Calcium/Calmodulin-Dependent Protein Kinase II and Its Endogenous Inhibitor α in Medullary Thyroid Cancer

Eleonora Russo, Marcella Salzano, Valentina De Falco, Caterina Mian, Susi Barollo, Agnese Secondo, Maurizio Bifulco, and Mario Vitale

Abstract

**Purpose:** Calcium/calmodulin-dependent kinase II (CaMKII) is involved in the regulation of cell proliferation. Its endogenous inhibitor (hCaKIINα) is expressed in some cell types. We determined the role of CaMKII in RET-stimulated proliferation and hCaKIINα in medullary thyroid carcinoma (MTC).

**Experimental Design:** We analyzed the role of RET mutants on CaMKII activation in NIH3T3 and in MTC cell lines, and determined the effect of CaMKII inhibition on RET/ERK pathway and cell proliferation. Then the expression of hCaKIINα mRNA was determined by real-time PCR in primary MTC and it was correlated with some clinicopathologic parameters.

**Results:** RET-C634V and RET-M918T mutants expressed in NIH3T3 cells induced CaMKII activation. CaMKII was activated in unstimulated MTC cells carrying the same RET mutants and it was inhibited by RET inhibition. Inhibition of CaMKII in these cells induced a reduction of Raf-1, MEK, and ERK phosphorylation, cyclin D expression, and cell proliferation. hCaKIINα mRNA expression in primary MTC was very variable and did not correlate with gender and age at diagnosis. Serum calcitonin, (R² = 0.032; P = 0.017), tumor volume (P = 0.0079), lymph node metastasis (P = 0.033), and staging (P = 0.0652) were negatively correlated with the hCaKIINα mRNA expression.

**Conclusions:** CaMKII is activated by RET mutants and is activated at baseline in MTC cells where it mediates the oncogenic pathway leading to cell proliferation. The mRNA expression of its endogenous inhibitor hCaKIINα inversely correlates with the severity of MTC. CaMKII might represent a new target for MTC therapy and hCaKIINα is a marker of disease extension. Clin Cancer Res; 20(6); 1–8. ©2014 AACR.

Introduction

Medullary thyroid carcinoma (MTC) arises from calcitonin-producing parafollicular (C) cells of the thyroid and accounts for 5%–8% of all thyroid cancers (1). MTC is sporadic in about 75% of the cases; in the remaining cases, it is familial MTC (FMTC) or occurs as a component of the autosomal dominant familial multiple endocrine neoplasia type 2 (MEN2A and MEN2B). Point mutations of the RET gene (REarranged during Transfection) are present in about 40% of sporadic MTC and in more than 95% of MEN2 and FMTC. RET point mutations are described mainly in exons 10, 11, and 16, and less frequently in exons 5, 8, 13, 14, and 15 (2–4). In 95% of patients with MEN2B, RET is mutated at codon 918 (Met918Thr; ref. 5). Multiple signals are generated by RET. Phosphorylation of tyrosine 1062 located in the RET carboxyl terminal tail is important for transforming activity of MEN2-mutant proteins (6). Tyr1062 acts as a docking site for many adaptor or effector proteins such as SHC, FRS2, DOK1/4/5, IRS1/2, Enigma, protein kinase C α (PKCa), and Shank (7). Among these, SHC binding plays a crucial role in activation of both the RAS/ERK and phosphoinositide 3-kinase (PI3)-K/AKT pathways (8). Phosphorylated tyrosine 1015 is docking site for phospholipase C γ (PLCγ; refs. 9, 10).

