2 cases with only lymph node metastases (LNM). We also studied 21 autologous LNM, matched to 21 primary MTCs. Staining was gradd on a 0 to 4 scale (S score) based on the percentage of positive cells. We also studied the functional relevance of the mTOR pathway by measuring cell viability, motility, and tumorigenicity upon mTOR chemical blockade.

Results: Phosphorylation of ribosomal protein S6 (pS6), a downstream target of mTOR, was evident (S ≥ 1) in 49 (96%) of 51 primary MTC samples. This was associated with activation of AKT (phospho-Ser473, S > 1) in 79% of cases studied. Activation of pS6 was also observed (S ≥ 1) in 7 (70%) of 10 hereditary C-cell hyperplasia specimens, possibly representing an early stage of C-cell transformation. It is noteworthy that 22 (96%) of 23 LNM had a high pS6 positivity (S ≥ 3), which was increased compared with autologous matched primary MTCs (P = 0.024). Chemical mTOR blockade blunted viability (P < 0.01), motility (P < 0.01), and tumorigenicity (P < 0.01) of human MTC cells.

Conclusion: The AKT/mTOR pathway is activated in MTC, particularly, in LNM. This pathway sustains malignant features of MTC cell models. These findings suggest that targeting mTOR might be efficacious in patients with advanced MTC. Clin Cancer Res; 18(13); 3532–40. ©2012 AACR.

Introduction

Medullary thyroid carcinoma (MTC) arises from neural crest–derived thyroid-C cells and represents 5% to 8% of thyroid cancer cases (1). MTC is sporadic in about 75% of cases and in the remainder it is inherited as a component of the multiple endocrine neoplasia type 2 (MEN2) syndromes [MEN2A, MEN2B, and familial MTC (FMTC); ref. 1]. C-cell hyperplasia (CCH), confined to the upper lobes of the thyroid, is a preneoplastic lesion in familial patients with MTC. Some investigators consider CCH an in situ carcinoma (2).

In early stages thyroidectomy is curative (3, 4), but unfortunately most patients with hereditary MTC and virtually all patients with sporadic MTC present with a clinically evident thyroid nodule and are frequently incurable because the cancer has already metastasized to regional lymph nodes or distant sites (1). There had been no effective conventional systemic treatment for metastatic MTC until recently, when clinical trials of tyrosine kinase inhibitors (TKI) showed promising results. The Food and Drug Administration in the United States recently approved the TKI, vandetanib, for the treatment of patients with advanced MTC (5–8).

Mutations of the RET (Rearranged during transfection) gene, which codes for the transmembrane receptor for growth factors of the glial-derived neurotrophic factor (GDNF) family, have been consistently associated with hereditary and sporadic MTC (5). Germ-line mutations of RET are present in virtually all patients with MEN2 and FMTC, and approximately half of the patients with sporadic MTC have somatic RET mutations (9, 10). MTC-associated mutations activate the RET kinase and its downstream signaling pathways. Very recently, mutations in RAS, a component of the RET signaling cascade, have been identified in RET-negative sporadic MTC samples (11).
Translational Relevance

Medullary thyroid carcinoma (MTC) responds poorly to conventional chemotherapy or radiation therapy. Understanding the molecular pathways driving MTC formation would be helpful in the design of targeted therapeutics for patients with advanced disease. Here, we show that the mTOR pathway is consistently activated in MTC, that such activation is robust in MTC metastases, and that the expression of neoplastic phenotype depends on mTOR activity. Targeting mTOR with rapamycin or its derivatives may be exploited as a therapeutic strategy for metastatic MTC.

RET promotes the activation of several intracellular signaling pathways, including the RAS/mitogen-activated protein kinase kinase/extracellular signal–regulated kinase (RAS/MEK/ERK) pathway and the phosphoinositide 3-kinase (PI3K)/mTOR pathway, which in turn promote cell proliferation, invasion, and survival (12–14). PI3K produces phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and AKT activation is stimulated through PIP3 binding, threonine 308 (Thr308) phosphorylation by PDK1, and, in a positive feed back circuit, through serine 473 (Ser473) phosphorylation by the serine/threonine kinase mTOR (12, 15). Inhibition of tuberous sclerosis complex 2 (TSC2) by AKT leads to the activation of Rheb, which, in turn, activates mTOR. mTOR triggers cell growth and proliferation by phosphorylating eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1; ref. 12). mTOR has received considerable attention as a therapeutic target. Its pharmacologic inhibition by rapamycin and its analogues (like Rad001/everolimus) has shown promising preclinical and clinical results in the treatment of many human malignancies (16).

