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ATM and LKB1 dependent activation of AMPK sensitizes cancer cells to etoposide-induced apoptosis

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ABSTRACT

The present study aims to determine the effect of AMPK on etoposide-induced apoptosis of cancer cells. Our results revealed that etoposide induced AMPK activation in prostate C4-2 cancer cells, an event that was attenuated by ATM siRNA. In A549 cells that lack LKB1, AMPK was unable to be activated by etoposide, which was restored by introduction of LKB1. Likewise, silencing LKB1 in C4-2 cells impaired AMPK activation. Finally, etoposide displayed a potent pro-apoptotic effect in cancer cells with functional LKB1 and AMPK. Thus, our results establish a linear relationship of ATM, LKB1 and AMPK in response to the DNA damage drug.

1. Introduction

AMP-activated protein kinase (AMPK) consists of three subunits, a catalytic α subunit (α1, α2), and two regulatory subunits, β (β1, β2) and γ (γ1, γ2, γ3) [1]. AMPK is activated in response to stresses such as hypoxia, ischemia, glucose deprivation, and increased reactive oxygen species (ROS) [2]. Under these circumstances, the intracellular level of AMP or AMP to ATP ratio is increased, such that AMP binds to the γ subunit and allosterically activates the enzyme. Upon activation, AMPK inhibits anabolic processes and stimulates catabolic processes, assuring acute cell survival program and repair of cell damages. In addition to its allosteric effect, AMP prevents dephosphorylation of T172 on the activation domain of the α subunit and enables phosphorylation of T172 by upstream kinases [3]. Several kinases have been reported to function upstream of AMPK, allowing its maximal activation. These include LKB1, CaMKK(α, β), TAK1 and ATM, among which LKB1 and CaMKK have been agreed to be the kinases that directly phosphorylate T172 [4]. While CaMKK is activated by increases in calcium levels and enriched in brain, LKB1 is constitutively active and ubiquitously expressed.

LKB1 is an established tumor suppressor, whose function was first depicted owing to the discovery of loss-of-function mutations in Peutz–Jeghers syndrome, an autosomal dominant genetic disorder. The genetic syndrome is characterized by multiple hamartomatous polyps in the gastrointestinal tract and a markedly increased risk of GI adenocarcinomas [5]. Most of mutations were found to impinge on the kinase domain. Secondly, somatic mutations of the LKB1 gene have been found in many other cancers, for example, in approximately 34% of lung adenocarcinomas, 19% of squamous cell carcinomas, and 20% cervical carcinomas and other cancers [6–9]. Thirdly, in vitro studies have revealed that LKB1 regulates cell polarity and inhibits tumor cell proliferation, migration and invasion [5].

LKB1 phosphorylates and regulates 12 additional kinases related to AMPK [5]. Thus, it has been an interesting topic with regard to which of them plays a critical role in mediating the tumor suppressive function of LKB1. AMPK has been recognized to be an important player in many, if not all aspects of the tumor suppressive function [4]. Sustained activation of AMPK attenuates cell cycle progression and induces autophagy and apoptosis of cancer cells [4]. In animal models, pharmacological AMPK activators

Abbreviations: AMPK, 5′ AMP-activated protein kinase; AICAR, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide; ATM, ataxia telangiectasia mutated kinase; LKB1, liver kinase B; CaMKK, calmodulin dependent kinase kinase; TAK1, TGF beta-activated protein kinase 1; TSC2, tuberous sclerosis complex 2; FoxO3, forkhead transcription factor O3.

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such as AICAR and metformin suppress tumor development [10,11]. Of more interest, clinical investigations have reported that treatment of patients with type 2 diabetes with metformin significantly reduces the risk of cancer as compared to other anti-diabetic drugs [12,13]. AMPK activity has been shown to be suppressed in human breast cancer and combination of metformin with other anti-cancer drugs in the treatment of breast cancer improves pathological responses [14,15]. At molecular levels, AMPK has been demonstrated to regulate such tumor suppressors as p53 [16], TSC2 [17], Raptor [18], p27 [19] and FoxO3 [20]. Collectively, these regulatory events coordinately impose a brake to uncontrolled behaviors of cancer cells.