The calcium/calmodulin-dependent kinase II (CaMKII) is a ubiquitous serine/threonine protein kinase involved in multiple signals and biologic functions. In some cell types, CaMKII participates with Ras to Raf-1 activation, phosphorylating Raf-1 at S338, a phosphorylation necessary for ERK activation upon different physiologic and pathologic stimuli in the mitogen activated protein kinase (MAPK) cascade (11–13). The biologic function of CaMKII is cell type and cell contest dependent. In neuronal cells, it is involved in regulating postsynaptic signaling complexes, neurotransmission and memory (14). In fibroblasts and myocytes, CaMKII modulates the insulin signaling (15, 16). In papillary thyroid carcinoma, CaMKII is activated by BRafV600E, oncogenic Ras, and by RET rearrangements (RET/PTC) and participates in the activation of the ERK

Authors’ Affiliations: 1Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Istituto di Endocrinologia e Oncologia Sperimentale del CNR Naples; 2Department of Neuroscience, Reproductive and Odontostomatological Sciences, University of Naples “Federico II”, Naples; 3Department of Medicine-DIMED, Unit of Endocrinology, University of Padua, Padova; and 4Department of Medicine and Surgery, University of Salerno, Salerno, Italy

Corresponding Author: Mario Vitale, Department of Medicine and Surgery, University of Salerno, Via Allende 84081, Baronissi, Salerno, Italy. Phone: 398-1746-4983; Fax: 398-1743-3668; E-mail: mavitale@unisa.it

doi: 10.1158/1078-0432.CCR-13-1683

©2014 American Association for Cancer Research.

www.aacrjournals.org
pathway by oncogenic Ras and RET/PTC, thus modulating tumor cell proliferation (17). Two endogenous CaMKII inhibitory proteins (hCaMKIIINα and β) have been identified in some cell types (18, 19). These proteins inhibit human colon adenocarcinoma cell growth and their expression is negatively correlated with the severity of human colon adenocarcinoma, suggesting a pivotal role of CaMKII in the development and progression of carcinomas. In this study, we investigated the role of CaMKII and its endogenous inhibitor an important role in MTC, suggesting that hCaMKIIINα might be used as a prognostic factor useful for tailoring the therapy of MTC, while CaMKII could represent a new therapeutic target for pharmacologic intervention in this cancer type.

**Materials and Methods**

**Cell cultures**

Parental murine NIH3T3 fibroblasts and NIH3T3 cells stably transfected with the RET mutants RETC634R and RETM918T were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% FBS. TT cells were from American Type Culture Collection. TT cells were derived from the primary tumor of sporadic MTC. TTs harbor a cysteine 634 to tryptophan exon 11 RET mutation (20) as well as a tandem duplication of the mutated RET allele (21). MZ-CRC1 cells were derived from a malignant pleural effusion from a patient with a metastatic MTC (22). MZ-CRC1 cells revealed a heterozygous (ATG to ACG) transition in RET exon 16 resulting in substitution of threonine 918 to methionine. TT cells were grown in RPMI-1640 supplemented with 16% FBS (Gibco). MZ-CRC1 cells were grown in DMEM supplemented with 10% FBS. All media were supplemented with 2 mM/L l-glutamine and 100 U/mL penicillin–streptomycin (Gibco). All cell lines were maintained at 37°C, 5% CO2.

**[Ca2+]i measurement**

[Ca2+]i was measured by Fura-2 AM single-cell computer-assisted video imaging (23). The equation of Grynkiewicz and colleagues was used for calibration (24). Ca2+ content into endoplasmic reticulum was evaluated as cytosolic Ca2+ release by using the irreversible and selective inhibitor of the sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA) thapsigargin (Tg) together with the purinergic receptor agonist ATP (100 μmol/L) in a Ca2+-free solution containing EGTA (1 mmol/L). Tg was able to induce a progressive and complete ion depletion from ER, thus providing an indirect measure of Ca2+ levels into the ER.

