Research Article

Gurcan Gunaydin* and Mustafa Emre Gedik

Effects of cellular energy homeostasis modulation through AMPK on regulation of protein translation and response to hypoxia

AMPK aracılı hücresel enerji homeostazı modülasyonunun protein translasyonu ve hipoksi yanıtı üzerine etkileri

https://doi.org/10.1515/tjb-2018-0338
Received August 10, 2018; accepted January 28, 2019; previously published online March 5, 2019

Abstract

Objective: AMP-activated-protein-kinase (AMPK) regulates cellular energy-homeostasis. Eukaryotic-elongation-factor-2-kinase (eEF2K) plays important roles in regulating protein-synthesis, functions similarly to AMPK to protect cancer-cells from metabolic stress. Tumor-hypoxia induces angiogenesis with activation of hypoxia-inducible-factor-1-alpha (HIF-1α). AMPK might be implicated both in regulation of protein-translation and response-to-hypoxia. We aimed to investigate the effects of AMPK modulation on regulation of protein translation and response-to-hypoxia by evaluating eEF2K and HIF-1α proteins in breast and hepatocellular cancers.

Materials and methods: Hepatocellular (Huh-7, HepG2) and breast (SKBR-3, MDA-MB-453) cancer-cells were incubated with AMPK-activator (A769662) or inhibitor (dorsomorphin) for 8/24 h. Alterations in eEF2K/HIF-1α protein expressions were examined.

Results: Cancer-cells slightly increased eEF2K expression after 24-h of AMPK-activation. Significant decreases in eEF2K expressions were observed with AMPK inhibition in all cancer-cells except Huh-7. Slight transient decrease in HIF-1α expression was observed after 8-h of AMPK-activation in all cancer-cells except MDA-MB-453. AMPK-inhibition decreased HIF-1α expression, especially in HepG2-cells.

Conclusion: The effects of AMPK modulation on eEF2K/HIF-1α protein expressions were investigated. Cells with varying molecular-expression-profiles demonstrate different metabolic activities. AMPK-activation may provide adaptive advantage to cancers and such an advantage may be reverted with an AMPK-inhibitor. The current study contributes to the literature in determining the effects of therapeutic strategies targeting AMPK on cancer-cell metabolic-pathway regulation.

Keywords: A769662; AMPK; Breast cancer; Dorsomorphin; eEF2K; Hepatocellular cancer; HIF-1α.

Öz

Amaç: AMP-aktive-protein-kinaz (AMPK), hücresel enerji homeostazını regüle etmektedir. Ökaryotik-elongasyon-faktör-2-kinaz (eEF2K), protein sentezinin düzenlenmesinde önemli role sahiptir ve kanser-hücrelerini metabolik stresten korumak için AMPK'a benzer şekilde fonksiyon gösterir. Tümör-hipoksisi, hipoksi-ile-indüklenen-faktör-1-alfa (HIF-1α) aktivasyonu aracılığıyla anjiogenezi uyarır. AMPK, hem protein-translasyonunun regülasyonu hem de hipoksi-yanıtı ile ilişkili olabilir. Bu çalışmada, meme ve hepatosellüler kanserlerde eEF2K ve HIF-1α proteinlerini inceleyerek, AMPK modülasyonunun protein translasyonu regülasyonu ve hipoksi-yanıtı üzerindeki etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Hepatosellüler (Huh-7, HepG2) ve meme (SKBR-3, MDA-MB-453) kanser-hücreleri, AMPK-aktivatörü

***Corresponding author: Asst. Prof. Gurcan Gunaydin,** MD, PhD, Department of Basic Oncology, Hacettepe University Cancer Institute, Sihhiye, Ankara 06100, Turkey, Phone: +90-312-305-4322, Fax: +90-312-324-2009, e-mail: gurcangunaydin@hacettepe.edu.tr. https://orcid.org/0000-0002-6751-2217

Mustafa Emre Gedik: Department of Basic Oncology, Hacettepe University Cancer Institute, Ankara, Turkey, e-mail: emregediktr@gmail.com. https://orcid.org/0000-0003-0522-0267

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

(A769662) veya inhibitörü (dorsomorphin) ile 8/24 saat inkübe edilmiştir. eEF2K/HIF-1α protein ekspresyon düzeylerindeki değişimler incelenmiştir.

Sonuçlar: 24-saatlik AMPK-aktivasyonu sonucunda kanser-hücrelerinde eEF2K ekspresyonu hafif düzeyde artış göstermiştir. Huh-7 hariç tüm kanser-hücrelerinde, AMPK inhibisyonu neticesinde eEF2K ekspresyon düzeyinde belirgin düşüş gözlenmiştir. MDA-MB-453 hariç tüm kanser-hücrelerinde, 8-saatlik AMPK-aktivasyonu neticesinde HIF-1α ekspresyon düzeyinde hafif geçici düşüş gözlenmiştir. Özellikle HepG2 hücrelerinde olmak üzere, AMPK-inhibisyonu HIF-1α ekspresyonunu azaltmıştır.

Tartışma: AMPK modülasyonunun, eEF2K/HIF-1α protein ekspresyonları üzerindeki etkileri araştırılmıştır. Farklı moleküler-ekspresyon-profiline sahip hücreler, farklı metabolik aktiviteler göstermektedir. AMPK-aktivasyonu, kanserlere adaptif avantaj sağlayabilir ve bu avantaj, bir AMPK-inhibitörü aracılığıyla engellenebilir. Bu çalışma, AMPK'ı hedefleyen terapötik yaklaşımların, kanser-hücre metabolik-yolak regülasyonu üzerindeki etkilerini belirleme konusunda literatüre katkı sağlamaktadır.

Anahtar Kelimeler: A769662; AMPK; Meme kanseri; Dorsomorphin; eEF2K; Hepatosellüler kanser; HIF-1α.

