The influence of the BRAF V600E mutation in thyroid cancer cell lines on the anticancer effects of 5-aminoimidazole-4-carboxamide-ribonucleoside

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Abstract

5-Aminoimidazole-4-carboxamide-ribonucleoside (AICAR) is an activator of 5′-AMP-activated protein kinase (AMPK), which plays a role in the maintenance of cellular energy homeostasis. Activated AMPK inhibits the protein kinase mechanistic target of rapamycin, thereby reducing the extent of protein translation and suppressing both cell growth and cell cycle entry. Recent reports indicate that AMPK-mediated growth inhibition is achieved via an action of the RAF–MEK–ERK mitogen-activated protein kinase pathway in melanoma cells harboring the V600E mutant form of the BRAF oncogene. In this study, we investigated the anti-cancer efficacy of AICAR by measuring its effects on proliferation, apoptosis, and cell cycle progression of BRAF wild-type and V600E-mutant thyroid cancer cell lines. We also explored the mechanism underlying these effects.

AICAR inhibited the proliferation of BRAF V600E-mutant thyroid cancer cell lines more strongly than was the case with wild-type cell lines. The suppressive effect of AICAR on cell proliferation was associated with increased S-phase cell cycle arrest and apoptosis. Interestingly, AICAR suppressed phosphorylation of ERK and p70S6K in BRAF V600E-mutant thyroid cancer cells, but rather increased phosphorylation in wild-type cells. Together, the results indicate that AICAR-induced AMPK activation in BRAF V600E-mutant thyroid cancer cell lines resulted in increases in apoptosis and S-phase arrest via downregulation of ERK and p70S6K activity. Thus, regulation of AMPK activity may be potentially useful as a therapy for thyroid cancer if the cancer harbors a BRAF V600E mutation.

Introduction

In eukaryotic cells, 5′-AMP-activated protein kinase (AMPK) serves as an energy sensor detecting the intracellular AMP:ATP ratio (Hardie & Carling 1997, Hardie et al. 1998, 2003). AMPK regulates metabolic processes, including fatty acid synthesis, glucose uptake, and biogenesis of glucose transporter 4 (Bergeron et al. 1999, Winder 2001, Ojuka 2004). AMPK activation has been implicated in regulation of cell proliferation and cell cycle progression and AMPK is considered to be potential target in cancer therapy (Luo et al. 2005, Guan et al. 2007, Fogarty & Hardie 2010). 5′-Aminoimidazole-4-carboxamide-ribonucleoside (AICAR), a cell-permeable nucleoside used as an AMPK activator, has pro-apoptotic effects in cancer cell lines (Meisse et al. 2002, Kefas et al. 2003, Dagon et al. 2006).

An important genetic alteration that occurs during progression of thyroid cancer is the BRAF T1799A mutation resulting in a V600E amino acid substitution. This mutation induces constitutive activation of mitogen-activated protein kinase (MAPK) signaling, and is associated with uncontrolled cell growth, proliferation, and tumorigenesis (Gray-Schopfer et al. 2005, Xing 2005, Dhomen & Marais 2007). Several studies found that mutation of BRAF was associated with cancer cell sensitivity to a BRAF/MEK blockade (Dignam et al. 1983, Cohen et al. 2003, Ball et al. 2007, Liu et al. 2007, Liu & Xing 2008), but the detailed mechanisms are not well understood. Recent reports have demonstrated that AMPK-mediated growth inhibition of melanoma cells harboring the BRAF V600E mutation involves the RAF–MEK–ERK (MAPK) pathway (Martin et al. 2009, Zheng et al. 2009).