**Western blot analysis**

For Western blot analysis, cells were lysed in Laemmli buffer [0.125 mol/L Tris (pH 6.8), 5% glycerc, 2% SDS, 1% β-mercaptoethanol, and 0.006% bromophenol blue]. Proteins were resolved by 7%–15% SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon P, Millipore Corp.). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine in PBS, washed, and incubated for overnight at 4°C with primary antibodies and then washed again and incubated for 1 hour with a horseradish peroxidase–conjugated secondary antibody. Finally, protein bands were detected by an enhanced chemiluminescence system (Amersham Biosciences). Anti-phosphorylated p44/42 ERK (ERK1/2) were rabbit polyclonal antibodies from Cell Signaling Technology. Anti-RET was a rabbit polyclonal antibody raised against the tyrosine kinase protein fragment of human RET (25). Anti-phosphorylated Y905 recognizes RET proteins that are phosphorylated at Y905 (26). Blots were incubated with primary antibodies for 1 hour at room temperature, followed by three washes in buffer (20 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, and 0.05% Tween 20). The blots were then incubated with the goat anti-rabbit secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour at room temperature followed by three washes in buffer (20 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, and 0.05% Tween 20). Monoclonal anti-phospho-CaMKII antibody (pT286-CaMKII) was from Cell Signaling Technology; anti-phospho Raf-1 S338 was from Biotechnology.

**Reagents and inhibitors**

The CaMK inhibitor KN93 and the CaM inhibitors N-(6 aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W7) were purchased from Sigma Aldrich. The selective inhibition of RET ZD6474 (Vandetanib) was kindly provided by AstraZeneca. Ionomycin and BAPTA-AM were provided by AstraZeneca.
Patients and tumors

Tumor tissues were promptly dissected immediately after operation and then snap frozen and stored at −80°C. MTCs were classified according to the American Joint Committee on Cancer (AJCC) TNM system. All patients gave consent to make their tumor tissue available for genetic analysis. The present study was approved by the Internal Reviewing Board. All MTCs were sporadic. The mean age at diagnosis was 54.9 years (range 37–81 years). Serum calcitonin concentrations were determined using an immunoradiometric assay in blood samples obtained 1 to 3 days before surgery.

RNA extraction and relative mRNA quantification

Total RNA was extracted from TT and MZ-CRC1 cell lines using 500 μL TRI Reagent, resuspended in 10 μL DEPC water, and reverse-transcribed with SuperScript III (Invitrogen) in a 20 μL reaction volume with random primers. mRNA was reverse-transcribed into cDNA and analyzed by real-time RT-PCR with the cDNA of the patients with MTC. Real-time PCR was performed using SYBR Green PCR reagents (Fermentas) and primers specific for hCaMKINα and β-actin. Forward and reverse primers for hCaMKINα were respectively: 5'-TACGGCGACGAGAAGTGC-3' and 5'-TCACGAGTCATCATCCTATC-3'. The β-actin forward and reverse primers were: 5'-TTGCTCTCTGGCGATTGAGT-3' and 5'-TACGGCGACGAGAAGCTGAG-3'. The samples were analyzed with Bio-Rad Cycler with the following protocol: 95°C for 5'; 95°C × 10⁶; 60°C × 60⁶ (× 40 cycles). The levels of relative mRNA expression were determined by normalizing to β-actin expression and adopting the 2−ΔΔCt method (27).

Statistical analysis

Results are presented as the mean ± SD. Statistical procedures included Student t test, ANOVA, χ² analysis, simple and rank correlation analysis, and logistic regression analysis. The P value was considered statistically significant when P < 0.05.

Results

RET mutants activate CaMKII by a Ca²⁺/calmodulin-mediated signal

Murine NIH3T3 fibroblast cells were transfected with an expression vector coding for the RET mutants C634R and M918T to generate stable cell mutants (NIH2A and NIH2B). To determine the CaMKII activation status in parental NIH3T3 and in cell mutants, serum-starved cells were treated with the calcium ionophore ionomycin and the phosphorylation of Thr 286 of CaMKII was evaluated by Western blot analysis (Fig. 1A). CaMKII phosphorylation was minimal in unstimulated parental NIH3T3 cells, and it was strongly induced by ionomycin. In untreated NIH2A and NIH2B cells, CaMKII phosphorylation was evident. The treatment with ionomycin had no effect, whereas the treatment with the calcium chelator BAPTA-AM decreased CaMKII phosphorylation in a dose-dependent manner. Accordingly, under resting conditions, intracellular Ca²⁺ concentrations ([Ca²⁺]) were higher in NIH2A and NIH2B mutants than in parental NIH3T3 cells (90 ± 2 and 89 ± 5 in NIH2A and NIH2B, respectively, versus 60 ± 9 in NIH3T3 cells; *P < 0.05; Fig. 1B). Similarly, the intracellular Ca²⁺ content into the endoplasmic reticulum was higher in NIH2A and NIH2B mutants than in parental NIH3T3 (143% ± 11% and 149% ± 15% in NIH2A and NIH2B, respectively, versus 98% ± 5% in NIH3T3 cells; *P < 0.05; Fig. 1C). These results demonstrate that CaMKII is activated by RET mutants in a calcium-dependent manner.

