Calcium/calmodulin-dependent protein kinase II and its endogenous inhibitor α in medullary thyroid cancer

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Running title: CaMKII in MTC

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Statement of Translational Relevance:

Medullary thyroid carcinoma (MTC) originates in the thyroid from the parafollicular C cells, frequently harboring point mutations of the RET gene. The calcium-calmodulin dependent kinase II (CaMKII) is an ubiquitous kinase involved in the MAPK cascade activation, while its endogenous inhibitor hCaMKIINα is a protein expressed in some tumor types. In this study we demonstrate that CaMKII is necessary to RET-induced activation of the MAPK pathway and stimulated cell proliferation. We determined the level of mRNA expression of hCaMKIINα and its correlation with clinicopathological features in primary MTC, observing a strong inverse correlation between relative hCaMKIINα expression levels and the disease extension. Our results assign to CaMKII and to its endogenous inhibitor an important role in MTC, suggesting that hCaMKIINα might be used as a prognostic factor useful for tailoring the therapy of MTC, while CaMKII could represent a new therapeutic target for pharmacological intervention in this cancer type.
ABSTRACT

Purpose: Calcium/calmodulin dependent kinase II (CaMKII) is involved in the regulation of cell proliferation. Its endogenous inhibitor (hCaKINα) is expressed in some cell types. We determined the role of CaMKII in RET-stimulated proliferation and hCaKINα 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 hCaKINα mRNA was determined by real-time PCR in primary MTC and it was correlated with some clinicopathological parameters.

Results: RETG634V and RETM918T 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. hCaKINα mRNA expression in primary MTC was very variable and did not correlate with gender and age at diagnosis. Serum calcitonin, ($R^2 = 0.032, p = 0.017$), tumor volume ($p = 0.0079$), lymph node metastasis ($p = 0.033$) and staging ($p = 0.0652$) were negatively correlated with the hCaKINα mRNA expression.

Conclusions: CaMKII is activated by RET mutants and is activated at baseline in MTC cells were it mediates the oncogenic pathway leading to cell proliferation. The mRNA expression of its endogenous inhibitor hCaKINα inversely correlates with the severity of MTC. CaMKII might represent a new target for MTC therapy, and hCaKINα is a marker of disease extension.
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 (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) (5). Multiple signalings 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, DOK1/4/5/6, IRS1/2, Enigma, protein kinase Cα (PKCα) and Shank (7). Among these, SHC binding plays a crucial role in activation of both the RAS/ERK and phosphatidylinositol 3-kinase (PI3)-K/AKT pathways (8). Phosphorylated tyrosine 1015 is docking sites for phospholipase Cγ (PLCγ) (9, 10).

The calcium-calmodulin dependent kinase II (CaMKII) is an ubiquitous serine/threonine protein kinase involved in multiple signalings and biological 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 biological function of CaMKII is cell type and cell context 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 B RAFV600E, oncogenic Ras and by RET rearrangements (RET/PTC) and participates to the activation of the ERK 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 our study, we investigated the role of CaMKII and its endogenous inhibitor hCaMKIIINα in MTC. We determined the ability of major RET mutants to activate CaMKII, the status of CaMKII activation in two MTC cell lines, the effect of CaMKII inhibition on RET/ERK signaling and cell proliferation. Then we determined the level of mRNA
expression of hCaMKIINα and its correlation with clinicopathological features in primary MTC. Our results assign to CaMKII an important role in MTC.

MATERIALS AND METHODS

Cell cultures
Parental murine NIH3T3 fibroblasts and NIH3T3 cells stably transfected with the RET mutants RET<sup>C634R</sup> and RET<sup>M918T</sup>, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. TT cells were from American Type Culture Collection (Manassas, VA, USA). TT 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, Grand Island, NY). MZ-CRC1 cells were grown in DMEM supplemented with 10% FBS. All media were supplemented with 2 mM L-glutamine and 100 U/ml penicillin–streptomycin (Gibco). All cell lines were maintained at 37°C, 5%CO2.

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

Western blot
For Western blot analysis, cells were lysed in Laemmli buffer [0.125 mol/liter Tris (pH 6.8), 5% 95 glycerol, 2% sodium dodecyl sulfate(SDS),1%βmercaptoethanol, and 0.006% bromophenol blue]. Proteins were resolved by 7–15% SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon P, Millipore Corp., Bedford, MA).
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 h with a horseradish peroxidase conjugated secondary antibody. Finally, protein bands were detected by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Anti-phosphorylated p44/42 ERK (ERK1/2) were rabbit polyclonal antibodies from Cell Signaling (Beverly, MA). 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 mM Tris-HCl at pH 7.5, 150 mM 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, Santa Cruz, CA) for 1 hour at room temperature followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Monoclonal antiphospho-CaMKII antibody (pT286-CaMKII) were from Cell signaling; anti-phospho Raf-1 S338 was from Biothechnology, Lake placid, NY.

