CLINICAL STUDY

AMP-activated protein kinase signaling is upregulated in papillary thyroid cancer

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Abstract

AMP-activated protein kinase (AMPK) is activated by the depletion in cellular energy levels and allows adaptive changes in cell metabolism and cell survival. Recently, our group described that AMPK plays an important role in the regulation of iodide and glucose uptake in thyroid cells. However, AMPK signaling pathway in human thyroid carcinomas has not been investigated so far.

Objective: To evaluate the expression and activity of AMPK in papillary thyroid carcinomas.

Methods: We examined total and phosphorylated AMPK (tAMPK and pAMPK) and phosphorylated acetyl-CoA-carboxylase (pACC) expressions through immunohistochemistry, using a tissue microarray block composed of 73 papillary thyroid carcinomas (PAP CA) or microcarcinomas (PAP MCA) and six adenoma (AD) samples from patients followed at the Federal University Hospital. The expression levels were compared with the non-neoplastic tissues from the same patient. Two different pathologists analyzed the samples and attributed scores of staining intensity and the proportion of stained cells. A total index was obtained by multiplying the values of intensity and the proportion of stained cells (INTxPROP).

Results: tAMPK, pAMPK, and pACC showed a predominant cytoplasmic staining in papillary carcinomas, adenomas, and non-neoplastic thyroid tissues. However, the intensity and the proportion of stained cells were higher in carcinomas, so that a significant increase was found in the INTxPROP score both in PAP CA and PAP MCA, when compared with their respective controls.

Conclusion: Our results show unequivocally that AMPK pathway is highly activated in papillary thyroid carcinomas; however, more studies are necessary to understand the pathophysiological significance of AMPK activation in thyroid carcinogenesis.

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Introduction

AMP-activated protein kinase (AMPK) is a metabolic stress-sensing cytoplasmic enzyme composed of an α -catalytic and two regulatory subunits (β and γ) (1). Stresses that deplete energy and increase the intracellular AMP-to-ATP ratio induce allosteric activation of AMPK (1, 2), promoting conformational changes that make the enzyme a better substrate for upstream kinases, which phosphorylate its Thr-172 residue and further activate AMPK. In its activated state, AMPK shuts down processes that consume energy and upregulates energy-producing pathways in an attempt to restore intracellular ATP levels (1, 2, 3). One of the

well-described effects of AMPK is the inhibition of acetyl-CoA-carboxylase (ACC), an enzyme responsible for the conversion of acetyl-CoA to malonyl-CoA in *de novo* fatty acid biosynthesis. Malonyl-CoA is a potent inhibitor of carnitine palmitoyl transferase-1, responsible for the transport of long-chain fatty acid to mitochondrial matrix. The reduction of malonyl-CoA induced by AMPK activation thus favors fatty acid translocation into the mitochondria to be oxidized, in an attempt to restore intracellular ATP levels (1, 2, 3, 4). Activation of AMPK can be evaluated by the use of a specific antibody that recognizes the catalytic subunits of AMPK when phosphorylated in Thr-172 residue (4). Also, a direct inference of AMPK activity is obtained through the

analyses of the phosphorylation in Ser-79 of its downstream target, ACC (4). In its activated state, AMPK allows cellular adaptive changes in order to maintain growth, differentiation, and metabolism under conditions of low intracellular energy availability (1, 2, 3, 4, 5).

Recently, we described the involvement of AMPK signaling in the regulation of iodide and glucose uptake in rat thyrocytes, both *in vitro* and *in vivo* (3, 6). We showed that the AMPK activator, 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR), decreases iodide uptake (*in vitro* and *in vivo*) and NIS protein and mRNA content in thyroid cells (4). Also, using the same methodological approach, we have recently demonstrated that AICAR produces a concentration-dependent increase in glucose uptake by thyroid PCCL3 cells (6). It is important to notice/emphasize that tumor cells are submitted to metabolically stressful conditions and a shift toward glucose metabolism, known as the 'Warburg Effect' occurs. Therefore, it is tempting to speculate whether AMPK activation could be implicated in this phenomenon.

Although the role of AMPK signaling in cancer cells is not completely understood, some evidence suggests that AMPK activation leads to anti-proliferative effects, with G1-S phase cell cycle arrest (7, 8, 9, 10). Further evidence for AMPK anti-proliferative effects on tumor cells relies on the fact that the major kinase that phosphorylates and activates AMPK has been identified to be the tumor suppressor kinase LKB1 (9). The previous findings showing that the oral anti-diabetic drug, metformin, inhibits proliferation of epithelial cells derived from breast, prostate, and ovarian cancers, effects that require both LKB1 and AMPK, are consistent with the concept that AMPK pathway might be implicated in tumor cell biology (1, 11, 12, 13). Also, several epidemiological studies demonstrated that the chronic use of metformin is associated with a lower incidence of cancer (1, 13). On the one hand, activated AMPK shows anti-proliferative effects on tumor cells; on the other hand, it leads to tumor cell survival, as cell adaption to adverse conditions, such as glucose deprivation and hypoxia, might require AMPK activation (1, 14, 15).