Ataxiatelangiectasia mutated (ATM) is serine/threonine protein kinase that is activated by ionizing radiation or other agents such as etoposide that induces DNA double strand breaks [21]. Consequently, ATM phosphorylates and activates p53, Brca1, Chk2, p95/nbs1, andSmc1, thereby initiating cell cycle checkpoint [21]. Function-deficient mutations of the ATM gene account for ataxia telangiectasia, an autosomal recessive disorder characterized by cerebella ataxia, oculocutaneous telangiectasia, immunodeficiency, radiation sensitivity, growth retardation, premature aging, and cancer predisposition [22]. Therefore, ATM also functions as a tumor suppressor. Recently, ATM has been implicated in metabolic pathways seemingly unrelated to DNA damage [23]. In addition, several reports have suggested this link may involve AMPK and IGF-1 signaling [24–32].

Recent studies have shown that several anti-cancer drugs can activate AMPK. However, it is controversial regarding the effect of AMPK activation on survival of cancer cells [33–40]. While a few studies report a protective effect of AMPK, the majority show that AMPK activation enhances the sensitivity of cancer cells to apoptosis. Thus, the present study attempts to assess the effect of AMPK on cancer cell survival when they are treated with etoposide, a commonly used anti-cancer drug. We found that AMPK is activated by etiposide. Surprisingly, this event occurs in ATM and LKB1 dependent fashions. Furthermore, inhibition of AMPK or disruption of LKB1 renders the cells less sensitive to etoposide-induced apoptosis.

2. Materials and methods

2.1. Reagents

Etoposide was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against phospho-ATM (S1981) and total ATM, phospho-AMPK α (T172) and total AMPKα, cleaved PARP, active Caspase 3, and β-actin were from Cell Signaling Technology, Inc. (Danvers, MA). Antibody againstLKB1 was from EMD Millipore Corporation (Billerica, MA). SiRNAs for LKB1 and ATM were from Ambion. Annexin-V FITC kit and Lipofactamine 2000 were purchased from Life Technologies (Grand Island, NY).

2.2. Cell culture

Prostate cancer C4-2 cells were cultured in RPMI-1640 supplemented in 10% FBS and lung adenocarcinoma A549 cells were cultured in DMEM supplemented with 10% FBS in 37 °C cell culture incubator containing 5% CO_2. The stable cells for the dominant negative AMPK α1 subunit were established as described previously [41].

2.3. Cell transfection

C4-2 cells were transfected with siRNA for ATM or LKB1 using Lipofectamine 2000 according to the protocol provided by manufacturer. A549 cells were transfected with human LKB1 in pCDNA3.1 (+) and selected with G418.

2.4. Western blot

C4-2 or A549 Cells were treated with etoposide and extracts prepared in lysis buffer (25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 and 25 mM β-glycerol-phosphate, 1 mM DTT, 1% NP-40 and protease inhibitors). Cell debris was removed by centrifugation at 14,000 × g at 4 °C for 15 min and protein concentrations were measured using Bio-Rad Protein Assay kit. Protein samples (20 μg) were subjected to SDS–PAGE and electrophoretically transferred to PVDF membranes (EMD Millipore). The membranes were sequentially blotted with the first and second antibodies, and developed by the enhanced chemiluminescence (ECL) method.

2.5. Fluorescence-activated cell sorting analysis (FACS)

Cells at 50% confluence were treated with etoposide (20 μM) for 24 or 48 h. They were then trypsinized, washed in ice-cold PBS and resuspended in Annexin V-FITC binding buffer at a concentration of ~5×10^5 cells/ml. Annexin-V FITC conjugate and propidium iodide were added according to the protocol of manufacturer. The samples were examined using FACS on a Becton-Dickinson flow cytometer with Cellquest software.

2.6. Statistical analysis

Averages with standard errors were tested with student t-test for significance between two groups.

3. Results

3.1. Etoposide activates ATM and AMPK

To explore the effect of AMPK on etoposide-induced apoptosis of cancer cells, we first examined if AMPK was regulated by this agent. In this experiment, we administrated etoposide to the cultured prostate cancer C4-2 cells, relatively advanced and androgen-independent, which was derived from bone-metastasized LNCaP cells. We then carried out Western blot analysis. As shown in Fig. 1A, when the cells were treated with different doses of etoposide for 2 h, ATM phosphorylation was increased in a dose-dependent manner, which was paralleled with changes in phosphorylation of AMPK. A similar trend was also found in a time course experiment (Fig. 1B). Next, we asked if activation of ATM and AMPK is a coincident event or dependent on each other. We transfected siRNA for ATM or scrambled siRNA into C4-2 cells, and 48 h later, treated them with different doses of etoposide. The results revealed that knockdown of ATM abrogated AMPK activation (Fig. 2), whereas SiRNA knockdown of LKB1 or its loss-of-function mutation did not affect ATM phosphorylation (Fig. 3). These results clearly indicate that ATM functions upstream of LKB1 that relays signal onto AMPK in response to etopoide.