In this study, we investigated the anti-cancer efficacy of AICAR in thyroid cancer cell lines with wild-type and V600E-mutant BRAF. In particular, we explored the effects of the compound on cell proliferation, apoptosis, and cell cycle progression. We further investigated the mechanisms underlying such changes.
**Materials and Methods**

**Reagents**

AICAR was purchased from Toronto Research Chemicals (North York, ON, Canada). DMEM, RPMI-1640, fetal bovine serum, PBS, trypsin–EDTA, and penicillin–streptomycin solution were obtained from Gibco. Propidium iodide was the product of Sigma–Aldrich. Activated- and pan-type antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), and a monoclonal antibody directed against p21 was from BD Pharmingen (San Jose, CA, USA). HRP-conjugated anti-mouse and rabbit antibodies were purchased from Vector Labs (Burlingame, CA, USA). Cell Death Detection ELISA Kit was purchased from Roche. All other chemicals (of biotechnology grade) were purchased from Amresco, Inc. (Solon, OH, USA).

**Cell culture**

Human thyroid cancer cell lines, including three wild-type lines for BRAF (CAL62, FTC133, and ML1) and three BRAF V600E-mutant lines (BCPAP, 8505C, and BHT-133), were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained under the recommended conditions. Cells were seeded at $1 \times 10^5$ cells/well in 96-well culture plates or at $5 \times 10^5$ cells/60 mm culture dish and incubated for 24 h before treatment with AICAR.

**Cell viability assay**

Cell viability was measured by the colorimetric CCK-8 (Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer’s instructions. Cells cultured in 96-well culture plates were treated with various concentrations of AICAR, for 48, 72, or 96 h. The amount of formazan dye generated by cellular dehydrogenase activity, an indicator of cell viability, was determined by measurement of absorbance at 450 nm using a SPECTRA MAX Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Untreated cells served as controls.

**Western blotting**

Cells were washed twice with cold PBS, harvested into cell lysis buffer (Cell Signaling Technology) containing a protease inhibitor cocktail, and lysed by sonication. Extracts were centrifuged at 16 000 $g$ value for 30 min at 4°C and protein concentrations were determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). Aliquots (30 µg) of total cellular proteins were separated by SDS–PAGE on 10–12% (w/v) gradient NuPAGE gels (Invitrogen), transferred to nitrocellulose membranes (Amersham Bioscience), and incubated with specific antibodies. Immunoreactive proteins were detected by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and relative protein band intensity was determined by densitometric scanning using a FluorS Multi-Imager (BioRad). Expression levels of target proteins were normalized to that of β-actin in each sample.

**Apoptosis assay**

Programed cell death was determined by relative quantification of histone–complexed DNA fragments using the Cell Death Detection Plus Kit (Roche). Cells were harvested using trypsin–EDTA and washed twice with cold PBS. After cell lysis, intact nuclei were pelleted by centrifugation and 20 µl amounts of the supernatant were transferred to wells of a streptavidin coated microplate, which was then incubated with immunoreagent and substrate according to the manufacturer’s instructions. The amount of colored product was measured by absorbance at 405 nm using a SPECTRA MAX Microplate Spectrophotometer. Absorbance was expressed as a percentage of that of untreated control cells.

**Cell cycle analysis**

Cells, plated at $5 \times 10^5$ per 60 mm culture dish and grown for 24 h, were treated with 300 µM AICAR for the indicated times. Cells were next harvested using trypsin–EDTA, washed twice with PBS, and fixed overnight in ice-cold 70% (v/v) ethanol at $-20\,^\circ C$. The next day, cells were washed twice with PBS and incubated with propidium

![Figure 1](image1.png)

**Figure 1** Effect of AICAR on the proliferation of six thyroid cancer cell lines. Upper panel: three BRAF V600E-mutant cell lines (BCPAP, BHT101, and 8505C) and lower panel: three wild-type cell lines (FTC133, CAL62, and ML1). Cells were exposed to different concentrations (0, 10, 30, 100, or 300 µM) of AICAR for 48, 72, or 96 h, and cell proliferation was determined by the CCK-8 assay. Data are the means ± S.D. from three independent experiments.
iodide (50 μg/ml) and RNase A (20 μg/ml) at 37 °C for 30 min. Flow cytometry employed a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), at least 1 × 10^6 cells were analyzed per sample.