Differentiated thyroid carcinomas (DTC) are slow-growing and usually curable forms of thyroid cancer (16). The adequate intervention for this type of cancer includes the combined effects of surgery, radioiodine ablation, and thyroid-stimulating hormone suppressive therapy (17, 18, 19). However, tumor recurrence can occur in about 20–30% of patients with DTC, which reinforces the importance of unraveling novel targets for thyroid cancer diagnosis and treatment (20, 21, 22). Previous reports show that thyroid tumor progression is accompanied by increased glucose uptake detected by 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) and decreased radioiodide uptake ability (23). Thus, based on our previous findings in normal thyroid cells, we hypothesized that the expression and activation of AMPK could be modulated in thyroid tumors.

Hence, our objective in this study was to evaluate the expression of total AMPK (tAMPK), phosphorylated AMPK (pAMPK), and phosphorylated ACC in papillary thyroid carcinomas and microcarcinomas, in relation to adenomas and the non-neoplastic tissue (NNT) of the same patient. We herein describe for the first time that the AMPK pathway is significantly upregulated in papillary thyroid carcinomas, while normally expressed in benign hyperplastic lesions of the thyroid.

Subjects and methods

Patients

This retrospective study used paraffin-embedded tissue blocks from 79 patients, accompanied at the Clementino Fraga Filho University Hospital from the Federal University of Rio de Janeiro, who underwent resection of papillary thyroid carcinomas ($n=73$) or thyroid follicular adenomas ($n=6$) between 1998 and 2008. After obtaining approval from the institutional review board, we retrospectively reviewed the electronic medical records of the patients. Each slide case was reviewed by the same two pathologists and classified according to the classification of the World Health Organization (22). The patients consisted of 12 men and 61 women, and the mean patient age at the time of surgery was 45.2 ± 15.3 years old (range: 14–87 years old). All available clinical, pathological, and follow-up data were collected from our database, reviewed and updated for all patients.

Tissue microarray

Thyroid tissue specimens were fixed in 10% buffered formaldehyde solution and embedded in paraffin. We reviewed the available slides and selected the paraffin-embedded tissue blocks. The diagnosis of carcinomas was confirmed using hematoxylin- and eosin (HE)-stained sections, following standard criteria according to the classification of the World Health Organization (22). Using a manual tissue microarray (TMA) instrument (Beecher Instruments, Sun Prairie, WI, USA), two blocks of high-density TMA were designed to include at least two samples of 57 papillary thyroid carcinomas (>1 cm, CAPAP) distributed as 50 classical and seven follicular subtypes, 16 papillary microcarcinomas (<1 cm, MCAPAP), and six follicular adenomas. All the samples were analyzed in relation to their corresponding NNT. A total of 236 spots were analyzed.

Immunohistochemistry

AMPK α , phospho-AMPK α (Thr-172)-(40H9), and phospho-acetyl-CoA carboxylase (Ser-79) primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies were

previously tested for immunohistochemistry analysis in paraffin-embedded tissue, as mentioned by the manufacturer. Optimal staining conditions such as epitope unmasking, antibody titer, and incubation and visualization methods were validated in our service using conventional whole tissue sections and TMA fragments. For the immunostaining reaction, DAKO autostainer using Vectastatin ABC kits (Vector Labs, Peterborough, UK) were used according to the manufacturer's protocol. The antigen retrieval was carried out in citrate buffer (pH 6.0) vaporized at 95–98 °C for 20 min. Tissues were subject to blocking of endogenous peroxidase activity with 3% hydrogen peroxide. Sections were then incubated with the primary antibody overnight, at 4 °C, in the dilution of 1:50. Ductal breast carcinoma was used as positive and negative controls of the reactions. Omitting the primary antibody was carried out to provide negative controls. Following the incubation with the specific primary antibodies, sections were incubated with either biotinylated anti-rabbit, anti-sheep, or anti-mouse antibodies for 30 min, followed by Vectastain Elite ABC reagent for another 30 min. Liquid diaminobenzidine (Dako, Glostrup, Denmark) was used as a chromogenic agent for 5 min and sections were counterstained with Mayer's hematoxylin. After each immunostaining step, the slides were briefly washed in PBS buffer, pH 7.6.