CaMKII is activated in MTC cell lines

The MTC-derived cell lines TT and MZ-CRC1 contain a cysteine 634 to tryptophan (C634W) transversion at exon 11 of the RET gene with concurrent tandem duplication of the mutated RET allele, or a methionine 918 to threonine (M918T) transversion at exon 11 of the RET gene, respectively. Both cell lines were starved from serum for 24 hours and incubated in suspension with the calcium chelator BAPTA-AM or the ionophore ionomycin for 30 minutes at different concentrations. Cells were left in

Figure 1. CaMKII is activated by RET mutants and in MTC cells harboring RET mutants. A, parental murine NIH3T3 fibroblasts and NIH3T3 cells stably transfected with the RET mutants RET/C634R (NIH2A) and RET/M918T (NIH2B) were starved for serum for 24 hours and then treated with the calcium ionophore ionomycin 2 μmol/L for 5 minutes or with BAPTA-AM at the indicated concentration for 30 minutes. CaMKII phosphorylation at Thr 286 (pCaMKII) was evaluated by Western blot analysis. The 3 bands visible correspond to different CaMKII isoforms. Numbers in italic are averages of relative bands intensity, determined by scanning densitometry of three independent immunoblots. SDs were less than 10%; *P < 0.05 versus first point. B, [Ca²⁺]i recorded under basal conditions in NIH2A and NIH2B mutants and parental NIH3T3 cells. C, quantification of ATP- and Tg-induced effects on [Ca²⁺]i increase, calculated as % of peak/basal value, in NIH2A and NIH2B mutants and parental NIH3T3 cells. For each experiment, 40 to 65 individual cells were monitored. Each bar represents the mean (± SEM) of the aforementioned experimental values studied in 3 independent experimental sessions; *P < 0.05 versus NIH3T3 cells.
OF4

Clin Cancer Res; 20(6) March 15, 2014

Clinical Cancer Research

Published OnlineFirst January 21, 2014; DOI: 10.1158/1078-0432.CCR-13-1683

Russo et al.

Figure 2. CaMKII activation is RET and PLCγ mediated in MTC cells. A, TT and MZ-CRC1 were starved from serum for 24 hours and incubated with BAPTA-AM at the indicated concentrations or 2 μmol/L ionomycin for 30 minutes. Phosphorylation of CaMKII at Thr 286 was evaluated by Western blot analysis. B, TT cells starved from serum for 24 hours and incubated in suspension with increasing concentration of the RET inhibitor ZD6474. The cells were then lysed in increasing concentration of the RET inhibitor ZD6474 serum for 24 hours and incubated in suspension with increasing concentration of the PLCγ inhibitor U73-122. CaMKII phosphorylation was determined by Western blot analysis (Fig. 2C). In MZ-CRC1 cells, 15 μmol/L U73-122 was sufficient to induce a dramatic CaMKII dephosphorylation, whereas in TT cells, the same effect was obtained with 45 μmol/L. The different result between the two cell lines evidences a major sensitivity of MZ-CRC1 cell line to calcium deprivation and confirm that in these cells CaMKII activation is RET/PLCγ/calcium mediated.