3.2. LKB1 is necessary for ATM-mediated activation of AMPK

To ascertain if etoposide-induced AMPK activation is dependent on LKB1, we transiently knocked down LKB1 with siRNA and stably expressed LKB1 in A549 cells. As shown in Fig. 3A, when LKB1 was silenced in C4-2 cells, AMPK activation by etoposide was diminished. Likewise, the response to A549 cells to etoposide was greatly suppressed, whereas it was restored by the introduction of wild type of LKB1 (Fig. 3B).

3.3. AMPK activation increases the sensitivity to apoptosis induced by etoposide

To gain an insight into the role of AMPK in etoposide chemotherapy, we stably infected C4-2 cells with lentivirus encoding the dominant negative mutant of AMPK α1 (C4-2 DN) or empty virus (C4-2 E) [41] and treated them with etoposide at different doses for 24 h. The cells were stained with Annexin-V FITC conjugates and subsequently propidium iodide and subjected to FACS analysis. This assay allows us to evaluate the percentage of cells that undergo apoptosis, necrosis and both. As shown in Fig. 4A, a considerable PI staining was observed even in the absence of etoposide (Q1 area, Fig. 4A) and the extent of PI staining is not proportional to the drug treatment. Thus, we suspected a

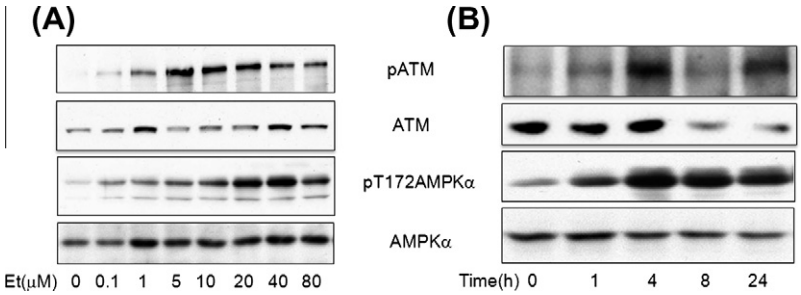

Fig. 1. Etoposide activates ATM and AMPK. C4-2 prostate cancer cells were treated with etoposide (Et) at different doses for 2 h (A) or at 20 μM for indicated time (B). Cell extracts (20 μg) were prepared and blotted with antibodies, as indicated.

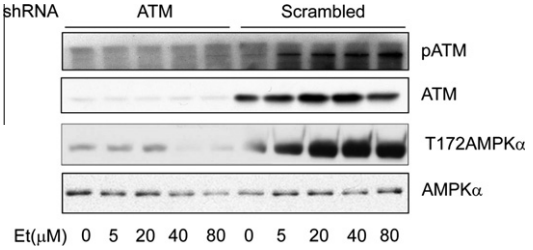

Fig. 2. Knockdown of ATM inhibits AMPK activation by etoposide C4-2 cells were transfected with ATM siRNA or scrambled siRNA as a control. Forty eight hours later, the cells were treated with different doses of etoposide for 2 h. Equal amounts of cell extracts (20 μg) were subjected to Western blot with different antibodies, as indicated.