Here, we studied the phosphorylation status of proteins of the RAS/MEK/ERK and PI3K/AKT/mTOR pathways in samples of primary MTC, lymph node metastases (LNM), and normal thyroid tissue. The aim of the study was to evaluate activation of these pathways as potential therapeutic targets in patients with MTC.

Materials and Methods

Human MTC samples

Archival, formalin-fixed, paraffin-embedded (FFPE), tissue blocks and slides from cases of primary and paired metastatic (when available) MTCs from 1989 to 2009 were retrieved from the Department of Pathology and Immunology at the Washington University School of Medicine (St. Louis, MO). The Institutional Review Boards at Washington University and at the NIH approved the study. Representative hematoxylin and eosin (H&E)-stained sections from each block were evaluated to determine suitability for inclusion in the study. The inclusion criteria were tumor size at least 1 to 2 mm in depth and 5 × 5 mm² in length and width, appropriate fixation, absence of electrosurgical device lesions, and absence of signs of acidic declacifying agents. By immunohistochemistry analysis all of the MTC samples were positive for expression of RET, calcitonin, and carcinoembryonic antigen (CEA; data not shown). The primary MTC was available in only 30 patients, both the primary MTC and the LNM were available in 21 patients, and LNMs were available in only 2 patients. For the majority (37 of 53) of the cases, we also examined normal thyroid tissue from the thyroid isthmus that is virtually free of C-cells. In 3 patients, we examined 2 independent primary MTC nodules, whereas in 1 patient we examined 3 independent MTC nodules. A summary of clinicopathologic features of the cases is shown in Supplementary Table S1 (Supplementary Information). Moreover, we examined CCH samples in 2 of the hereditary MTCs and 8 independent cases in which only CCH was evident histologically (Supplementary Table S2, Supplementary Information).

RET and RAS genotyping

Cores of 0.6 mm in diameter were sampled from FFPE blocks. Fibrotic or amyloid-rich samples with less than 50% of tumor material were excluded from the analysis. DNA extraction was carried out from deparaffinized FFPE specimens using the DNeasy Blood and Tissue Kit (Qiagen). Targeted analysis of RET mutations in exons 10, 11, and 13 to 16 was done by pyrosequencing on a PyroMark Q24 instrument (Qiagen). Primers for the RET gene pyrosequencing reactions were designed in the Laboratory of Pathology, Center for Cancer Research, NCI, NIH and are available upon request. Germline RET mutations were present in all of the 18 familial MTC samples and in one CCH sample. The presence of RET mutations in the remaining 7 CCH only samples was documented previously at the Washington University School of Medicine (data not shown). Targeted analysis of RAS mutation was conducted in 28 samples, including 11 hereditary and 17 sporadic patients with MTC (7 RET mutant cases and 10 RAS wild-type cases). RAS mutations were searched for the 3 RAS genes (H-, N-, and K-RAS) by exome sequencing. Briefly, 3 µg of genomic DNA from each sample were sheared in 150 to 250 bp fragments using the CovarisTM S220 sonicator (Covaris Inc.). The ends of the DNA fragments were repaired using T4 and Klenow DNA polymerases (NEB) and an A-tail was added by using Klenow enzyme (NEB). Nucleotide adapters of about 100 bp (NEXTflex—BioScientific) specific for each sample were added at the ends of DNA fragments in a reaction buffer containing T4 DNA ligase (Enzymatics Inc.). DNA fragments ranging from 250 to 400 bp were purified using the Caliper LabChip XT fractionation system (Caliper) according to manufacturer’s instructions. The DNA fragments were amplified by PCR using NEXTflex primers according to manufacturer’s instructions. PCR products were purified by using magnetic beads (Agencourt AMPure XP; Beckman) according to manufacturer’s instructions.
Finally, the quality of the DNA libraries was assayed by using the 2100 Bioanalyzer Agilent. Equal amounts of each DNA library were mixed in a hybridization buffer containing biotinylated RNAs complementary to the exons of RAS genes (SureSelect—target enrichment system, Agilent) according to manufacturer’s instructions, and the hybridization reaction was run for 48 hours. Streptavidin magnetic beads (Dynabeads Myone streptavidin C1; Invitrogen) were used for DNA/RNA capturing. The captured DNA was amplified by PCR using NEXTFlex primers and quantified by real-time PCR. Finally, DNA fragments were sequenced in an Illumina sequencer (Illumina GA II) according to manufacturer’s protocol. The results of the sequences were analyzed with the IGV 2.0 software.