Introduction

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is an important serine/threonine protein kinase which potently activates the cellular energy homeostasis in various metabolic stress conditions [1]. The amount of cellular AMP is increased when the amount of energy in the organism is decreased or the energy consumption is increased under stress conditions such as low nutrient and hypoxic environment. Catabolic reactions such as fatty acid oxidation and glycolysis increase via AMPK activation. In addition, anabolic reactions such as fatty acid, cholesterol and protein synthesis are suppressed [2]. AMPK plays a crucial role in regulating cellular growth as well as specifically reprogramming metabolism [3]. AMPK has long been known to have important roles in type-2 diabetes. Recently, it has also been implicated in cancer and investigated for possible effects in carcinogenesis. AMPK was reported to be associated with several tumor suppressors such as liver kinase B1 (LKB1) and P53. As a result, AMPK inhibits cellular growth and induces cell cycle arrest by modulating survival signaling pathways like mechanistic target of rapamycin (mTOR) and Akt [4]. It was also demonstrated that AMPK regulates Nanog gene expression via p53 in stem cells and AMP might be implicated in stem cell pluripotency [5]. AMPK is also crucial for T-cell functions; thus; implicated in anti-tumor immune responses. T-cell-receptor activation causes AMPK activation [6]. This activation seems to be independent of changes in ATP levels and provides T-cells the energy necessary for proliferation required during an immune response [6]. Given these findings, the notion that "AMPK keeps tumor cells from starving to death" seems to be gaining importance [7]. Therefore, targeting AMPK may prove to be useful in cancer treatment.

Eukaryotic elongation factor-2 kinase (eEF2K) is a calcium-calmodulin-dependent protein kinase belonging to the α-kinase group, which plays an important role in the regulation of protein synthesis [8]. eEF2K phosphorylates eukaryotic elongation factor 2 and inhibits its function in the elongation stage of protein synthesis, which is a high energy consuming process. This inhibition also provides protection against proteotoxicity. Activation of eEF2K occurs by the phosphorylation of eEF2K from serine 500 (Ser500) residue [9]. As a result, the eEF2 protein is phosphorylated from the threonine 56 residue and the protein translation is suppressed [10]. Therefore, eEF2K decreases protein synthesis and provides cancer cells a way of adaptation against metabolic stress. In line with these, eEF2K is overexpressed in various cancers. In fact, eEF2K was also shown to inhibit apoptosis in cancer cells by regulating the expressions of apoptotic proteins [11]. In summary, it functions in a way similar to AMPK to protect cancer cells from metabolic stress; since AMPK activity is increased in order to reduce the energy consumption of the cell in starvation under conditions of insufficient nutrient resources for the cell as well as increased level of intracellular cyclic AMP (cAMP). Thus, eEF2K might also be implicated in carcinogenesis and it may be linked to AMPK. The association of eEF2K and AMPK activities should therefore be further investigated. Phosphorylation of eEF2K may be mediated by AMPK activation in cellular stress conditions where cellular energy is depleted. Activation of the p38/MAPK signaling pathway can also control AMPK and eEF2K regulation independent of PI3K/Akt/mTOR pathway for regulating the cell metabolism under stress conditions [12].

A common feature of tumor microenvironment is hypoxia [13]. Oxygen level decreases and angiogenesis is triggered as a result of excessive and dysregulated growth of tumor cells. Under hypoxic conditions, angiogenesis is induced as a result of activation of hypoxia-inducible factor 1-alpha (HIF-1α) [14]. Transcriptional activation of HIF-1α provides important properties to cancer cell such as epithelial-mesenchymal transition, cell invasion, metastasis as well as drug resistance [15, 16]. PI3K pathway regulates growth signals of the cell. Activation

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

of mTORC1 protein complex, which regulates the PI3K pathway, is regulated by AMPK phosphorylation. In addition, mTORC1 drives HIF-1α, which regulates cellular response to hypoxia, and inhibits eEF2K, which regulates translation elongation. Given these pathway associations, AMPK might be implicated in the blockage of the cellular growth signal, in the suppression of the translation elongation, in the response to hypoxia, and in the regulation of energy status of the cell [10].

Cancer cells need to acquire crucial metabolic and oxidative adaptations in order to survive such as increased glucose uptake and catabolism; decreased oxidative phosphorylation; adequate responses to hypoxia, increased environmental acidosis and proteotoxicity [17]. The metabolic activity of the cell substantially decreases in situations of low energy, which is highly required for cell proliferation, or cellular stress such as hypoxia [17, 18]. Therefore, it might be aimed to control the excessive and dysregulated growth of cancer cells by increasing AMPK activation in those cancer cells, given the reported negative regulatory mechanism of AMPK activation on cell growth [19].

It is of utmost importance to investigate such metabolic mechanisms in different types of cancers. For this reason, we investigated liver and breast cancers. The liver plays an important role in controlling carbohydrate metabolism, while breast does not have such a prominent role in carbohydrate homeostasis. In this study, hepatocellular carcinoma (Huh-7, HepG2) and breast cancer (SKBR-3, MDA-MB-453) cell lines were incubated separately with either AMPK inhibitor (dorsomorphin) or activator (A769662). The effects of drug incubations on possibly-AMPK-associated eEF2K and HIF-1α protein expressions have been investigated. The expressions of such proteins that play crucial roles in the constitution of the cancer biological responses have been compared, through the inhibition and activation of AMPK, thus providing insights into AMPK, eEF2K and HIF-1α interactions. In this respect, it was also aimed to compare the correct strategy for AMPK modulation in terms of cancer treatment in two types of cancers, four different cancer cell lines.

Materials and methods

Cell culture

The studies performed with the cell lines of hepatocellular carcinoma (Huh-7, HepG2) and breast cancer (SKBR-3, MDA-MB-453) have been carried out using a vertical laminar flow hood for cell culture practices. Cell lines were grown in DMEM (Huh-7, HepG2 and SKBR-3) or RPMI 1640 (MDA-MB-453) medium supplemented with 10% fetal bovine serum, 2.1 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin and incubated at 37°C, 5% CO_2.

Drug treatments

The aforementioned cells were incubated with either 100 μM AMPK activator "A769662 (Abcam, Cambridge, UK)" or 10 μM AMPK inhibitor "Dorsomorphin (Compound C) (Abcam, Cambridge, UK)" agents for either 8 or 24 h in their respective culture mediums. New direct AMPK activators, such as "A769662", are used to get over the wide effects of metformin, which is also an AMPK activator. "A769662" is an effective, reversible AMPK activator. It mimics AMP effects and activates AMPK directly. "A769662" activates AMPK both allostericly and by inhibiting AMPK dephosphorylation [20]. On the other hand, "Dorsomorphin (Compound C)", is an effective and reversible AMPK inhibitor [21].