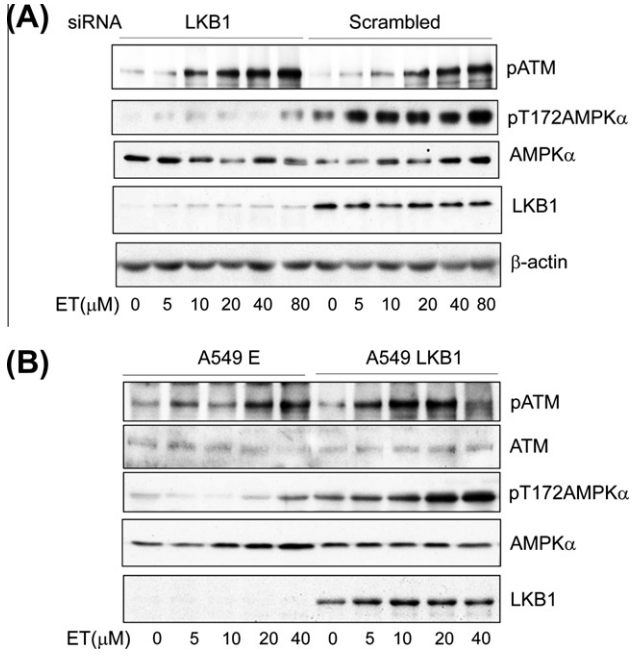

Fig. 3. Etoposide activation of AMPK is dependent on LKB1. (A) C4-2 cells were transfected with LKB1 siRNA or scrambled siRNA as a control. Forty eight hours later, the cells were treated with different doses of etoposide for 2 h. Equal amounts of cell extracts (20 μg) were subjected to Western blot with different antibodies, as indicated. (B) A549 cells were stably transfected LKB1 (A549 LKB1) or empty (A549 E) plasmid as a control. Both cells were treated with etoposide and extracts blotted with the indicated antibodies.

certain portion of the staining was attributable to damage of the cell membranes due to trypsinization. Thus, we neglected necrotic cell population, but instead focused on apoptotic cells (i.e. Q4) by plotting apoptotic cells against the total cell population. As shown in Fig. 4B, with increasing doses of etoposide, more C4-2 E cells underwent apoptosis, whereas the C4-2 DN cells were relative resistant.

To examine if caspases were activated, we carried out Western blot with antibodies against active caspase 3 and cleaved PARP, a substrate of caspase 3. As shown in Fig. 5, caspase 3 was more evidently activated by etoposide in C4-2 E cells, where AMPK was able to be activated. In contrast, the activation was severely inhibited in C4-2 DN cells or absence of LKB1 (A549 E). The cleavage of PARP precisely followed changes in caspase 3 activation. Interestingly we observed a similar change in C4-2 cells treated with another DNA damage agent, doxorubincin (data not shown). All these findings demonstrate that AMPK activation makes cancer cells more vulnerable to apoptotic insults induced by DNA-damage anti-cancer drugs.

4. Discussion

AMPK is activated under stress conditions such as nutrient deprivation, hypoxia, ischemia and ROS, where the intracellular level of AMP or AMP to ATP ratio is increased. As such, activation of AMPK stimulates fatty acid oxidation to generate more ATP in coping with energy crisis, and inhibits ATP-consuming anabolic processes to preserve energy for acute cell program, a phenomenal event called metabolic stress response [4]. Altogether, these effects confer a protection to those cells under stress, by which the cells are arrested at a certain stage of the cell cycle to initiate a repair program [16,17]. AMPK executes such checkpoint function through regulating several important molecules including mTOR, p53 and other cell cycle regulators [4,16,17,42]. Cancer cells that confront metabolic stress and checkpoint can have two fates, repair the damage, leading to survival or otherwise, fail to do so and thus undergo programmed cell death. Sustained activation of AMPK by pharmacological agents can mimic metabolic stress and drive them to the second fate.

It is well accepted that LKB1 is the master upstream kinase for AMPK. However, studies in recent years have pointed to several other kinases. Of peculiar interest is the recent finding of the link between ATM and AMPK [24–32]. ATM was first shown by Suzuki et al to be involved in phosphorylation of AMPK at threonine 172 in response to IGF-1 [24]. Their study suggests that the phosphorylation could be achieved by direct incubation of immunoprecipitate of ATM from the cells treated with IGF-1. A second study has shown that AICAR activates AMPK in mouse embryonic fibroblasts (MEF), which is completely abolished by deletion of the ATM alleles or KU-55933, a chemical inhibitor of ATM [25]. Interestingly, in Hela cells lacking LKB1, AICAR-induced AMPK activation is blunted by KU-55933. A similar observation is reported by chronic incubation of cells with AICAR [29]. A third study has reported that ionizing radiation induces AMPK activation in A549 and H23 cells, both of which lack LKB1, an event that is inhibited