**Tissue microarrays**

We constructed a tissue microarray (TMA) including tissue samples from 53 patients with MTC (18 hereditary and 35 sporadic; including 30 primary tumors, 21 pairs of primary tumors and LNM, and 2 independent LNM) and 37 tissue samples of normal C-cell–free tissue from the thyroid isthmus of patients with MTC (Supplementary Table S2, Supplementary Information). From each FFPE block, one representative core was sampled and manually embedded in a recipient TMA paraffin block using 1 to 2 mm skin biopsy punches. TMA cores featuring extensive loss of tissue or damage were excluded from the analysis. In the 21 pairs of primary tumor and autologous metastatic lymph nodes, TMA immunohistochemical data were thereafter confirmed by immunohistochemistry for pS6, p-AKT473, and p-ERK in regular 5-μm tissue sections. The 10 CCH samples were all studied by immunohistochemistry on regular 5-μm tissue sections. Finally, for another 20 primary MTC samples pS6, p-AKT473, and p-ERK immunohistochemical data were thereafter confirmed and correlated by Spearman test on regular 5-μm tissue sections.

**Immunohistochemistry**

The primary antibodies used in this study are listed in Supplementary Table S3, Supplementary Information. All antibodies were tested first in representative MTC samples by immunohistochemistry on regular 5-μm tissue sections to set optimal staining conditions (antibody dilution and antigen retrieval conditions). Immunohistochemical staining of the sections, either cut from the TMA block or from the original FFPE blocks, was carried out as follows. The tissue slides were immersed in SafeClear II for dewaxing 3 × 30 minutes, hydrated through graded alcohol and distilled water solution, and then washed with PBS. Antigen retrieval was carried out by using 10 mmol/L citric acid in a microwave for 20 minutes (2 minutes at 100% power and 18 minutes at 10% power). Slides were allowed to cool for 30 minutes at room temperature, rinsed twice with PBS, and incubated in 3% hydrogen peroxide for 30 minutes to quench the endogenous peroxidase. The sections were then washed in distilled water and PBS and incubated in blocking solution (2.5% bovine serum albumin in PBS) for 1 hour at room temperature. Excess solution was discarded, and the sections were incubated with the primary antibody diluted in blocking solution at 4°C overnight. After washing with PBS, the slides were sequentially incubated with the biotinylated secondary antibody (1:400; Vector Laboratories) for 30 minutes, followed by the avidin–biotin complex method (Vector Stain Elite, ABC kit; Vector Laboratories) for 30 minutes at room temperature. Finally, slides were washed and developed in 3,3′-diaminobenzidine (Sigma FASTDAB tablet; Sigma Chemical) under microscopic control. The reaction was stopped in tap water, and the tissues were counterstained with Mayer hematoxylin, dehydrated, and mounted. In all cases, the specificity of the reaction was validated in parallel control sections omitting the primary antibody. Digital image acquisition was conducted from each stained slide by the Aperio ScanScope CS System (Aperio, Inc.). The percentage of stained cells was quantified in representative areas by the algorithm Pixel Count. The results were also visually evaluated to check consistency of the automated analysis. The results of the quantification were converted into a 0 to 4 scale (S score) based on the percentage of cells which stained positively as follows: 0, less than 10%; 1, between 10% and 24%; 2, between 25% and 49%; 3, between 50% and 74%; and 4, between 75% and 100%. We conducted a Pearson correlation test to exclude that variation in staining intensity was due to the age of the samples. The test showed no correlation between the variables “age of the samples” and “immunohistochemical value” for any of the markers studied.

