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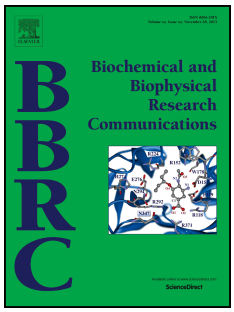

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AICAR activates ER stress-dependent apoptosis in gallbladder cancer cells

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Abstract. AICAR (5-Aminoimidazole-4-carboxamide riboside or acadesine) is an AMP-activated protein kinase (AMPK) agonist, its activity in human gallbladder cancer cells was evaluated here. We show that AICAR provoked significant apoptosis in human gallbladder cancer cell lines (Mz-ChA-1, QBC939 and GBC-SD) and primary gallbladder cancer cells. AICAR-induced cytotoxicity in gallbladder cancer cells appears independent of AMPK activation. Inhibition of AMPK, via AMPKα shRNA knockdown or dominant negative mutation (T172A), failed to rescue GBC-SD cells from AICAR. Further, forced-activation of AMPK, by adding two other AMPK activators (A769662 and Compound 13), or expressing a constitutively-active mutant AMPKα (T172D), didn't induce GBC-SD cell death. Remarkably, AICAR treatment in gallbladder cancer cells induced endoplasmic reticulum (ER) stress activation, the latter was tested by caspase-12 activation, C/EBP homologous protein (CHOP) expression and IRE1/PERK phosphorylation. Contrarily, salubrinal (the ER stress inhibitor),

z-ATAD-fmk (the caspase-12 inhibitor) or CHOP shRNAs significantly attenuated AICAR-induced gallbladder cancer cell apoptosis. Together, we conclude that AICAR-induced gallbladder cancer cell apoptosis requires ER stress activation, but is independent of AMPK.

Keywords: AICAR; ER stress; AMPK; Apoptosis and Gallbladder cancer cells

1. Introduction

The prognosis of patients with bile duct gallbladder cancer is still poor [1,2]. Surgical resection is available for early-stage and well-qualified gallbladder cancer patients [1,2]. Therefore, chemotherapy and other adjuvant therapies are vital for the treatment of gallbladder cancers [1,2]. Our group [3] and others are dedicated to search for more effective therapeutic agents for this disease [4].

The low-energy mimetic AICAR (5-aminoimidazole-4-carboxamide riboside) is a known agonist of adenosine monophosphate (AMP)-activated protein kinase (AMPK) [5,6]. Growing evidences have implied that this compound could also induce profound cytotoxicity in a variety of cancer cells [7,8,9,10], therefore AICAR may function as a promising anti-cancer agent [7,8,9,10]. The potential effect of AICAR in human gallbladder cancer cells, and the underling signaling mechanisms, have not been fully studied.

Endoplasmic reticulum (ER) is responsible for new protein synthesis, properly folding, and post-translationally modifications [11,12]. It is also critical for calcium homeostasis [11,12]. Various anti-cancer drugs, however, were shown to interrupt normal ER functions, causing pathological ER stress [11,12]. Unresolved ER stress could lead to several unfolded protein responses (UPR) and up-regulation of ER chaperones, including C/EBP homologous protein (CHOP) and several others [13,14]. Prolonged or severe ER stress will further initiate cell apoptosis [13]. Our previous study has shown that ginseng Rg3 activates ER stress to provoke gallbladder cancer cell apoptosis [3]. In this current research, we discover that AICAR-induced gallbladder cancer cell apoptosis is surprisingly independent of AMPK, but is associated with ER stress activation.

2. Material and methods

2.1. Chemicals and reagents. The AMPK activators AICAR and A769662 as well as the ER stress inhibitor salubrinal (Sal) [15] were obtained from Sigma (St. Louis, MO). Compound 13, a novel AMPK activator, was from Dr. Lv [16]. The pan-caspase inhibitor (z-VAD-fmk) and the specific caspase-12 inhibitor (z-ATAD-fmk) [17] were purchased from Calbiochem (Darmstadt, Germany). All antibodies [3] used in this study were obtained from Cell Signaling Tech (Danvers, MA).

2.2. Cell lines and culture. GBC-SD, Mz-ChA-1 and QBC939 transformed human gallbladder cancer cells were cultured as described [3].