ERK activation and cell cycle are CaMKII mediated in MTC cells

To determine the role of CaMKII in ERK activation, serum-starved TT and MZ-CRC1 cells were treated with ionomycin or the inhibitor of calmodulin W7 at different times and concentrations, and ERK phosphorylation was determined by Western blot analysis (Fig. 3A). ERK phosphorylation was evident in unstimulated cells and remained unchanged following ionomycin treatment. W7 displayed a powerful reproducible inhibitory effect of ERK phosphorylation, demonstrating that CaMKII activation in these cells is calmodulin dependent. The cells were starved from serum for 24 hours and incubated with the pharmacologic CaMKII inhibitor KN93 10 μmol/L for 24 and 48 hours. Then, phosphorylation of RET-Y1062, CaMKII, S338-Raf1, ERK, total cyclin D, and p27Kip1 were evaluated by Western blot analysis (Fig. 3B). KN93 was ineffective on RET phosphorylation, ensuring that the effects on the phosphorylation status of the other kinases was not a direct effect of KN93 on RET. A time-dependent inhibition of CaMKII phosphorylation confirmed the inhibitory effect of KN93. A substantial dephosphorylation of Raf1 at Ser 338 and ERK occurred by 24 hours. A remarkable decrease of cyclin D expression was evident in both cell lines, with a time-dependent fashion. Accumulation of p27Kip1, more evident in TT cells, could also be observed. Phosphorylation of Raf-1 at Ser338 and cyclin D expression level were evaluated also upon CaMKII inhibition by the synthetic peptide antCaNdte (Fig. 3C). This peptide is derived from the endogenous hCaMKII inhibitor protein and was made cell permeable by N-terminal addition of an Antennapedia-derived sequence (28). CaMKII inhibition by antCaNdte confirmed the effects observed by KN93. These results demonstrate that ERK activation and the cell cycle are CaMKII mediated in MTC cells.

Effect of CaMKII inhibition on MTC cell proliferation

MTC cell lines were cultured in the presence of antCaNdte at different concentrations for up to 9 days. Every three
days, the medium and the inhibitor were renewed and the cell number was determined (Fig. 4). In TT cells, antCaNtide at both 25 and 50 μmol/L induced a significantly reduced number of cells respect to control after 3 days of treatment, then an effect was significant only with the higher concentration of inhibitor. Microscopic observation of the cells treated, revealed a considerable number of floating cells, and adherent round shaped cells already by 6 days of culture, indicating a toxic effect of the treatment. MZ-CRC1 exhibited a more resistant phenotype at lower peptide concentration. The number of MZ-CRC1 cells after 9 days of treatment with 50 μmol/L antCaNtide was 23% of the untreated cells.

hCaMKIINα expression in primary MTC and MTC cell lines

The hCaMKIINα mRNA relative expression was assessed by real-time PCR in 49 tissue samples of primary MTC, TT, and MZ-CRC1 cells. Relative mRNA expression level was determined as previously reported (27) and normalized to an internal standard (β-actin). hCaMKIINα mRNA expression was remarkably variable (Fig. 5). hCaMKIINα mRNA expression in MZ-CRC1 was about two-fold than in TT cells. These results pursued us to investigate about a possible correlation between hCaMKIINα mRNA expression and clinicopathologic features in patients with MTC.

hCaMKIINα expression and clinicopathologic characteristics of MTC

We determined the association between clinicopathologic characteristics of 49 MTC at surgery time and hCaMKIINα mRNA expression. hCaMKIINα mRNA expression inversely correlated with the serum calcitonin concentration ($R^2 = 0.032$ in Spearman rank correlation, $P = 0.017$; Fig. 6A). hCaMKIINα mRNA expression was inversely correlated with the local tumor extension ($T$; $F = 5.276$; $P = 0.0079$ by ANOVA; Fig. 6B). Data also indicated a borderline significantly negative trend between hCaMKIINα mRNA expression and tumor staging ($F = 5.158$; $P = 0.0652$ by ANOVA; Fig. 6D). Inverse correlation was also observed between hCaMKIINα mRNA expression and lymph node metastasis...
at surgery time (Student t test, P = 0.033; Fig. 6C). These results indicate that hCaMKIINα expression is inversely correlated with a more aggressive disease at diagnosis, suggesting that inhibition of CaMKII by its endogenous inhibitor protects patients from a more aggressive MTC.