**Cell culture and reagents**

MZ-CRC-1 cells, kindly provided by Dr. Robert F. Gagel (M.D. Anderson Cancer Center, Houston, TX), were derived from a malignant pleural effusion from a patient with a metastatic MTC (17). The cells were originally given to one of the coauthors (M. Santoro) who provided them to the lead author (A. Tamburrino) in August of 2010. RET cDNA sequencing for MZ-CRC-1 revealed a heterozygous (ATG to ACG) transition in RET exon 16 resulting in MEN2B-associated substitution of threonine 918 for methionine (M918T; ref. 18). The TT cells were obtained from American Type Culture Collection in December 2011. TT cells harbor the MEN2A-associated RET C634W mutation in exon 11, as confirmed by genomic DNA sequencing. Each of the cell lines is checked periodically (and within the last 6 months) by Western blotting for RET presence and activation and by sequencing for the RET M918T mutation (MZ-CRC-1) and for the RET C634W mutation (TT). MZ-CRC-1 cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FCS, TT cells were grown in RPMI-1640 supplemented with 16% fetal calf serum. Media were supplemented with 2 mmol/L L-glutamine and 100 U/mL penicillin–streptomycin (Gibco, Invitrogen). Rapamycin was from Calbiochem, everolimus (RAD001) was from LC Laboratories, and Torin 2 was from Tocris.
Bioscience. Vandetanib was provided by AstraZeneca. EGF was purchased from Sigma.

Cell viability and motility assays

For viability, MZ-CRC-1 and TT cells were seeded in quadruplicate in 96-well plates (10,000 cells per well) in culture media with 2.5% and 4% FBS, respectively. After 24 hours, cells were treated with the indicated compounds. At the indicated time point, cells were incubated for 3 hours with 10 µL of CellTiter96 AQueous One solution in 100 µL of culture media (Promega) and absorbance was measured at 490 nm. For migration assays, Transwell inserts with membranes of 8 µm/L pore size (Corning-Costar) were used. Membranes were coated with 10 µg/mL collagen and kept at 4°C overnight. Cells were serum starved for 8 hours, detached by trypsin, and counted by hemocytometer. A total of 1 × 10^5 cells were plated in the upper chambers in serum-free medium, containing the vehicle or the indicated compounds (rapamycin: 100 nmol/L; everolimus/Rad001: 100 nmol/L, and Torin 2: 100 nmol/L). The lower chambers were filled with MZ-CRC-1 and TT culture media containing 2.5% and 4% FBS, respectively, supplemented with 20 ng/mL EGF (Sigma). After 24 hours, cells were fixed in methanol and stained with hematoxylin. Cells on the top surface of the membranes were wiped off with cotton swabs. Membranes were removed from the inserts, placed on microscope slides, and images acquired by Scanscope. Migrated cells were counted and the number of migrated cells per mm^2 was calculated.

Tumorigenicity assay

Animal studies were carried out according to NIH-approved protocols in compliance with the NIH Guide for the Care and Use of Laboratory Animals. A total of 5 × 10^5 MZ-CRC-1 cells were injected subcutaneously into both flanks of female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (n = 12). When tumor volume reached approximately 200 mm^3 (after 30 days from injection), animals were randomly assigned to receive intraperitoneal (i.p.) vehicle or rapamycin (5 mg/kg/d). Tumor size was assessed twice a week by caliper and tumor volume was calculated according to the formula: \( V = \frac{1}{2} \times L \times W^2 \) (where \( L = \) length and \( W = \) width of tumor). At day 25 of treatment, 2 hours before sacrifice, mice were given an intraperitoneal injection of bromodeoxyuridine (BrdUrd; BD Pharmingen, 100 mg/kg). Animals were euthanized, and tissue was fixed in 4% paraformaldehyde and embedded in paraffin for histologic evaluation and BrdUrd immunohistochemistry.