Reagents and inhibitors
The CaMK inhibitor KN93 and the CaM inhibitors N-(6 aminohexyl)-5-chloro-1-nafthalene-sulfonamide (W7) were purchased from Sigma Aldrich (St. Louis, MO). The selective inhibition of RET ZD6474 (Vandetanib) was kindly provided by AstraZeneca (Macclesfield, UK). Ionomycin and BAPTA-AM were provided by Sigma.

Patients and tumors
Tumor tissues were promptly dissected immediately after operation and then snap-frozen and stored at −80 °C. MTC 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 MTC were sporadic. The mean age at diagnosis was 54.9 years (range 37-81). Serum CT concentrations were determined using an immunoradiometric assay in blood samples obtained 1-3 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, Milan, Italy) 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 MTC patients. Real-time PCR was performed using SYBR Green PCR reagents (Fermentas) and primers specific for hCaMKIINα and β-actin. forward and reverse primers for hCaMKIINα were respectively: 5’-TACGGCGACGAGAAGCTGAG-3’ and 5’-TCAGCACGTCATCAATCCTATC-3’. The β-actin forward and reverse primers were: 5’-TTCCCTCCTGGGCATGGAGT-3’ and 5’-TACAGGTCTTTGCGGATGTC-3’. The samples were analyzed with Biorad Cycler with the following protocol: 95°C x 5’; 95°C x 10”; 60°C x 60” (x 40 cycles). The levels of relative mRNA expression were determined by normalizing to β-actin expression and adopting the ΔΔCt method (27).

**Statistical analysis**

Results are presented as the mean ± SD. Statistical procedures included Student’s 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 (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, while 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 suspension to reduce the integrin signalings, and CaMKII phosphorylation was evaluated by Western Blot. (Fig. 2A). Untreated cells exhibited a strong CaMKII phosphorylation. Ionomycin treatment produced a paradoxical effect reducing CaMKII phosphorylation. The calcium sensitivity of CaMKII was preserved as demonstrated by the dose-dependent inhibition induced by the calcium chelator BAPTA.

CaMKII activation is RET and PLC-γ mediated in MTC cell lines. To determine whether CaMKII activation was RET dependent in MTC cell lines, TT cells were starved from serum for 24 hours and incubated in suspension with increasing concentration of the RET inhibitor ZD6474 (Vandetanib) for 60 minutes. The cells were then lysed in RIPA buffer, and the phosphorylation of RET-Y1062, CaMKII-T286 and MEK, were evaluated by Western Blot. (Fig. 2B). Abrogation of RET phosphorylation by ZD6474 was accompanied by down-phosphorylation of CaMKII and MEK. Similar results were obtained in MZ-CRC1 cells (not shown). PLCγ is activated by RET and increases intracellular calcium concentration. To determine whether CaMKII activation by RET was PLCγ mediated, serum starved TT and MZ-CRC1 cells were treated in suspension with the PLC-γ inhibitor U73-122. CaMKII phosphorylation was determined by Western Blot (Fig. 2C). In MZ-CRC1 cells, 15 μM U73-122 was sufficient to induce a dramatic CaMKII dephosphorylation, whereas in TT cells the same effect was obtained with 45 μM. 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 (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 pharmacological CaMKII inhibitor KN93 10μM for 24 and 48 hours. Then, phosphorylation of Y1062-RET, CaMKII, S338-Raf1, ERK, total cyclin D and p27Kip1 were evaluated by Western Blot (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 h. 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 ERK, and cyclin D expression level were evaluated also upon CaMKII inhibition by the synthetic peptide antCaNtide (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 antCaNtide 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 antCaNtide 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 μM 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 μM antCaNtide was 23% of the untreated cells.

**hCaMKIIα mRNA expression in primary MTC and MTC cell lines.**

The hCaMKIIα 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). hCaMKIIα mRNA expression was remarkably variable (Figure 5). hCaMKIIα mRNA expression in MZ-CRC1 was about two fold than in TT cells. These results pursued us to
investigate about a possible correlation between hCaMKII\(\alpha\) mRNA expression and clinicopathological features in MTC patients.