Discussion

Previous studies demonstrated that RET/PTC3 activates CaMKII through two pathways: Y1062/RAS/PLCγ/Ca2+ /CaMKII and Y1015/PLCγ/Ca2+ /CaMKII (17). This conclusion arises from the signaling analysis of RET/PTC mutants in COS-7 cells and the observation that inhibition of RET/PTC-induced CaMKII activation is achieved by both RAS and PLCγ inhibitors and by calcium chelators. Based upon these data, we hypothesized that RET mutants in MTC cells might activate CaMKII and participate in C-cell carcinogenesis. The NIH3T3 cells carrying the RET mutants G634W and M018T provided us with convincing evidence that oncogenic RET activates CaMKII though a calcium-mediated signal. In these cell mutants, CaMKII resulted in an active state of a magnitude comparable with that obtained by the calcium ionophore ionomycin and was inhibited by the calcium chelator BAPTA. However, although the NIH3T3 is a good cell model, suitable to study signal transduction pathways, the biologic role of CaMKII is cell type–dependent and results obtained in NIH3T3 need a direct experimental validation in MTC cells. Indeed, inhibition of CaMKII does not prevent cell proliferation in NIH3T3 and in some prostate cancer cells (unpublished personal observations), while it occurs in other cell types such as normal thyroid cells, thyroid cancer cells, or fibroblasts (15–17). Thus, the role of CaMKII in the MAPK pathway was studied in two MTC cell lines harboring G634W and M018T RET mutants. In both TT and MZ-CRC1 cell lines, CaMKII was in an active state also in the absence of extracellular stimuli. BAPTA, W7, and U73-122 abrogated the kinase phosphorylation, indicating that CaMKII in these cells was depended by a calcium/calmodulin/PLCγ-mediated signaling. RET inhibition by ZD6474 demonstrated that CaMKII activation was induced by the RET mutants present in these cell lines. The effect of PLCγ inhibitor U73-122 was more evident in MZ-CRC1 than in TT cells, suggesting some difference between the two RET mutants or the existence in TT cells of an additive factor modulating the intracellular calcium concentration. This result is not surprising, as there

---

**Figure 5.** hCaMKIINα expression in primary MTC and MTC cell lines. hCaMKIINα mRNA relative expression was assessed by real time PCR in 49 tissue samples of primary MTC, TT, and MZ-CRC-1 cells. Relative mRNA expression level was normalized to β-actin and relative mRNA expression in each sample was calculated according to the 2 ΔΔCT method (27). For each sample, the relative expression is reported as relative mRNA expression/lower relative mRNA expression. Data, mean ± SDs of triplicates. MZ-CRC1 and TT cells are indicated.

---

**Figure 6.** hCaMKIINα expression and clinicopathologic parameters in primary MTC. hCaMKIINα mRNA relative expression was assessed by RT-PCR in 49 tissue samples of primary MTC and correlated with serum calcitonin (A), T classification of the pTNM system (B), lymph node metastasis (C), and tumor staging (D). The ordinates in B, C, and D report relative hCaMKIINα mRNA expression. CT, calcitonin; Horizontal bars are averages.
are many factors that modulate the intracellular calcium concentration. Among these, there are the oncogenes- 
\[BRaf^{V600E}\] and \[Ras^{V12}\] in papillary thyroid carcinomas (17). Like in other cell types investigated previously, also in MTC cells, active CaMKII phosphorylated RAF-1 at Ser 338, participating in the MAPK pathway, leading to cell proliferation (11, 13). Phosphorylation of Ser338 only potentiates RAF-1 activation, whereas full Raf-1 activation is achieved by concurrent S338 and Y341 (i.e., by Src) phosphorylation (29, 30). Accordingly, direct evidence in COS-7 cells demonstrated that expression of constitutively activated CaMKII-induced phosphorylation of Ser338 RAF-1, but induced neither RAF-1 nor ERK activation (13). The demonstration that RET-induced RAF-1, MEK and ERK phosphorylation, and cell proliferation requires CaMKII indicates the involvement of RAF-1 in MTC cells, while the effective role of BRAF remains unknown. The participation of RAFl to the proliferative signaling in MTC can be an important limitation in the therapy with BRAF inhibitors. Overall, these data indicate that CaMKII is a pivotal component of the RET signaling and is necessary for ERK activation induced by RET mutants in MTC cells.