Two independent pathologists (V A P and C B A) analyzed the immunostaining reactions. The observers were unaware of the clinical history and the follow-up of the patients. Each staining was assessed using a scoring system based on the Quick Score Method (23). Immunoreactivity was scored semiquantitatively for both the staining intensity and the proportion of cytoplasmic cell staining. Intensity evaluation used scores that ranged from 0 to 3 (0 = negative; 1 = light; 2 = moderate; 3 = strong) and the proportion scores ranged from 1 to 6 (1 = 0–4%; 2 = 5–20%; 3 = 21–40%; 4 = 41–60%; 5 = 61–80%; 6 = 81–100%). The two scores were then multiplied to obtain a total index of staining. Average score was taken as the final score between duplicate spots of the same patient. The staining analyses were scored at the magnification of 40× to estimate the proportion of positive cells. The two pathologists, using a double-headed microscope, reevaluated all cases with discrepant scores and a consensus was reached.

Statistical analysis

The statistical analysis was done using GraphPad Prism (La Jolla, CA, USA) 5.0 software. For the histological quantification of intensity and proportion, we analyzed data using the nonparametric Wilcoxon matched pairs test for the distribution frequency of the scores. Data shown in Table 1 correspond to the differences in ranks in two conditions (NNT and CAPAP; NNT and MCAPAP). Data are expressed in number and percentiles of those

Table 1 Difference between paired samples of NNT and CAPAP/MCAPAP in intensity score calculated by Wilcoxon test for total AMPK, phospho-AMPK, and phospho-ACC.

	Difference between NNT and CAPAP n (%)	P	Difference between NNT and MCAPAP n (%)	P
Total AMPK				
Reduced	17 (30)	0.04	6 (37)	NS
Maintained	33 (58)		8 (50)	
Increased	7 (12)		2 (13)	
Phospho-AMPK				
Reduced	8 (14)	0.01	3 (18)	NS
Maintained	26 (46)		7 (44)	
Increased	23 (40)		6 (38)	
Phospho-ACC				
Reduced	7 (12)	0.001	1 (6)	0.007
Maintained	27 (48)		5 (31)	
Increased	23 (40)		10 (63)	

NS, non-significant.

conditions in the ranks of the test that were denominated as reduced, maintained, and increased. For the analysis of the total index obtained in tumor and NNT, data were expressed as mean $\pm$ S.E.M. and analyzed using the Wilcoxon matched pairs test. *P* values <0.05 were considered statistically significant.

Results

Immunohistochemistry analysis of tAMPK, pAMPK, and pACC in CAPAP of thyroid

tAMPK, pAMPK, and phospho-ACC (pACC) showed a cytoplasmic staining in papillary carcinomas and non-neoplastic thyroid tissues (Fig. 1, center and right columns). Apart from cytoplasmic staining, membrane and nuclear stainings were also present. Ductal breast carcinoma was used as positive and negative controls of the reactions (Fig. 1, left column). Strong and diffuse staining was observed for both tAMPK and pAMPK (Fig. 1, first and second lines) in the majority of papillary carcinomas. Most spots of non-neoplastic thyroid tissue showed a focal positivity for either tAMPK or pAMPK (Fig. 1, first and second lines). Diffuse staining was observed for pACC in papillary carcinoma, while a focal pattern was observed in NNT (Fig. 1, third line). Increased AMPK phosphorylation signal was seen in the vast majority of papillary thyroid cancer specimens, as a diffuse and strong staining in the cytoplasm compared with the focal expression in normal epithelium with a significant positive association.

Comparison of tAMPK, pAMPK, and pACC expression between CAPAP and MCAPAP of thyroid

In order to quantify the expression pattern of AMPK pathway in thyroid cancer, we analyzed the intensity

Figure 1 Photomicrographs of papillary thyroid carcinomas and non-tumoral tissue. Photomicrographs in the left column are representatives of positive immunostaining controls (40 $\times$) with breast cancer samples with a corresponding negative control spot (40 $\times$) in total AMPK, phospho-AMPK, and phospho-ACC; center column is representative of papillary carcinoma immunostaining in 40 $\times$ magnification and the respective spots at 10 $\times$ magnification; right column represents non-neoplastic tissue (NNT). Photomicrographs on the first line are representative of total AMPK immunostaining in controls, papillary carcinoma, and NNT; photomicrographs in the centerline are representative of phospho-AMPK and in the third line they represent phospho-ACC.

and proportion of immunostaining, which represent the level and number of cells expressing tAMPK, pAMPK, and pACC.