Western blot analyses

When the cells reached to a confluency of >70% in the culture conditions, 2×10^5–1×10^6 cells were added to each of the T25 flasks. Cells were cultured for 24 h at 37°C, 5% CO_2 in order to achieve proper adhesion of the cells to the flasks. Subsequently, 8- and 24-h drug incubations were performed, as described previously. Untreated samples were used as negative controls for both of the drug treatments.

At the end of the designated drug treatment period, culture medium was withdrawn. Cell lysates were obtained by lysing the cells in the flask with RIPA (Thermo Fisher Scientific, Waltham, MA, USA) lysis buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein quantitation was performed to quantify the protein content of the cell lysates that were used in the Western blot experiments and protein denaturation was performed by incubating the cell lysates at 95°C for 5 min.

After this procedure, eEF2K (Cell Signaling Technology, Danvers, MA, USA) and HIF-1α (Cell Signaling Technology, Danvers, MA, USA) protein expressions in drug treated and negative control groups were examined using the Western blot method. GAPDH (Cell Signaling

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

Technology, Danvers, MA, USA) was used as the Western blot loading control. Finally, densitometry analysis of the protein bands obtained by chemiluminescence imaging were determined.

Comparative analysis of gene expressions

Comparative analysis of basal *PRKAA1* gene expressions in human cell lines of hepatocellular carcinoma (Huh-7, HepG2) and breast cancer (SKBR-3, MDA-MB-453) have been carried out using the Morpheus web-tool available from the Broad Institute (https://software.broadinstitute.org/morpheus/). This gene encodes the protein which is the catalytic subunit of AMPK. This protein belongs to the serine/threonine protein kinase family.

Statistical analyses

IBM SPSS Statistics for Windows Version 23 software was used for the statistical analysis of the data. Student's t-test was used to examine differences in protein expressions. A 5% type-I error level was used to infer statistical significance.

Results

Basal *PRKAA1* gene expressions and drug treatments

Comparative analysis of *PRKAA1* gene expressions in hepatocellular carcinoma (Huh-7, HepG2) and breast cancer (SKBR-3, MDA-MB-453) cell lines revealed that the basal gene expressions of the catalytic subunit of AMPK (*PRKAA1*) of Huh-7 and SKBR-3 cells are higher than that of HepG2 and MDA-MB-453 cells, respectively (Figure 1A, B).

One hundred micromole AMPK activator (A769662) or 10 μM AMPK inhibitor (Dorsomorphin) was applied to breast and hepatocellular cancer cells as the final concentration. Cells were incubated with the agents for 8 and 24 h. Each experiment setting was repeated three times for each condition. Cells that were not exposed to any agents were used as negative control. Normalization of target proteins was performed with GAPDH protein, as the Western blot loading control.

Modulation of AMPK in hepatocellular carcinoma

There seems to be a time-dependent increase in the expressions of eEF2K and HIF-1α proteins in the Huh-7 hepatocellular carcinoma cells as a result of AMPK activation with 8 and 24 h of A769662 treatment (Figure 2A). However, such increases were not statistically significant (Figure 2B, C). A statistically significant increase was observed in the expression of eEF2K protein as a result of 24-h AMPK inhibition with dorsomorphin treatment, as compared to control and 8-h dorsomorphin incubation (Figure 2D, E). There also seems to be a time dependent gradual decrease in HIF-1α protein expression as a result of AMPK inhibition with 8 and 24 h of dorsomorphin treatment (Figure 2D). However, this decrease was not statistically significant (Figure 2F).

There were no time-dependent changes in the expressions of eEF2K and HIF-1α proteins in the HepG2 hepatocellular carcinoma cells as a result of AMPK activation for 8 and 24 h with A769662 treatment (Figure 3A–C). There is a gradual decrease in eEF2K protein expression as a result of AMPK inhibition for 8 and 24 h with dorsomorphin incubation (Figure 3D). In line with this finding, the decrease of

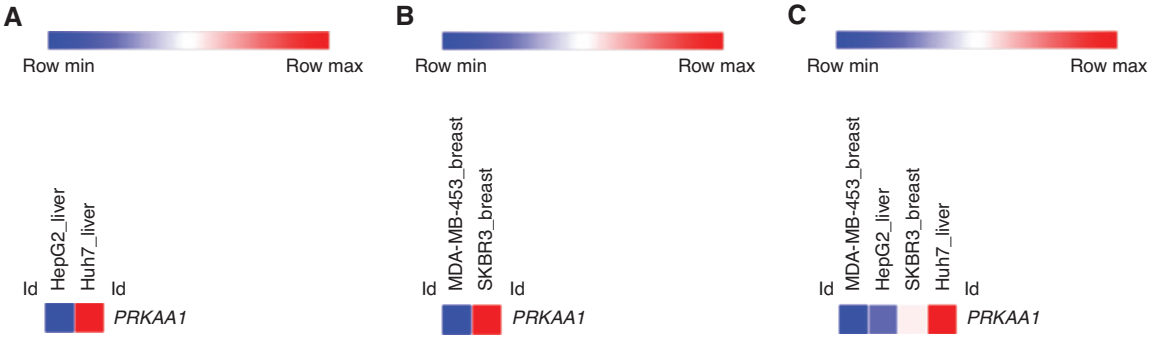


Figure 1: Heatmaps showing comparative expressions of *PRKAA1* gene in human hepatocellular carcinoma (A), breast cancer (B) and all of the (C) cell lines.
PRKAA1: Protein Kinase AMP-Activated Catalytic Subunit Alpha 1 [catalytic subunit of AMPK].