**hCaMKII\(\alpha\) expression and clinicopathological characteristics of MTC**

We determined the association between clinicopathological characteristics of 49 MTC at surgery time and hCaMKII\(\alpha\) mRNA expression. hCaMKII\(\alpha\) mRNA expression inversely correlated with the serum calcitonin concentration (\(R^2=0.032\) in Spearman rank correlation \(p=0.017\)) (Fig. 6A). hCaMKII\(\alpha\) 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 hCaMKII\(\alpha\) mRNA expression and tumor staging (\(F=5.158, P=0.0652\) by ANOVA) (Fig. 6D). Inverse correlation was also observed between hCaMKII\(\alpha\) mRNA expression and lymph node metastasis at surgery time. (Student’s t-test; \(p=0.033\)) (Fig. 6C). These results indicate that hCaMKII\(\alpha\) 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 medullary thyroid carcinoma.

**DISCUSSION**

Previous studies demonstrated that RET/PTC3 activates CaMKII through two pathways: Y1062/RAS/PLC\(\gamma\)/Ca\(^{2+}\)/CaMKII and Y1015/PLC\(\gamma\)/Ca\(^{2+}\)/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 PLC\(\gamma\) inhibitors and by calcium chelators. Based upon these data, we hypothesized that also RET mutants in MTC cells, might activate CaMKII and participate to C cell carcinogenesis. The NIH3T3 cells carrying the RET mutants C634W 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, while the NIH3T3 is a good cell model, suitable to study signal transduction pathways, the biological 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 C634W 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/PLC\(\gamma\) mediated signaling. RET inhibition by ZD6474, demonstrated that CaMKII activation was induced by the RET mutants present in these cell lines. The effect of PLC\(\gamma\) 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 many are the factors that modulates the intracellular calcium concentration. Among these, 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 to the MAPK pathway, leading to cell proliferation (11, 13). Phosphorylation of Ser338 only potentiates RAF-1 activation, while 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 RAF-1 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 modulates 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 signalings (17). Similarly, if such calcium modulating signalings 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 (CaMKIIIN\(\alpha\) and \(\beta\)) were first identified from brain rat extracts, in Cos-7 cell line and in neurons. It has been hypothesized that CaMKIIIN competes with the CaMKII region around T286 in an isoforms independent manner (31). The endogenous CaMKII inhibitor hCaMKIIIN\(\alpha\) induces accumulation of
p27^Kip1, ERK dephosphorylation and cell cycle arrest in colorectal carcinoma (19). hCaMKIINα expression was negatively correlated with the severity of human colon adenocarcinoma, while 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 suggest 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 hCaMKIINα, 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 pharmacological intervention in this cancer type.

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REFERENCES


Legends to figures

**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 from serum for 24 hours and then treated with the calcium ionophore ionomycin 2 μM 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. 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. S.D. were less than 10%. *, p <0.05 vs. first point. B) [Ca^{2+}]i recorded under basal conditions in NIH2A and NIH2B mutants and parental NIH3T3 cells. C) quantification of ATP- and Tg-induced effects on [Ca^{2+}]i increase, calculated as Δ% 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.

**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 μM ionomycin for 30 minutes. Phosphorylation of CaMKII at Thr 286 was evaluated by Western Blot. B) TT cells starved from serum for 24 hours and incubated in suspension with increasing concentration of the RET inhibitor ZD6474. The proteins were resolved on SDS PAGE, and the phosphorylation of Y1062-RET, CaMKII and MEK, were evaluated by Western Blot. C) TT and MZ-CRC1 cells were starved from serum for 24 hours and incubated for 2 hours with increasing concentration of the PLC-γ inhibitor U73-122 (15-30-45 μM). The cells were then lysed and CaMKII phosphorylation was evaluated by Western Blot. Numbers in italic are averages of relative bands intensity, determined by scanning densitometry of three independent immunoblots. S.D. were less than 10%. *, p <0.05 vs. first point.

**Figure 3. ERK activation and cell cycle are mediated by CaMKII in MTC cell lines.**

TT and MZ-CRC1 cells were serum starved for 24 hours and treated with the ionomycin or BAPTA-AM for 30 minutes, or with the calmodulin inhibitor W7 for 15 or 30 minutes (A). The cells were then lysed in RIPA buffer.
and ERK phosphorylation was evaluated by Western Blot. The cells were starved from serum for 24 h and then treated with the CaMKII inhibitor KN93 (10 μM) for 24 or 48 hours (B) or starved from serum for 24 hours and incubated with the CaMKII inhibitor antCaNtide (ant), 25 or 50 μM (C). Phosphorylation of Y1026-RET, pCaMKII, S338-RAF1 and ERK, and total cyclin D and p27^kip1 were evaluated by Western blot. Numbers in italic are averages of relative bands intensity, determined by scanning densitometry of three independent immunoblots. S.D. were less than 12%. *, p <0.05 vs. first point.