Statistical analysis

Correlation among the clinicopathologic parameters and RET or RAS genotyping was carried out by Fisher exact test. Correlation among the immunohistochemical markers was carried out by Spearman rank correlation coefficient test. Pearson correlation test was run to assess correlation between age of samples and immunohistochemical data. Student t test was carried out to analyze cell culture and mice experiments. The software used for all analyses was Prism 5 (GraphPad Software, Inc.).

Results

Screening of the patients’ tumors for RET and RAS mutations

The presence of the most common RET mutations in exons 10, 11, and 13 to 16 was evaluated in tumor (or LNMs) DNA in 53 patients with MTC (Supplementary Table S2, Supplementary Information; ref. 19). All the 18 MTC hereditary cases were positive for RET mutations. Among the 35 sporadic cases, 20 were positive for somatic RET mutations (Supplementary Table S2, Supplementary Information). RAS (N-, H-, and K-RAS) mutations were analyzed in 28 out of the 53 MTCs, including 11 hereditary and 17 sporadic (7 RET mutant and 10 RET wild-type) cases. RAS mutations (4 HRAS and 2 KRAS) were found in 6 patients, among the 10 sporadic RET-negative samples. RAS and RET mutations were mutually exclusive (Supplementary Table S2, Supplementary Information). Neither the presence nor the type of RET and RAS mutation was correlated with gender, age, histologic variant (spindle cells, epithelioid), or other histologic features (fibrosis, amyloid, or necrosis; data not shown).

Ribosomal protein S6 phosphorylation in primary MTC

We measured by immunohistochemistry the level of phosphorylation of proteins involved in 2 major signaling cascades: RAS/MEK/ERK, using phospho-ERK Thr202/ Tyr204 (p-ERK202/204 antibodies) and PI3K-AKT-mTOR, using phospho-AKT Ser473 or Thr308 (p-AKT473 and p-AKT308) and phospho-ribosomal protein S6 Ser235/236 (pS6235/236) antibodies. AKT Thr308 is phosphorylated by PDK1, which in turn is stimulated by PI3K, therefore phosphorylation at this site indicates upstream pathway stimulation. AKT Ser473 is phosphorylated by an mTOR containing protein complex (TORC2), therefore, phosphorylation at this site is a marker of mTOR activity (12, 15).

p-ERK202/204, p-AKT473, AKT308 scored negative in all normal thyroid tissues, whereas pS6 scored weakly positive (S = 1) in 7 (21%) of 33 cases of normal thyroid tissue (the remaining 4 normal thyroid tissues were excluded from the analysis due to extensive tissue loss). In contrast, a significant proportion of MTCs was positive for p-ERK202/204, p-AKT473, and pS6. Indeed, 29 (58%) of 50 primary MTCs and 9 (50%) of 18 LNMs stained positively (S > 1) for p-ERK202 (1 primary MTC and 5 LNMs were not evaluable due to tissue loss). Notably p-AKT473 staining was mostly nuclear, as shown by the representative images in Fig. 1B. Only 39% of primary MTC samples were p-AKT308-positive (S ≥ 1; Supplementary Table S2, Supplementary Information). The reduced fraction of p-AKT308-positive cases was likely due to reduced sensitivity of this antibody; indeed, antibodies to p-AKT308 exerted a reduced reactivity compared with antibodies to p-AKT473, when AKT phosphorylation was...
assessed by Western blotting in HEK293 cells stimulated by insulin (data not shown). To confirm the TMA data, whole sections from 20 representative primary MTC samples were stained with pS6, p-ERK, and p-AKT473 antibodies. Spearman correlation analysis showed a good correlation ($P < 0.01$) between TMA and whole section data for all of the tested antibodies (Supplementary Table S2, Supplementary Information).

The upregulation of p-ERK, p-AKT, and p-S6 in MTC compared with normal thyroid was statistically significant ($P < 0.001$). The highest percentage of positive samples was seen with pS6 (Fig. 1A). Indeed, the majority of primary MTCs (49 of 51, 96%) and, notably, all of the LNMs (23 of 23) were pS6-positive ($S \geq 1$; Fig. 1A and Supplementary Table S2, Supplementary Information). Representative staining of pS6 from 6 MTC samples is shown in Fig. 1B. Overall, pS6 phosphorylation was homogeneous among different nodules from the same patient, with the exception of one case in which it was strong ($S = 4$) in one nodule and negative ($S = 0$) in the other one (data not shown).