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

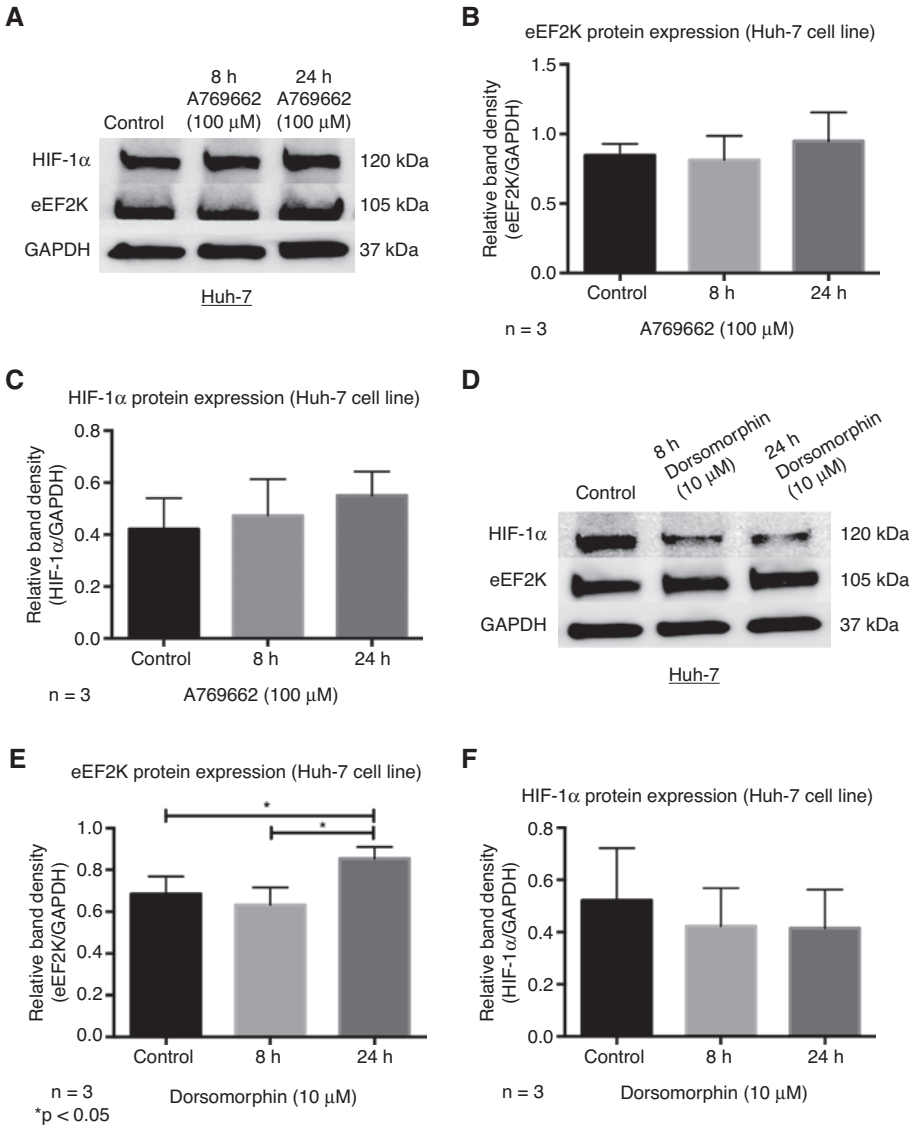


Figure 2: Effects of AMPK modulation in Huh-7 cells.
Western blot protein band images of eEF2K and HIF-1α proteins in Huh-7 cells incubated with A769662 for 0, 8 or 24 h (A). Relative band densities of eEF2K (B) and HIF-1α (C) proteins in Huh-7 cells incubated with A769662 for 0, 8 or 24 h. Western blot protein band images of eEF2K and HIF-1α proteins in Huh-7 cells incubated with dorsomorphin for 0, 8 or 24 h (D). Relative band densities of eEF2K (E) and HIF-1α (F) proteins in Huh-7 cells incubated with dorsomorphin for 0, 8 or 24 h.

protein expression level of eEF2K after 24-h incubation was statistically significant compared to the control samples (Figure 3E). Statistically significant time dependent gradual decreases were observed in HIF-1α protein expression levels as a result of 8 and 24 h of dorsomorphin treatments compared to control samples (Figure 3D, F).

Modulation of AMPK in breast cancer

There were decreases in the protein expression levels of eEF2K and HIF-1α in SKBR-3 breast cancer cells as a result of AMPK activation for 8 h with A769662 treatment compared to the control samples (Figure 4A). However, after 24 h of treatment, such transient inhibitions on protein expressions disappeared (Figure 4A). Such decreases of eEF2K and HIF-1α protein levels in SKBR-3 breast cancer cells were not found to be statistically significant (Figure 4B, C). Statistically significant time dependent gradual decreases were observed in eEF2K protein expression levels as a result of 8 and 24 h of dorsomorphin treatment compared to control samples (Figure 4D, E). There also seems to be a gradual decrease in HIF-1α protein expression levels as a result of AMPK