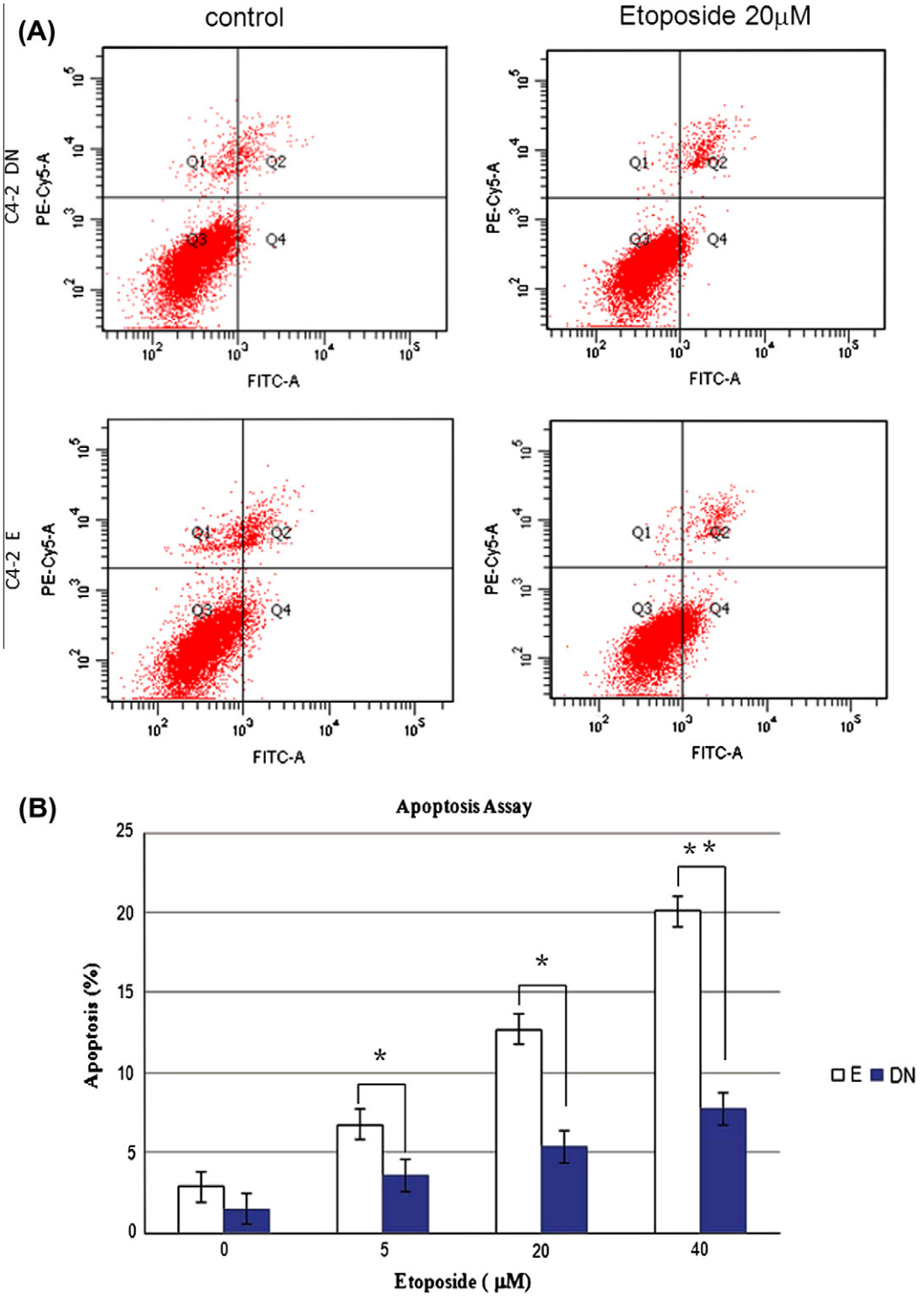

Fig. 4. AMPK activation increases the sensitivity of cancer cells to apoptosis. C4-2 cells expressing the dominant negative mutant of AMPK α1 subunit (DN) or empty (E) virus were treated with different doses of etoposide for 24 h and subjected to FACS analysis. (A) The result at 24 h was shown as a representative. (B) The percentage of apoptotic cells against whole cell population was plotted. The graph represents averages of values from three independent experiments (means ± SD). *designates $P < 0.05$, **$P < 0.01$ (student t test).