A Spearman test was carried out to verify whether there was a correlation between the different markers. A significant correlation ($P = 0.006$) was found between pS6 and p-AKT473 immunostain; moreover, staining for pS6 and p-AKT473 correlated with p-ERK ($P < 0.0002$ and $P < 0.0001$, respectively). The latter finding is consistent with previous reports showing that ERK contributes to mTOR activation via inhibitory phosphorylation of TSC2, an mTOR-negative regulator (20). However, some samples (10 of 47) scored positive for pS6 but not for p-AKT473. It is possible that in these samples mTOR activation is dependent on genetic lesions affecting pathways other than PI3K/AKT. There was no correlation between positivity of p-AKT308 and the other markers ($P > 0.05$).

Finally, no significant difference in pS6, p-AKT, and p-ERK staining was detected between familial/sporadic, RET wild-type/mutant, germline/somatic RET mutant carriers, M918T/cysteine (609/618/620/630/634) RET mutants, or RAS wild type/mutant samples. However, we cannot exclude that the lack of correlation was due to the reduced sample size. A power calculation showed that to have a good discrimination ($\alpha = 0.05$) for pS6 ($S > 2$) the difference of the 2 proportions (RET mutant/wild-type samples) should be at least 0.357, whereas in our series it was less than 0.1.

Phosphorylated S6 ribosomal protein is detected in CCH

Seven of 10 CCH samples analyzed were pS6-positive ($S \geq 1$). Representative cases are shown in Fig. 2. In 2 patients, we examined both the CCH and the primary MTC nodule: the pS6 positivity was increased for both cases in the MTC nodule compared with the CCH. One of these cases (WU20) is shown in Figs. 2 (CCH) and 3 (MTC). These results suggest that activation of the mTOR pathway is an early event in C-cell transformation and that it further increases along with tumor progression.
Ribosomal protein S6 is prominently phosphorylated in lymph node metastases

The positivity for pS6 was high in the LNMs: S score $\geq 3$ was found in 20 (95%) of 21 LNMs compared with 31 (61%) of 51 primary MTCs (for 2 additional patients only the LNMs were available; Supplementary Table S2, Supplementary Information, Figs. 1B and 3). A Spearman rank correlation test showed that the increase in the S score of LNMs compared with matched primary MTC was statistically significant ($r = 0.5$, $P = 0.024$). These results suggest that activation of the mTOR pathway is particularly strong in MTC metastases.

Inhibition of mTOR decreases viability and motility of MTC cells

To assess the effect of mTOR inhibition in vitro we treated 2 human MTC cell lines, MZ-CRC-1 and TT, with 2 inhibitors mainly active on TORC1 (rapamycin and Rad001/everolimus) as well as a double TORC1/TORC2 inhibitor (Torin 2). We could show that in these RET mutant MTC cells, mTOR is active and its activity depends on RET, being effectively inhibited by a RET kinase inhibitor (vandetanib; ref. 18; Supplementary Fig. S1, Supplementary Information). We measured viability at 3 and 5 days of mTOR blockade by MTS assay. After 5 days, the 3 compounds caused a significant reduction in viability of both MZ-CRC-1 and TT cells (Fig. 4A). We measured cell motility by Transwell motility assay. At 24 hours, Torin 2 exerted a significant reduction of migration of both cell types ($P < 0.01$); also rapamycin and Rad001 reduced cell migration, but this effect reached statistical significance ($P < 0.05$) only for MZ-CRC-1 cells (Fig. 4B).