Table 1 shows the frequency distribution of staining intensity for tAMPK, pAMPK, and pACC. We observed a significantly different pattern of distribution of intensity score for tAMPK, pAMPK, and pACC in CAPAP vs their respective NNT. The pattern of distribution indicates that the majority of CAPAP samples maintained a similar intensity score when paired with their NNT, and the second most frequent pattern of intensity score was an increased staining in CAPAP when compared with NNT. Interestingly, there was no significant difference when tAMPK and pAMPK intensity scores were compared with NTT and MCAPAP, although the pACC intensity score was more frequently found to increased rank in MCAPAP when compared with their NTT counterparts.

Figure 2A, B, and C shows the results of proportion scores of tAMPK, pAMPK, and pACC in CAPAP, MCAPAP, and NNT. There are significantly higher frequency distributions for all the three targets in cancers when compared with the respective normal

tissue, clearly showing an increase in score 6 in cancer samples, for all the proteins analyzed. Corroborating with the description of heterogeneity in normal thyroid follicles, all the different scores of proportion for AMPK expression were detected in NNT. These results suggest that AMPK also plays a physiological role in human thyroids, although this has to be confirmed. In papillary thyroid cancers however, tAMPK, pAMPK, and pACC the proportion of stained cells with a score of 6 predominated, representing more than 95% of cancer cells. This finding suggests that AMPK may probably be implicated in thyroid cancer cell transformation.

Comparing intensity and proportion of tAMPK, pAMPK and pACC in cancer vs NNT of the same patient

To further confirm the increased expression and activity of AMPK in thyroid cancer, we multiplied the scores of intensity and proportion and compared the values of the total index found in paired samples of cancers and NNT of the same patient. As shown in Fig. 3A, B, and C, the expression of tAMPK, pAMPK, and pACC is significantly

Figure 2 Distribution pattern of the proportion of total AMPK, phospho-AMPK, and phospho-ACC in papillary ($n=57$) and micro-papillary ($n=16$) carcinomas and their respective non-neoplastic thyroid tissue samples. The staining proportion was scored using a range from 1 to 6 regarding the number of cells stained in each TMA spot. (A) Total AMPK; (B) phospho-AMPK; (C) phospho-ACC. Results are shown as percent of the total number of cases in each score. * $P < 0.0001$ vs control (χ^2 test).

increased in both CAPAP and MCAPAP compared with their respective NNT samples. These results initially suggest that AMPK is not only more expressed but also highly active in thyroid cancer cells than in NNT. In order to correlate the increase in expression and activity of AMPK with carcinogenesis, we analyzed six samples of thyroid adenomas. Interestingly, there was no significant difference in the expression of tAMPK, pAMPK, and pACC in adenomas when compared with the NNT of the same patients (Fig. 3C).

Indirect analysis of AMPK activity through the ratio between pACC and the relationship between pAMPK and tAMPK expression in cancers

In order to evaluate the activity of AMPK in human papillary thyroid cancer, we calculated the ratio

between pACC and AMPK phosphorylation rate (pAMPK/tAMPK). This analysis is necessary in order to exclude the possibility that the observed increase in pAMPK could only be related to the increase in tAMPK expression and not to a greater activity of this kinase. Indeed, Fig. 4A and B showed and confirmed that papillary and micro-papillary cancer samples have increased AMPK activity compared with their respective NNT and adenomas (Fig. 4C).

Discussion

In this study, we describe for the first time that the metabolic sensor AMPK is expressed in normal human thyroid gland and that its expression and activity are significantly higher in papillary thyroid carcinomas. As shown herein, a strong and diffuse cytoplasmic staining is observed for tAMPK and pAMPK in the majority of papillary carcinomas in contrast with the focal positivity found in non-tumor thyroid tissue cells. It is interesting to notice that in the previous studies regarding the molecular signature of PAPCA through microarray, AMPK mRNA has not been identified as differentially expressed. The differences between those studies and our present results could be related to the different sensitivity of the methods used and/or due to increased translation of AMPK mRNA, with no differences in its expression levels (24).

A significantly higher expression and activity of AMPK were found in thyroid cancer cells in relation to a higher total index (intensity $\times$ proportion) of immunostaining. Furthermore, there was a significant difference in the proportion of cells stained for the three proteins: tAMPK, pAMPK, and pACC, suggesting that when we compare normal vs papillary thyroid carcinomas, a strong stimulus for AMPK expression and activation is observed.