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

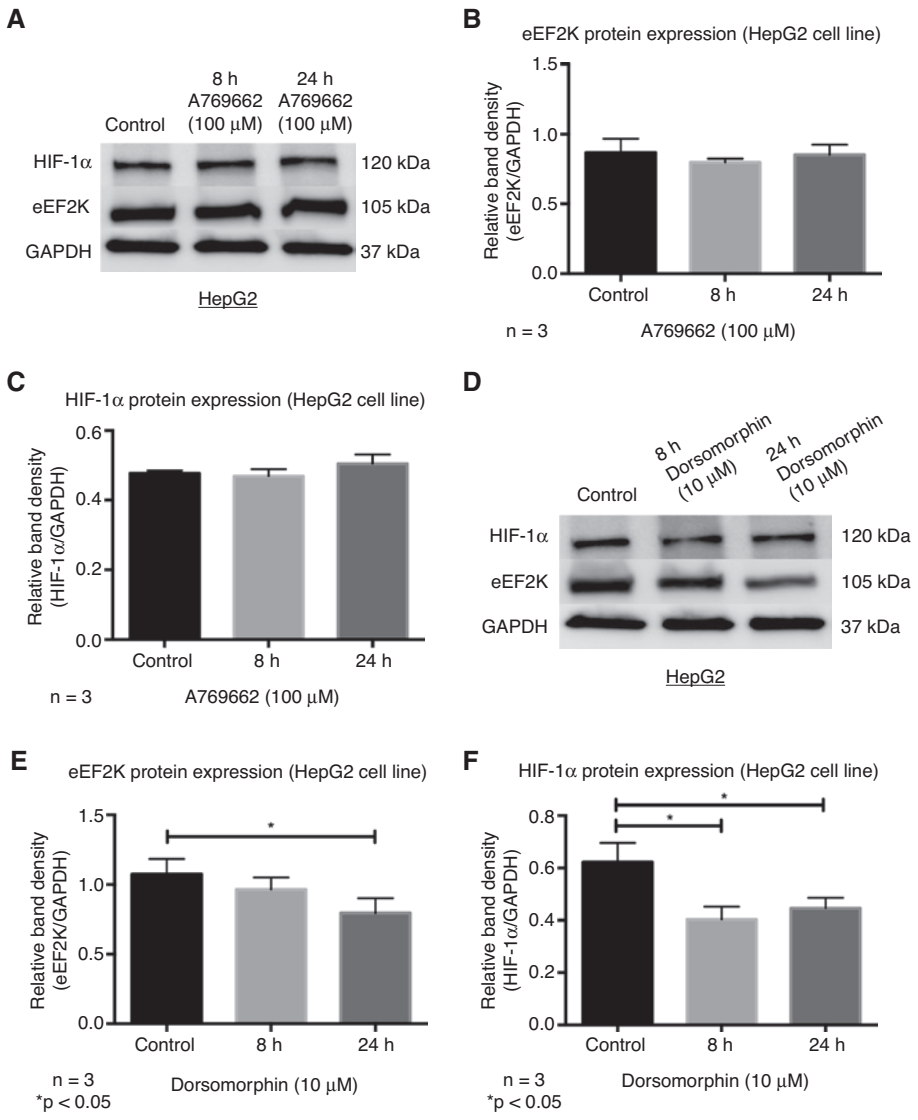


Figure 3: Effects of AMPK modulation in HepG2 cells.
Western blot protein band images of eEF2K and HIF-1α proteins in HepG2 cells incubated with A769662 for 0, 8 or 24 h (A). Relative band densities of eEF2K (B) and HIF-1α (C) proteins in HepG2 cells incubated with A769662 for 0, 8 or 24 h. Western blot protein band images of eEF2K and HIF-1α proteins in HepG2 cells incubated with dorsomorphin for 0, 8 or 24 h (D). Relative band densities of eEF2K (E) and HIF-1α (F) proteins in HepG2 cells incubated with dorsomorphin for 0, 8 or 24 h.

inhibition for 8 and 24 h with dorsomorphin treatment (Figure 4D). However, this decrease was not found to be statistically significant (Figure 4F).

There was a decrease in the protein expression level of eEF2K in MDA-MB-453 breast cancer cells as a result of AMPK activation for 8 h with A769662 treatment compared to the control samples (Figure 5A). However, after 24 h of treatment, the transient inhibition on protein expression disappeared (Figure 5A). Such a decrease of eEF2K protein level in MDA-MB-453 breast cancer cells was not found to be statistically significant (Figure 5B). There seems to be a time-dependent increase in the expression of HIF-1α protein in MDA-MB-453 breast cancer cells as a result of AMPK activation with 8 and 24 h of A769662 treatment (Figure 5A). However, this increase was not statistically significant (Figure 5C). Statistically significant time dependent gradual decreases were observed in eEF2K protein expression levels as a result of 8 and 24 h of dorsomorphin treatment compared to control samples (Figure 5D, E). The observed decrease in eEF2K protein expression level after 8 h of AMPK inhibition with dorsomorphin compared to control samples was enhanced further after 24 h of inhibition (Figure 5D, E). Both 8 h and 24 h of AMPK inhibition with dorsomorphin significantly decreased eEF2K protein expression. Such changes for two different durations were found to be statistically significant compared to the control samples. In addition, the decrease in eEF2K protein level after 24 h of dorsomorphin treatment compared to 8 h of