Figure 4. Effects on cell proliferation of CaMKII inhibition.
TT and MZ-CRC1 cells were plated at 500,000 cells/well in DMEM supplemented with 5% fetal bovine serum. The number of attached cells was determined after overnight culture (day 0). Then the cells were treated with the CaMKII inhibitor antCaNtide at different concentration (25 or 50 μM) for up to 9 days. The medium and the inhibitor were renewed every three days. Cell number was determined by a Biorad TC10 Automated Cell Counter. *, P < 0.05; **, P< 0.001. Data are reported as the mean ± standard deviation from triplicate experiments.

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 are presented as mean +/- standard deviations of triplicates. MZ-CRC1 and TT cells are indicated.

Figure 6. hCaMKIINα expression and clinicopathological 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.
Figure 1

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<td>3T3</td>
<td>80</td>
</tr>
<tr>
<td>2A</td>
<td>80*</td>
</tr>
<tr>
<td>2B</td>
<td>80*</td>
</tr>
</tbody>
</table>

C

ATP-Tg-induced [Ca^{2+}]_i increase (D%)

<table>
<thead>
<tr>
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<th>ATP-Tg诱导增加</th>
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<tbody>
<tr>
<td>3T3</td>
<td>120</td>
</tr>
<tr>
<td>2A</td>
<td>120</td>
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<tr>
<td>2B</td>
<td>120*</td>
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Figure 2

A

<table>
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<tr>
<th>TT</th>
<th>MZCRC-1</th>
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<tr>
<td>1</td>
<td>0.4*</td>
</tr>
<tr>
<td>0.1*</td>
<td>0.3*</td>
</tr>
<tr>
<td>1</td>
<td>0.4*</td>
</tr>
<tr>
<td>0*</td>
<td>0.1*</td>
</tr>
</tbody>
</table>

- + - 10 5
- - 10 5

Ionomycin BAPTA (μM)

Actin

B

<table>
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<tr>
<td>1</td>
</tr>
<tr>
<td>0.8</td>
</tr>
<tr>
<td>0.3*</td>
</tr>
<tr>
<td>0.1*</td>
</tr>
<tr>
<td>0*</td>
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</table>

pY1062-RET

pCaMKII

pMEK

tubulin

ZD6474 0 50 100 500 1000 nM

C

<table>
<thead>
<tr>
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<th>MZ-CRC1</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.7</td>
<td>0.1*</td>
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<tr>
<td>1</td>
<td>0.3*</td>
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<tr>
<td>0*</td>
<td>0*</td>
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</tbody>
</table>

pCaMKII

tubulin

U73-122 0 15 30 45 0 15 30 45 μM

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Figure 3

A

TT

MZ-CRC1

Ionomycin

- + - -

W7

- - 15 30 Min

1 1 0.1* 0*

pERK

1 1.1 0* 0*

ERK

- + - -

- 15 30 μM

B

TT

MZ-CRC1

pY1062-RET

1 1 0.2* 0.3*

pCaMKII

1 1 0.9 0*

pS338-RAF1

1 1 1.4 2.2*

RAF1

pERK

1 0.5* 0.3*

ERK

1 0.1* 0.1*

Cyclin D

1 1 1.8 1.1

p27Kip1

1 1 1 1

tubulin

KN93

- 24 48

- 24 48 hours

C

TT

MZ-CRC1

ant

- 25 50

- 25 50 μM

pCaMKII

1 1.1 0.3*

pS338RAF1

1 0.7* 0.2*

pERK

1 1.1 0.3*

1 0.9 0.4*

Cyclin D

1 1 1 1

tubulin

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Figure 4

TT

MZ-CRC1

Number of cells x 10^5

Days of culture

antCaNTide (μM) 0 25 50

**

*
Figure 6

A. Scatter plot showing the relationship between CT (ng/mL) and hCaMKIIINα. The regression line has an R^2 value of 0.032 and a P-value of 0.017.

B. Box plot showing the distribution of hCaMKIIINα across different pT stages. The P-value for the comparison between pT stages is 0.0079.

C. Box plot showing the distribution of hCaMKIIINα across different pN stages. The P-value for the comparison between pN stages is 0.033.

D. Box plot showing the distribution of hCaMKIIINα across different tumor stages. The P-value for the comparison between tumor stages is 0.0652.
Calcium/calmodulin-dependent protein kinase II and its endogenous inhibitor α in medullary thyroid cancer

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

Clin Cancer Res Published OnlineFirst January 21, 2014.

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