Reduction of MZ-CRC-1 cell tumorigenicity by rapamycin

We injected MZ-CRC-1 cells subcutaneously in both flanks of NOD/SCID mice ($n = 12$). When tumors of approximately 200 mm$^3$ were detected, animals were randomly assigned to receive i.p. rapamycin or vehicle; tumor growth was monitored by calipers. After 25 days of...
treatment, average tumor volume was significantly ($P < 0.01$) reduced by rapamycin treatment compared with control (Fig. 5). In the examined time frame of the experiment, metastases were not formed and therefore antimetastatic activity of the treatment could not be tested (not shown). DNA synthesis rate was measured in vivo by BrdUrd incorporation. Rapamycin treatment caused a significant reduction ($P < 0.0001$) of proliferating cells compared with control.

**Discussion**

In this study, we analyzed molecular pathways that are potentially involved in MTC formation and metastatic dissemination. We found that the AKT/mTOR/pS6 pathway was active in the majority of MTC cases. The pathway was active early as evidenced by positive staining in familial CCH, suggesting a functional link to the RET mutation, which initiates the familial disease.

Rapa and colleagues recently reported activation of the mTOR pathway in MTC (21). The present study confirms their observation and provides valuable information about the clinical relevance and functional consequences of mTOR pathway activation. Indeed, not only did our present analysis reveal that mTOR activation represents an early event in C-cell transformation, but it also shows that AKT/mTOR/pS6 activation was stronger in LNMs than in matched primary MTCs, suggesting a role of this pathway in MTC tumor progression in addition to initiation. Furthermore, our data show that inhibition of mTOR pathway causes significant reduction of MTC cell proliferation, motility, and tumorigenicity. Rad001 effects on TT cell proliferation were consistent with those recently reported (22).

In MTC and CCH, activation of the mTOR/pS6 pathway is likely mediated by AKT. Indeed, AKT inactivates TSC formed by TSC1 and TSC2 (15). Once associated to TSC1, TSC2 acts as a GTPase-activating protein (GAP) to shut off the Rheb1 GTPase. Thus, upon AKT activation, GTP-bound Rheb1 promotes mTOR activation (15). In turn, mTOR activates S6K1, that phosphorylates S6, and also feedbacks...
1. Schlumberger M, Carlomagno F, Baudin E, Bidart Jm, Santoro M. New phosphorylation depends on mTOR) it is feasible that this explanation of this difference (Thr308 phosphorylation) depends on the PDK1 kinase, whereas Ser473 phosphorylation (12, 15). RET is able to stimulate AKT through the recruitment of multiple protein adaptors, such as IRS1/2, FRS2, or SHC-C (23–25), or signaling effectors, such as SRC (26). Similarly, RAS is also able to positively on AKT by promoting its activation through Ser473 phosphorylation (12, 15). RET is able to stimulate PI3K and therefore AKT (12–14). We noted that in MTC, AKT was more consistently phosphorylated on Thr308 than on Thr308; while there are possible biochemical explanations of this difference (Thr308 phosphorylation depends on the PDK1 kinase, whereas Ser473 phosphorylation depends on mTOR) it is feasible that this difference is simply linked to the reduced efficiency of p-AKT,

Deregulated activation of the AKT/mTOR cascade is involved in many human malignancies and its pharmacologic inhibition with selective drugs such as rapamycin or its derivatives has shown efficacy in cancer treatment (15). Our data may provide the basis for future studies aimed at testing mTOR inhibitors for the treatment of metastatic MTC and may also suggest suitable biomarkers to monitor the biochemical consequences of mTOR inhibitors in MTC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: A. Tamburrino, J.S. Gutkind, M. Santoro Development of methodology: A. Tamburrino, M. Raffeld, L. Xi Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.D. Chernock, M. Raffeld, L. Xi, J.S. Gutkind, J.F. Moley Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Tamburrino, A.A. Molinolo, M. Raffeld, L. Xi, J.S. Gutkind, M. Santoro, S.A. Wells Writing, review, and/or revision of the manuscript: A. Tamburrino, A.A. Molinolo, R.D. Chernock, M. Raffeld, L. Xi, J.S. Gutkind, J.F. Moley, M. Santoro, S.A. Wells Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Tamburrino, P. Salerno, L. Xi, S. A. Wells

Study supervision: J.S. Gutkind, M. Santoro Execution of the immunohistochemistry and in vitro and in vivo experiments: A. Tamburrino DNA sequencing: P. Salerno

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**References**

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