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

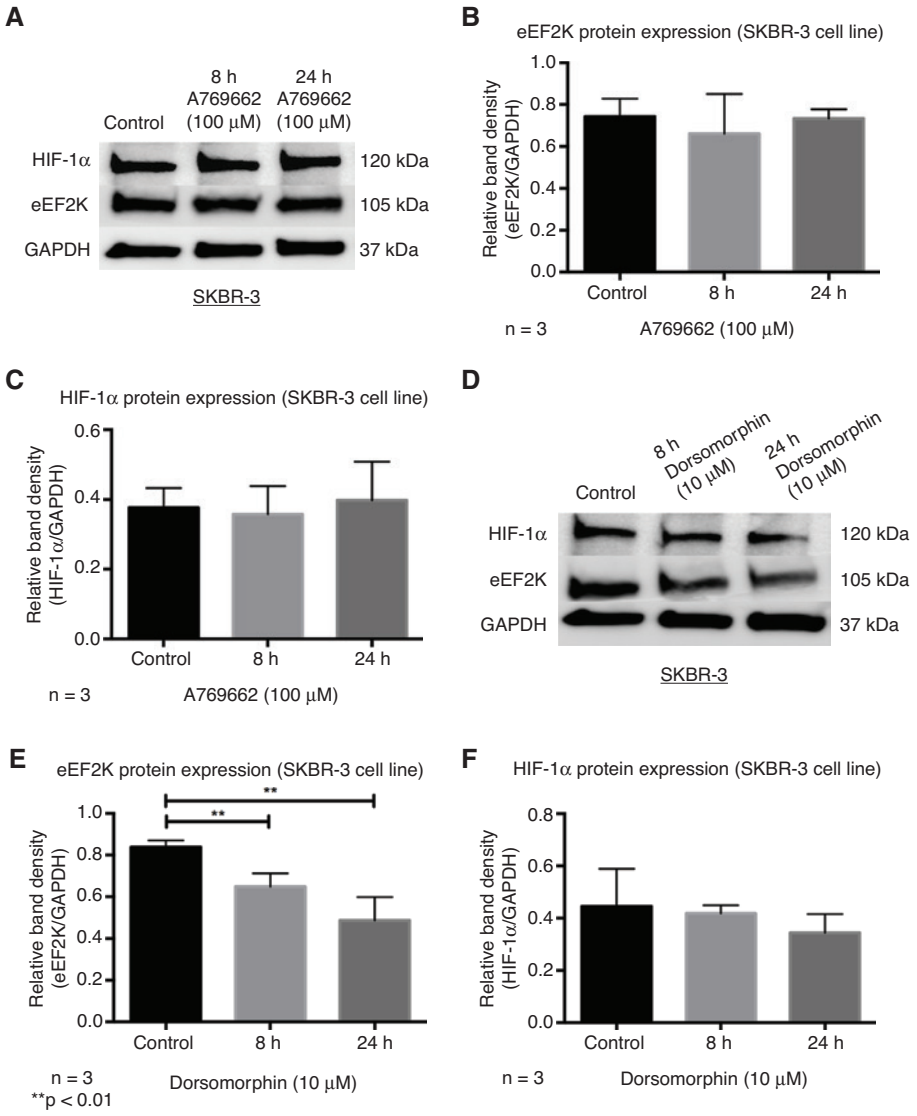


Figure 4: Effects of AMPK modulation in SKBR-3 cells.
Western blot protein band images of eEF2K and HIF-1α proteins in SKBR-3 cells incubated with A769662 for 0, 8 or 24 h (A). Relative band densities of eEF2K (B) and HIF-1α (C) proteins in SKBR-3 cells incubated with A769662 for 0, 8 or 24 h. Western blot protein band images of eEF2K and HIF-1α proteins in SKBR-3 cells incubated with dorsomorphin for 0, 8 or 24 h (D). Relative band densities of eEF2K (E) and HIF-1α (F) proteins in SKBR-3 cells incubated with dorsomorphin for 0, 8 or 24 h.

treatment was also statistically significant (Figure 5E). There were no changes in the expression of HIF-1α protein as a result of AMPK inhibition for 8 and 24 h with dorsomorphin treatment (Figure 5D, F).

Discussion

AMPK was reported to be dysregulated in various types of cancer, as well as diabetes, heart failure, inflammatory diseases and viral infections. It has been suggested that activation of AMPK, the main regulatory protein of the metabolic signaling pathway of the cell, leads to a number of changes in processes such as cell proliferation and suppression of protein translation [2, 22]. Bonini and Gantner proposed that AMPK is activated during malignant transformation [23]. AMPK activation in turn may provide cancer cells the capability to survive under conditions of hypoxia and nutrient deprivation. This study investigated the effects of direct activation or inhibition of AMPK on regulation of protein translation (eEF2K) and response to hypoxia (HIF-1α). Both of these proteins may be regulated in the downstream of AMPK, which plays an important role in the metabolic changes resulting from the transformation of healthy cells into cancer cells. For this reason, such effects of AMPK were evaluated in two