Results

*BRAF V600E-mutant thyroid cancer cells are susceptible to AICAR-induced cell death*

We first investigated the effects of AICAR on several wild-type or BRAF V600E-mutant cell lines. Cells were incubated with AICAR at different concentrations for 48, 72, or 96 h, and cell proliferation was determined by colorimetric CCK-8 assay (Fig. 1). AICAR significantly decreased the proliferation of BRAF-mutated thyroid cancer cells (BCPAP, BHT101, and 8505C lines) compared with wild-type cells (FTC133, CAL62, and ML1 lines), in a dose- and time-dependent manner. Notably, the anti-proliferative effect of AICAR at 300 μM was significantly increased in BRAF V600E-mutant thyroid cancer cells compared with wild-type cells. These results showed that thyroid cancer cells harboring a BRAF V600E mutation were more susceptible to the anti-proliferative effect of AICAR than were wild-type cells.

Analysis of AICAR-induced effects on signal transduction in thyroid cancer cells

AICAR is a cell-permeable activator of AMPK, which is involved in regulation of the cell cycle, cell proliferation, and energy homeostasis (Hardie & Carling 1997, Hardie et al. 1998, 2003, Hardie 2005). Recently, Meisse et al. (2002) reported that AICAR induces apoptosis independently of AMPK status in chronic lymphocytic leukemia cells (Santidrian et al. 2010). This finding encouraged us to identify the signaling molecules involved in mediating the anti-proliferative effects of AICAR in thyroid cancer cells harboring the BRAF V600E mutation. Cells were treated with 300 μM AICAR for the indicated times, and changes in the phosphorylation status of several signaling molecules were assessed by western blot analysis. We first tested whether AICAR-stimulated phosphorylation of AMPK and other signaling molecules in BRAF V600E-mutant (Fig. 2) and wild-type (Fig. 3) thyroid cancer cell lines. AICAR-stimulated AMPK phosphorylation at relatively early time points in all six thyroid cancer cell lines, and no significant difference in phosphorylated AMPK level was evident when wild type and mutant cells were compared. To further analyze the molecular mechanisms of AICAR, we explored the effects of AICAR on ERK, which is constitutively activated in BRAF mutant cells, and on other signaling pathways.

![Figure 2](image-url) Expression of signaling molecules and apoptotic markers in three BRAF V600E-mutant thyroid cancer cell lines (BCPAP, BHT101, and 8505C) exposed to AICAR. Cells were incubated with 300 μM AICAR for the indicated times, and changes in the phosphorylation status of the signaling molecules AMPK, AKT, p70S6 kinase, ERK, 4E-BP1, and p21 were assessed by western blot analysis using antibodies specific for the phosphorylated forms of each protein. β-Actin was used as a loading control.
molecules, including AKT, p70S6 kinase, S6 ribosomal protein, and 4-EBP1. Interestingly, AICAR treatment promoted ERK and p70S6 kinase phosphorylation in wild-type cells, but cells harboring the BRAF V600E mutation showed a decrease in phosphorylated ERK levels as well as a late reduction in p70S6 kinase phosphorylation after AICAR treatment. The phosphorylation of AKT, S6 ribosomal protein, and 4-EBP1 were unaffected by BRAF mutation status. We also examined whether the differential effect of AICAR was dependent on LKB1 depletion, but found no effect of AICAR treatment on the expression of endogenous LKB1 (Fig. 4). These results suggest that ERK and p70S6 kinase are involved in the AICAR resistance of wild-type cells and further imply that BRAF V600E-mutated cancer cells lack the defense mechanism by which ERK and p70S6 kinase activation oppose the anti-proliferative effects of AICAR.

**AICAR induces S-phase arrest in BRAF V600E-mutant thyroid cancer cells**

We next assessed the effects of AICAR on cell cycle progression of BRAF wild-type or V600E-mutant thyroid cancer cell lines. Cells were treated with 300 μM AICAR for 48 or 72 h and cell cycle progression was assessed. In Fig. 5A and B, AICAR treatment increased the size of the S-phase population in BRAF-mutant thyroid cancer cells compared with wild-type cells. After 48 h incubation with AICAR, the percentage of cells in S-phase increased from 19.72 to 26.8% (BHT101), 28.28 to 49.83% (8505C), and 23.05 to 39.86% (BCPAP) in BRAF-mutated cells. Similar results were obtained when cells were incubated with AICAR for 72 h (data not shown).