Although the cytoplasmic staining in both NNT and papillary carcinoma thyroid tissues were predominant, we also observed membrane and nuclear staining (Fig. 1, center and right columns). The relevance of these findings, however, is not known. It has been demonstrated that AMPK α 2 is localized in the nuclei of many cells and might be involved in the regulation of gene expression (25). The physiological significance of the increase in AMPK expression and activity still has to be elucidated. Recently, Faustino *et al.* (2012) (25) published a study describing the pattern of expression and activity of mTOR in thyroid cancer lesions. The authors showed that the PI3K/AKT/mTOR pathway is activated and that there is a correlation between this pathway activation and BRAF^{V600E} mutations in thyroid carcinomas (26). The interrelationship between AMPK and mTOR is well described for tissues other than the thyroid (27). Our group has previously described that mTOR plays an important role in the regulation of iodide uptake in rat thyrocytes (28, 29). Although not

Figure 3 Expression pattern of total AMPK, phospho-AMPK, and phospho-ACC in papillary ($n=57$) and micro-papillary ($n=16$) carcinomas, adenomas ($n=6$), and non-neoplastic thyroid paired samples. The antigen expression is represented by a score that corresponds to the multiplication of the value attributed to the staining intensity (from 0 to 3) by the value attributed to the staining proportion (0–6) (maximum value is therefore $3 \times 6 = 18$), comparing carcinoma vs the non-neoplastic tissue of the same patient. (A) Papillary carcinoma; (B) micro-papillary carcinoma; (C) adenoma. Results are shown by mean $\pm$ S.E.M. * $P < 0.001$ vs control; & $P < 0.05$ vs control (Wilcoxon paired test).

yet demonstrated for the thyroid tissue, under normal physiological conditions, AMPK activation inhibits mTOR through different mechanisms (27). Nevertheless, as our study clearly demonstrates increased expression and activity of AMPK in papillary thyroid carcinomas, and taking into consideration the previous report of Faustino *et al.* (2012) (25), one might speculate that at least in papillary thyroid carcinomas both mTOR and AMPK pathways are stimulated. The apparent controversy might be explained by the finding of Esteve-Puig *et al.* (2009) (27), who showed that *BRAF*^{V600E} mutation in melanoma cells leads to uncoupling in LKB1–AMPK pathway, also changing its relationship with the mTOR signaling pathway (30). However, the relationship between AMPK and mTOR pathways in normal thyroid cells and in thyroid cancers needs to be evaluated in future studies, preferably using fresh tumor samples and a more quantitative analysis.

Previous studies from our group have described that AMPK activation in normal thyroid gland and PCCL3 rat thyroid cell lineage leads to decreased iodide uptake

and increased glucose uptake (2, 5). This phenomenon (so called ‘flip-flop’) is commonly observed in thyroid oncology and seems to be well correlated with gain in tumor aggressiveness (20, 21, 31). Therefore, it is tempting to speculate whether changes in AMPK expression could play a role in thyroid tumor progression, although this has to be further evaluated. Buzzai *et al.* (2005) (14) demonstrated that AMPK is necessary for the survival of LN-229 cells during glucose deprivation protocol, indicating that this pathway might be involved in the control of tumor cell survival under stressful conditions. No matter the significance of AMPK activation for cancer prognosis, we can conclude that the higher expression and activity of AMPK in well-differentiated thyroid cancer could be related to the lower iodide uptake ability of thyroid cancer cells. As some clinical data point to the possibility that metformin – an AMPK agonist – might reduce cancer incidence, it is important to better understand the role of this kinase in tumorigenesis, cancer progression, and the reduced iodide uptake that impairs radioiodine therapeutically approach.

Figure 4 Ratio of expression of phospho-ACC in relation to (total AMPK/phospho-AMPK) in papillary ($n=57$) and micro-papillary ($n=6$) carcinomas, adenomas ($n=6$), and non-neoplastic thyroid paired samples. The ratio of expression was calculated through the division of the value attributed to phospho-ACC and the ratio of phospho-AMPK and total AMPK pattern of expression, comparing carcinoma vs normal tissue of the same patient. (A) Papillary carcinoma; (B) micro-papillary carcinoma; (C) adenoma. Results are shown by mean $\pm$ S.E.M.. * $P<0.0001$ vs control; # $P<0.001$ vs control; NS, not significant (Wilcoxon paired test).

In conclusion, our data show that AMPK expression and phosphorylation are increased in papillary thyroid cancer specimens when compared with the non-neoplastic

counterpart tissues and benign lesions. This finding suggests that AMPK may probably be implicated in thyroid cancer cell transformation. More data are now required to give us a comprehensive understanding about the role of AMPK pathway in thyroid carcinoma.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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