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

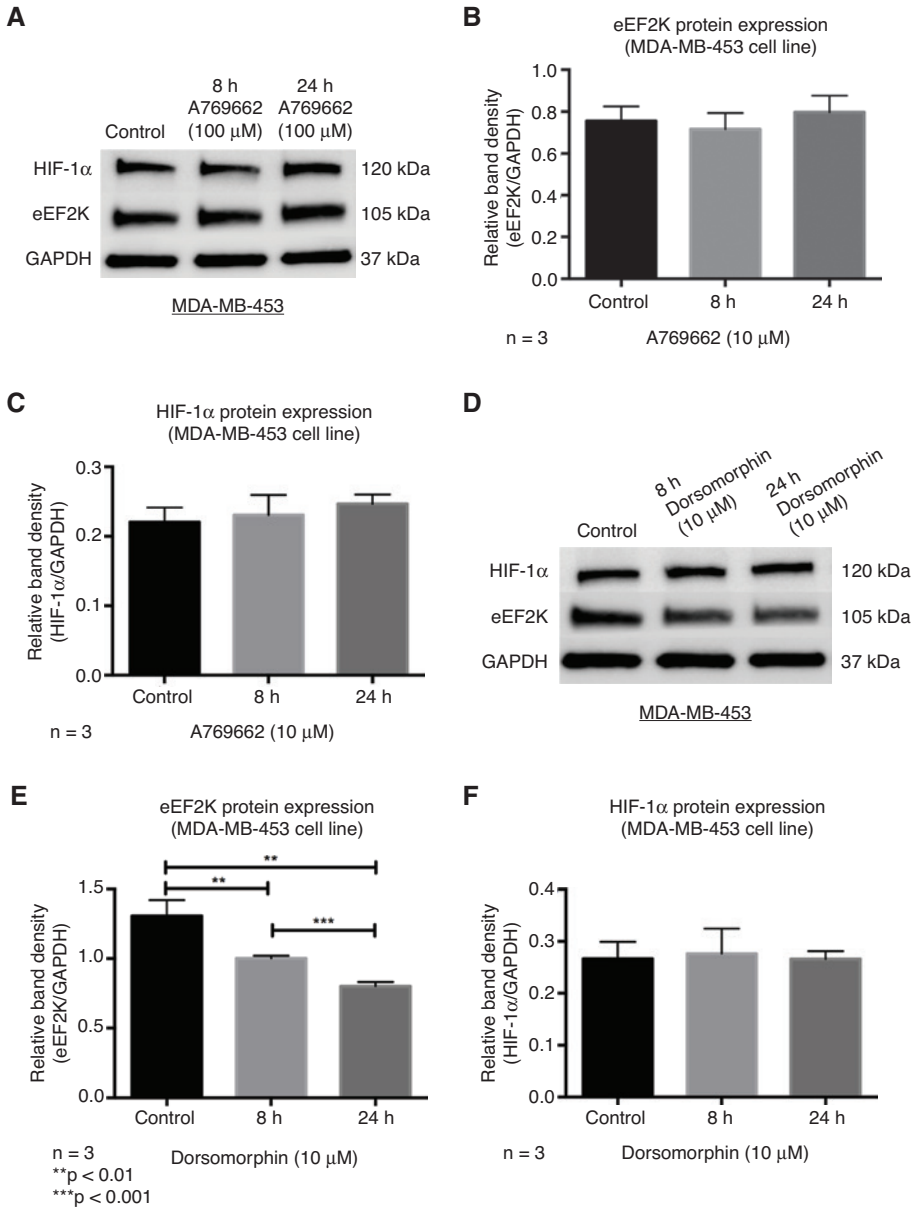


Figure 5: Effects of AMPK modulation in MDA-MB-453 cells.
Western blot protein band images of eEF2K and HIF-1α proteins in MDA-MB-453 cells incubated with A769662 for 0, 8 or 24 h (A). Relative band densities of eEF2K (B) and HIF-1α (C) proteins in MDA-MB-453 cells incubated with A769662 for 0, 8 or 24 h. Western blot protein band images of eEF2K and HIF-1α proteins in MDA-MB-453 cells incubated with dorsomorphin for 0, 8 or 24 h (D). Relative band densities of eEF2K (E) and HIF-1α (F) proteins in MDA-MB-453 cells incubated with dorsomorphin for 0, 8 or 24 h.

types of cancers (hepatocellular and breast) and four different cell lines in the current study. Two of those cell lines (Huh-7&SKBR-3) had higher basal AMPK gene expression than the other (HepG2&MDA-MB-453) (Figure 1).

We observed increased protein expression of eEF2K in both breast cancer and hepatocellular carcinoma cells as a result of 24-h treatment of cells with 100 μM AMPK activator A769662; however, such increases were not statistically significant. eEF2K is located downstream of AMPK and can be regulated by AMPK. These results are in agreement with the AMPK pathway regulatory studies of the cells under stress conditions in the literature [9, 10]. The increase in expression of eEF2K protein as a result of AMPK activation, may suppress the expression of eEF2 protein, thus repressing protein translation. By this way, the adaptation of the metabolic signal regulation of the cell to stress conditions seems to be ensured.

The activation of AMPK suppresses mTORC1, which induces protein translation, proliferation and cell growth in the cell [24]. HIF-1α is located downstream of mTORC1

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

and it is degraded by the ubiquitin protease pathway under normoxic conditions, while it is translocated to the nucleus under hypoxic conditions in order to stimulate angiogenesis in cancer cells. As a result of AMPK activation mediated mTORC1 suppression, HIF-1α protein was decreased in the current study. A decrease in HIF-1α protein expression was observed after 8 h of incubation with 100 μM AMPK activator in all cancer cells except in MDA-MB-453 cells. The decrease in HIF-1α protein expression was diminished after 24 h of incubation. This finding may indicate that the transient effects of the AMPK activator is due to the indirect regulation between AMPK and HIF-1α protein and there may also exist other compensatory pathways to overcome such an inhibition in the cancer cell. Although we observed alterations in eEF2K and HIF-1α protein expressions levels as a result of AMPK activation, such changes were slight and not statistically significant. We believe that the reason for such less-than-significant alterations in eEF2K and HIF-1α protein expressions levels as a result of AMPK activation might be due to the fact AMPK is already activated in those cancer cells [23]. Therefore, the activation of AMPK, which has already been activated during malignant transformation [23], could only result in slight downstream effects.

As a result of 24-h treatment with dorsomorphin, the decrease in eEF2K protein expression was statistically significant in all cell lines except Huh-7 cells compared to control samples. In Huh-7 cells, we observed a slight transient decrease in eEF2K after 8 h which was not statistically significant. Due to the decrease of eEF2K expression, protein translation in the cancer cell cannot be regulated. There are several studies in the literature that aim to kill cancer cells by the suppression of AMPK activity. By this way, cancer cells would not be able to control the metabolic stress in the cell [23, 25]. Suppression of eEF2K expression of Huh-7 cells after 8 h of incubation is diminished after 24 h of incubation. There is a significant increase in expression of eEF2K protein in Huh-7 cells after 24 h of dorsomorphin treatment compared to control cells. The evaluation of basal AMPK gene expression levels of the four different cancer cell lines used in the current study via the Broad Institute Cancer Cell Line Encyclopedia (CCLE) database revealed that Huh-7 cells have the highest basal AMPK gene expression [26] (Figure 1C). Therefore, it is reasonable to conclude that Huh-7 cells are the most resistant to AMPK inhibition, since their basal AMPK expression is the highest. This finding may explain the transient inhibitory effect of AMPK inhibition on expression of eEF2K protein in Huh-7 cells. In addition, other compensatory pathways to overcome such an AMPK inhibition in Huh-7 cells should not be disregarded.

There is a gradual decrease in HIF-1α protein expression levels as a result of AMPK inhibition in all cancer cells except in MDA-MB-453 cells. The decrease was statistically significant in HepG2 cells. As a result, the metabolic pathway is dysregulated in cancer cells, so they cannot adapt to adverse conditions due to the inhibition of AMPK. Thus, the cells are unable to suppress protein translation and cannot cope with cellular metabolic stress so that apoptosis may be involved. In addition, cancer cells are expected to be unable to induce adaptive mechanisms such as HIF-1α mediated angiogenesis under these conditions [27]. This result is confirmed by the decreases in the expressions of eEF2K and HIF-1α proteins as a result of AMPK inhibition in our study. MDA-MB-453 cells have the lowest basal AMPK gene expression according to CCLE database [26] (Figure 1C). Since their basal AMPK expression is very low, further inhibition of already-low AMPK might not be resulting in a significant change in HIF-1α protein expression level.