As stated previously, CaMKII activation is a frequent occurrence in tumors, including colon adenocarcinoma, ovarian cancer, and many tumor cell lines, as many are the factors that modulate the intracellular calcium concentration. Among the different tumors we analyzed so far, CaMKII activation was demonstrated in all papillary thyroid carcinomas (n = 8) and prostate cancer (n = 12), regardless the presence of RET/PTC, BRAF or Ras mutations, by other concurring intracellular calcium-modulating signals (17). Similarly, if such calcium-modulating signals are present in MTC, they can potentiate or replace the RET-mutant signaling. Hence, CaMKII can be activated in MTC cells even in the absence of RET-activating mutations. A role for CaMKII in the regulation of cell cycle and cell proliferation in some tumors has emerged by studying its endogenous inhibitors. The endogenous inhibitors of CaMKII (CaMKIINα and β) were first identified from rat brain extracts, in Cos-7 cell line and in neurons. It has been hypothesized that CaMKIIN competes with the CaMKII region around T286 in an isoform-independent manner (31). The endogenous CaMKII inhibitor hCaMKIINα induces accumulation of p27\(^{\text{G1p1}}\), ERK dephosphorylation, and cell-cycle arrest in colorectal carcinoma (19). hCaMKIINα expression was negatively correlated with the severity of human colon adenocarcinoma, whereas hCaMKIINβ expression was negatively correlated with the severity of ovarian adenocarcinoma (31). The analysis of hCaMKIINα expression in primary MTC revealed a broad variability. Notably, a strong inverse correlation between relative hCaMKIINα expression levels and the disease extension in patients affected by MTC was observed. Although the sample number was relatively low, a highly significant inverse correlation was found between hCaMKIINα mRNA expression in the tumors and the serum calcitonin levels and tumor dimension. A less evident inverse correlation was found between hCaMKIINα mRNA expression and lymph node metastasis and staging. All these findings strongly indicate that hCaMKIINα counteracts the aggressive clinical behavior of MTC, while suggesting that attenuation of CaMKII activity is correlated with a less aggressive tumor. A direct evidence of the endogenous CaMKII activity in the tumors studied could only be hypothesized on the basis of the expression of its inhibitor. However, until other functions will be assigned to hCaMKIIN, its CaMKII inhibitory effect is the mechanism that can explain its negative correlation with tumor extension. These results suggest that hCaMKIINα might be used as a prognostic factor useful for tailoring the therapy of MTC. As a final consideration, CaMKII could represent a new therapeutic target for pharmacologic intervention in this cancer type.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Salzano, M. Vitale
Development of methodology: E. Russo, M. Salzano, V. De Falco, S. Barollo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Russo, M. Salzano, C. Mian, A. Secondo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Russo, M. Salzano, A. Secondo, M. Vitale
Writing, review, and/or revision of the manuscript: V. De Falco, M. Vitale
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Russo, C. Mian, M. Vitale
Study supervision: V. De Falco, M. Bifulco, M. Vitale

Acknowledgments
The authors thank Prof. Massimo Santoro for his critical reading of the manuscript, and Dr. Valeria Ursini and Maria Teresa Paciolla for their technical support.

Grant Support
This study was partly supported by Ministero Italiano della Università e della Ricerca (to M. Vitale)
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 19, 2013; revised November 25, 2013; accepted December 22, 2013; published OnlineFirst January 21, 2014.


Calcium/Calmodulin-Dependent Protein Kinase II and Its Endogenous Inhibitor \(\alpha\) in Medullary Thyroid Cancer

Eleonora Russo, Marcella Salzano, Valentina De Falco, et al.

Clin Cancer Res  Published OnlineFirst January 21, 2014.

Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-1683

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.