**AICAR stimulates apoptosis in BRAF V600E-mutant thyroid cancer cells**

To determine whether AICAR-induced cell death was associated with apoptosis, we analyzed caspase-3 and PARP activation and p21 expression level by western blotting. As shown in Figs 2 and 3, p21 expression was stimulated by AICAR in all six thyroid cancer cell lines, and no significant difference was observed when BRAF wild type and mutant cell lines were compared. However, the levels of cleaved caspase-3 and PARP increased in BRAF V600E-mutant thyroid cancer cells in a time-dependent manner, whereas no such activation was observed in wild-type cells. The increase in caspase-3 and PARP activation in AICAR-treated BRAF V600E-mutant thyroid cancer cells paralleled the reduction in cell proliferation observed in these cells (please compare the data to those of Fig. 1).

![Figure 3](image_url)
and PARP. Cells, but also induces apoptosis via activation of caspase-3, only inhibits proliferation of BRAF-mutant thyroid cancer cells, thus indicating that AICAR not increased levels of cleaved caspase-3 and PARP in BRAF mutant thyroid cancer cells, respectively. These findings are consistent with those of our previous experiment (Figs 2 and 3) showing increased in apoptosis and the extent of S-phase cell cycle arrest, following AICAR treatment.

To confirm that AICAR triggered apoptosis, we measured the levels of DNA–histone complex fragments, which increase during apoptosis. Cells were treated with 300 µM AICAR for the indicated times and apoptosis was measured using the Cell Death Detection Plus Kit as described in Materials and Methods section. As shown in Fig. 5C, AICAR treatment increased the apoptosis ratio of thyroid cancer cells between 24 and 48 h, in a time-dependent manner. This increase in apoptotic ratio was clearly more evident in BRAF V600E-mutant thyroid cancer cells than in wild-type cells. In thyroid cancer cells harboring the BRAF V600E mutation, treatment with AICAR for 48 h increased the apoptosis ratio >3.3-, >1.5-, and >2-fold in BCPAP, BHT101, and 8505C cells respectively. These findings are consistent with those of our previous experiment (Figs 2 and 3) showing increased levels of cleaved caspase-3 and PARP in BRAF mutant thyroid cancer cells, thus indicating that AICAR not only inhibits proliferation of BRAF-mutant thyroid cancer cells, but also induces apoptosis via activation of caspase-3 and PARP.

**Discussion**

In this study, we investigated the relevance of AMPK activation with respect to BRAF-mutation status in thyroid carcinoma cell lines. We found that inhibition of cell proliferation by AICAR was significantly greater in thyroid cancer cells harboring the BRAF V600E-mutation than in wild-type cells, wherein AICAR treatment promoted ERK and p70S6 kinase phosphorylation. In contrast, cells harboring the BRAF V600E mutation showed a decrease in the levels of phosphorylated ERK and p70S6 kinase, and an increase in apoptosis and the extent of S-phase cell cycle arrest, following AICAR treatment.

The BRAF V600E mutation is one of the most important oncogenic changes in human cancer (Davies et al. 2002) and is a prevalent somatic mutation in thyroid cancers, particularly in papillary thyroid carcinomas and some anaplastic carcinomas (Kimura et al. 2003, Nikiforova et al. 2003). Mutated BRAF activates the MEK–ERK kinase pathway, which is an essential regulator of cell growth, differentiation, cell cycle progression, and oncogenic transformation (Sebolt-Leopold & Herrera 2004). BRAF-targeting compounds and MEK inhibitors, designed to disrupt the RAF–MEK–ERK pathway have been developed and are currently being tested in clinical trials for treatment of various human cancers (Beeram et al. 2005, Gray-Schopfer et al. 2007, Haass et al. 2008). Several studies have shown that BRAF mutant cancer cells may be susceptible to anti-cancer drugs, such as AZD6244 (used to treat melanoma) (Smalley & Flaherty 2009). Furthermore, Zheng et al. (2009) described the relationship between AMPK and the RAF–MEK–ERK signaling pathway in human melanoma cells harboring a BRAF mutation.