In this study, the effects of modulation of AMPK, which is known to be a crucial regulator of cancer cell metabolism, on the expressions of eEF2K and HIF-1α proteins, which are probably located downstream of AMPK, have been investigated in breast and hepatocellular cancer cells. Our findings suggest that AMPK activation may provide adaptive advantage to cancer cells and such an advantage may be reverted by treating the cancer cells with an AMPK inhibitor. On the contrary, the use of metformin, an AMPK activator, was reported to be associated with decreased risk of the occurrence of various types of cancers [28]. However, recent studies demonstrated that the anti-tumorigenic effects of metformin are not dependent on AMPK [29]. Moreover, the activation of AMPK induces resistance to the cytotoxic effects of metformin, underlining the pro-tumorigenic effect of AMPK [29]. Angiogenesis was predicted to be induced as a result of the incubation of cancer cells with an AMPK activator, due to our finding of increased HIF-1α protein expression. It has been reported that AMPK displays "multifaceted activities" in tumor progression [23] and it can act like a friend or a foe in cancer depending on the context [27]. In addition, Jeon and Hay proposed that the role of AMPK cannot be merely identified as either anti- or pro-tumorigenic, and instead it seems to have two faces similar to a double-edged sword [29]. Naive $CD4^+$ T-cells can differentiate into effector or regulatory T-cells (Treg) and effector-T-cells are crucial for anti-tumor immune responses, while Tregs usually act against anti-tumor immune responses. Treg differentiation was shown to be associated with increased AMPK activity [30]. Moreover, Tregs express higher AMPK and *in vivo* metformin administration is associated with increased Treg

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM

generation [30]. Manipulation of the AMPK signaling may provide new therapeutic advantages in augmenting antitumor immune responses. In line with these reports, our findings also indicate that the cells with different molecular expression profiles (even of the same tissue origin) may demonstrate different metabolic activities. In this context, the current study contributes to the literature in determining the effects of therapeutic strategies targeting AMPK on cancer cell metabolic pathway regulation.

Acknowledgements and Funding: This study was supported in part by Hacettepe University Scientific Research Projects Coordination Unit.

Conflicts of interest statement: The authors have no conflict of interest regarding this study.

Author contributions: G.G. and M.E.G. carried out the experiments. G.G. wrote the manuscript with support from M.E.G. G.G. and M.E.G. conceived the original idea. G.G. supervised the project.

References

1. Russo GL, Russo M, Ungaro P. AMP-activated protein kinase: a target for old drugs against diabetes and cancer. Biochem Pharmacol 2013;86:339–50.
2. Ross FA, MacKintosh C, Hardie DG. AMP-activated protein kinase: a cellular energy sensor that comes in 12 flavours. FEBS J 2016;283:2987–3001.
3. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat Cell Biol 2011;13:1016–23.
4. Wang Z, Wang N, Liu P, Xie X. AMPK and cancer. In: Cordero MD, Viollet B, editors. AMP-activated protein kinase. Springer International Publishing: Cham., 2016:203–26.
5. Chae HD, Broxmeyer HE. AMPK regulates nanog gene expression Via p53 in mouse embryonic stem cells. Blood 2009;114:997–8.
6. Tamas P, Hawley SA, Clarke RG, Mustard KJ, Green K, Hardie DG, et al. Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca2+ in T lymphocytes. J Exp Med 2006;203:1665–70.
7. Shaw RJ. AMPK keeps tumor cells from starving to death. Cell Stem Cell 2015;17:503–4.
8. Proud CG. Regulation and roles of elongation factor 2 kinase. Biochem Soc Trans 2015;43:328–32.
9. Leprivier G, Remke M, Rotblat B, Dubuc A, Mateo AR, Kool M, et al. The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. Cell 2013;153:1064–79.
10. Liu R, Proud CG. Eukaryotic elongation factor 2 kinase as a drug target in cancer, and in cardiovascular and neurodegenerative diseases. Acta Pharmacol Sin 2016;37:285–94.
11. Fu LL, Xie T, Zhang SY, Liu B. Eukaryotic elongation factor-2 kinase (eEF2K): a potential therapeutic target in cancer. Apoptosis 2014;19:1527–31.
12. Smith EM, Finn SG, Tee AR, Browne GJ, Proud CG. The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. J Biol Chem 2005;280:18717–27.
13. Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. Nat Rev Cancer 2014;14:430–9.
14. Semenza GL. Hypoxia-inducible factors in physiology and medicine. Cell 2012;148:399–408.
15. Zhang H, Wong CC, Wei H, Gilkes DM, Korangath P, Chaturvedi P, et al. HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. Oncogene 2012;31:1757–70.
16. Rausch LK, Netzer NC, Hoegel J, Pramsohler S. The linkage between breast cancer, hypoxia, and adipose tissue. Front Oncol 2017;7:211.
17. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med 2010;49:1603–16.
18. Carling D. AMPK signalling in health and disease. Curr Opin Cell Biol 2017;45:31–7.
19. Dandapani M, Hardie DG. AMPK: opposing the metabolic changes in both tumour cells and inflammatory cells? Biochem Soc Trans 2013;41:687–93.
20. Goransson O, McBride A, Hawley SA, Ross FA, Shpiro N, Foretz N, et al. Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. J Biol Chem 2007;282:32549–60.
21. Liu X, Chhipa RR, Nakano I, Dasgupta B. The AMPK inhibitor compound C is a potent AMPK-independent antiglioma agent. Mol Cancer Ther 2014;13:596–605.
22. Grahame Hardie D. AMP-activated protein kinase: a key regulator of energy balance with many roles in human disease. J Intern Med 2014;276:543–59.
23. Bonini MG, Gantner BN. The multifaceted activities of AMPK in tumor progression – why the "one size fits all" definition does not fit at all? IUBMB Life 2013;65:889–96.
24. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer 2009;9:563–75.
25. Hardie DG. AMPK: positive and negative regulation, and its role in whole-body energy homeostasis. Curr Opin Cell Biol 2015;33:1–7.
26. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 2012;483:603–7.
27. Hardie DG. Molecular pathways: is AMPK a friend or a foe in cancer? Clin Cancer Res 2015;21:3836–40.
28. Kasznicki J, Sliwinska A, Drzewoski J. Metformin in cancer prevention and therapy. Ann Transl Med 2014;2:57.
29. Jeon SM, Hay N. The double-edged sword of AMPK signaling in cancer and its therapeutic implications. Arch Pharm Res 2015;38:346–57.
30. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, MacIver NJ, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. J Immunol 2011;186:3299–303.

Brought to you by | Turkish Biochemical Society (TBD)
Authenticated
Download Date | 12/7/19 11:59 AM