by KU-55933 [27]. All these lines of evidence coincidently support that ATM regulates AMPK independent of LKB1. However, Alexander et al [28] have recently demonstrated that reactive oxygen species activates ATM, leading to phosphorylation of T172 and activation of AMPK. Furthermore, the phosphorylation of T172 is dependent on the presence of LKB1. In keeping with this, our present study leads to the conclusion that LKB1 is necessary for ATM activation of AMPK in response to etoposide. It is conceivable that etoposide causes an increase in ROS, which in turn activates ATM. What puzzles us is the discrepancy regarding the role of LKB1 in ATM activation of AMPK and the mechanism underlying how ATM regulates AMPK. The discrepancy could be reconciled if ATM regulates a modulator for AMPK, which may be common to all these scenarios and cooperate with any of kinases that directly

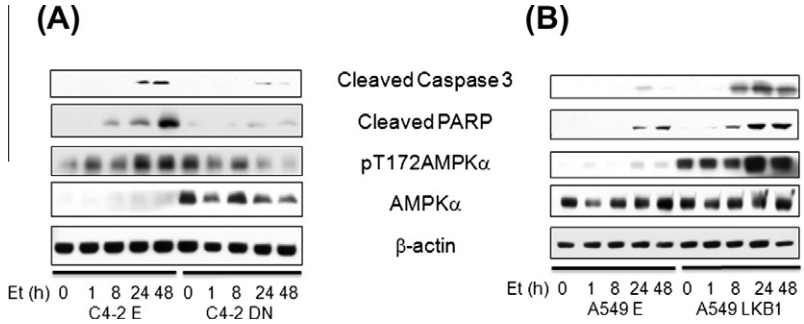

Fig. 5. AMPK activation enhances Caspase 3 activation. C4-2 DN or E cells (A) and A549 cells with or without wild type LKB1 (B) were treated with etoposide (20 μM), respectively, for different time. The cell extracts were immunoblotted with cleaved PARP and activated Caspase 3, as well as other antibodies, as indicated.

phosphorylate T172. A candidate could be AMP or phosphatase toward T172.

The role of ATM in the regulation of metabolism is emerging as a very interesting topic. In addition to checkpoint regulation, ATM has been implicated in metabolic pathways. Schneider et al. [23] have shown that transplantation of bone marrow with $ATM^{-/-}$ into $ApoE^{-/-}$ mice increases atherosclerosis, whereas activation of ATM in $ATM^{+/+}ApoE^{-/-}$ mice alleviates the vascular disease. A second interesting study reveals that both etoposide and AICAR stimulate mitochondria biogenesis in an ATM-dependent manner [26]. Cancer cells have adopted aerobic glycolysis, a trait referred to as Warburg effect. Thus, glycolysis becomes a major fuel source regardless of much less effect than oxidative phosphorylation in mitochondria. It has been an idea to inhibit glycolysis and restore oxidative phosphorylation as a cancer therapeutic approach [43]. It is tempting to speculate that increased biogenesis of mitochondria may render cancer cells more sensitive to apoptotic insults provoked by chemotherapy, as it is possible that apoptotic factors such as cytochrome C might also be increased during the course.

In sum, our present study has shown that etoposide, a widely used anti-cancer drug, activates AMPK in an ATM and LKB1 dependent manner. Although the data on the ATM activation of AMPK are reproduced by several labs, it is debatable as to if LKB1 is required. Our results definitely add more weight to one side. Furthermore, our data show that AMPK activation enhances the sensitivity of cancer cells to apoptosis induced by etoposide. This finding suggests that AMPK could be a therapeutic target for cancer. As an AMPK activator, metformin is a commonly used anti-diabetic drug and being tested in clinical trials for cancer therapy [44], it will be intriguing to explore if this drug can enhance the anti-cancer effect of etoposide. To be noteworthy, AMPK activation has also been reported to have a protective effect on some cancer cells against chemotherapy. Thus, caution should be taken to scrutinize sets of cancer cells that have opposite outcomes. Especially important is to find the mechanisms by which different responses are produced.

Conflicts of interest statement

All authors declare no financial and personal relationships with other people or organizations that could inappropriately influence (bias) the work described in this paper.

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