In this study, the anticancer effect of AICAR was investigated in six human thyroid cancer cell lines, of which three carried a BRAF V600E mutation and three were wild type. The anti-proliferative effect of AICAR was more prominent in BRAF-mutant thyroid cancer cells than in wild-type cells (Fig. 1). We investigated the molecular mechanism underlying this anti-proliferative effect, focusing on mediators of RAF–MEK–ERK signaling and triggers of apoptosis. We found that AICAR stimulated phosphorylation of AMPK in all six thyroid cancer cell lines, independently of BRAF-mutation status. Interestingly, AICAR treatment promoted ERK and p70S6 kinase phosphorylation in wild-type cells, but exerted the opposite effect in cells harboring the BRAF V600E mutation, resulting in marked drops in the levels of phosphorylated ERK and p70S6 kinase (Figs 2 and 3). p70S6 kinase, a serine–threonine kinase involved in mitogenic responses, including protein synthesis and cell cycle progression (Couch et al. 1999), is rapidly activated in response to certain stimuli and can synthesize oncogenic products. Furthermore, activation of ERK1/2, an established player in the anti-apoptotic defense mechanism, promotes cell survival via inhibition of cell death machinery components, such as Bcl-2-associated death promoter, and upregulation of pro-survival genes, including CREB (Bonni et al. 1999, Fang et al. 1999). These observations suggest that activation of ERK and p70S6 kinase contributes to the mechanism responsible for protection of wild-type cells against AICAR-induced cell death. The fact that endogenous ERK is fully activated by mutant BRAF helps explain the absence of a stimulatory effect of AICAR on ERK in BRAF-mutated thyroid cancer cells. These results suggest that the anti-proliferative effect of AICAR is mediated via inhibition of ERK and p70S6 kinase activity in BRAF V600E-mutant thyroid cancer cells.

In addition, AMPK activation mediated by the serine/threonine kinase, LKB1, has been shown to suppress cell growth, proliferation, cell cycle progression, and survival, and...
is also involved in regulation of energy metabolism in cancer cell lines (Hardie 2005, Luo et al. 2005, Motoshima et al. 2006). Carretero et al. (2007) found that activation of AMPK by AICAR exerted differential effects on survival and killing of cancer cells, dependent on LKB1-mutation status. Thus, as AICAR-induced activation of AMPK may be regulated by LKB1 in thyroid cancer cells, we also explored whether the differential effect of AICAR was dependent on LKB1 expression. Interestingly, we found that AICAR did not affect the expression of endogenous LKB1 (Fig. 4).

In this study, we found that AICAR treatment not only inhibited cell proliferation but also induced apoptosis in BRAF-mutant thyroid cancer cells via activation of caspase-3 and PARP. Cell proliferation or apoptosis is regulated by cell cycle progression. In some cancer cells, AICAR controls components of the cell cycle machinery, such as CDK1, p21, and p27, leading to cell cycle arrest at the G1-phase (Rattan et al. 2005). In other cancer cells (e.g. CaSki cells), AICAR significantly decreases G1 and G2 phase populations, but the proportion of cells in S-phase rises (Guan et al. 2007). In line with the findings of the cited report, we found that AICAR treatment increased the S-phase population in BRAF-mutant thyroid cancer cells, compared with wild-type cells, after 48 h of incubation with AICAR (Fig. 5B).

Thus, we suggest that thyroid cancer cells harboring the BRAF V600E mutation are more susceptible to the anticancer effects of AICAR than are wild-type thyroid cancer cells. We have identified the potential for crosstalk between AMPK activation- and BRAF mutation-induced changes in the signal transduction pathways of thyroid cancer cells. Hence, regulation of AMPK activity may be potentially useful as therapy for thyroid cancers carrying the BRAF V600E mutation.

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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References