2.3. Primary culture of human gallbladder cancer cells and non-cancerous epithelial cells. The procedures of isolation and culture of above patient-derived primary cells were described in detail in our previous study [3]. The protocol was approved by the institutional review board of all authors' institutions, and written informed consent was obtained from all participated patients.

2.4. Cell viability assay. Following the treatment/s, cell viability was examined by the routine 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) assay as described [3].

2.5. Clonogenicity assay. GBC-SD cells were suspended in complete DMEM plus agar (Sigma). Following the AICAR treatment, the cell suspension was then added on top of a pre-solidified culture dish. After ten days, the surviving colonies were counted.

2.6. Cell death assay. The percentage (%) of "dead" (trypan blue positive) cells [3] was recorded by an automated cell counter (Merck Millipore, Shanghai, China).

2.7. ssDNA ELISA assay of cell apoptosis. The ApoStrandTM single-stranded DNA (ssDNA) ELISA apoptosis detection kit (BIOMOL International, PA) was applied to quantify cell apoptosis as described [3]. ELISA optic density (OD) at 450 nm was utilized as a quantitative meter of cell apoptosis.

2.8. TUNEL assay of cell apoptosis. After applied treatment/s, cell apoptosis was also examined by TUNEL ApopTag fluorescence cell apoptosis kit (Millipore, Billerica, MA) [3].

2.9. Annexin V assay of cell apoptosis. As previously described [3], Annexin V-propidium iodide (PI) assay was performed to further test cell apoptosis, Annexin V positive cells were gated via the FACSCalibur machine (BD Biosciences, Shanghai, China) as the apoptotic cells.

2.10. Caspase-12 activity assay. Twenty μg of cytosolic extracts (per treatment) were added to the caspase assay buffer [3] with the caspase-12 substrate ATAD-7-AFC (Invitrogen) [17,18]. The amount of liberated AFC was then measured using a spectrofluorometer (Thermo-Fisher, Shanghai, China) with excitation of 380 nm.

2.11. Western blot assay. Cytosolic extracts (40 μg/sample) were resolved by SDS-PAGE, and were transferred to PVDF membranes. The blots were milk-blocked and incubated with designated primary and second antibodies, followed by detection with ECL system (Roche, Shanghai, China) [3]. Western blot assay results were quantified through Image J software [3].

2.12. shRNA-knockdown. The two non-overlapping lentiviral human CHOP shRNAs and the non-sense scramble shRNA were reported previously [3,17]. The AMPKα1 shRNA lentiviral particles were purchased from Santa Cruz Biotech (sc-29673-V, Shanghai, China). The lentiviral shRNA (15 μL/mL medium) was added to the cultured cells. After 24 hours, the medium was replaced by fresh complete medium, and cells were further cultured for additional 72 hours. Knockdown of targeted proteins (CHOP or AMPKα) in the infected cells was confirmed by Western blot assay.

2.13. AMPKα dominant negative mutation. A dominant negative mutant AMPKα ("dn-AMPKα", T172A) pSuper-neo-GFP vector was a gift from Dr. Lu [19]. The dn-AMPKα cDNA (0.10 μg/mL) was transfected to cultured cells via the Lipofectamine 2000 protocol [19], and stable cells were selected via neomycin (1.0 μg/mL, Sigma) for 6-7 days, until 90% of cells were GFP positive. Expression of AMPKα in the stable cells was tested by Western blot assay.

2.14. AMPKα constitutively active mutation. The adenoviral vector expressing a constitutively active mutant of AMPKα1 (T172D, Ad-ca-AMPKα1-GFP-puromycin) and the

empty vector (Ad-GFP-puromycin) were gifts from Dr. Zheng [20,21]. The ca-AMPKα1 (0.1 μg/mL) or the control vector ("Ad-GFP", 0.1 μg/mL) was transfected by the Lipofectamine 2000 protocol. The stable cells were selected via puromycin (1 μg/mL) for 6-7 days, until 90% of cells were GFP positive. Western blot assay was also applied to test AMPKα expression in stable cells.

2.15. Statistical analysis. All quantified results were expressed as mean ± SD. Statistical analyses were performed using the SPSS 18.0 software. Differences were considered significant at $P < 0.05$.

3. Results

3.1. AICAR induces human gallbladder cancer cell death

In order to study the potential effect of AICAR in gallbladder cancer cells, GBC-SD gallbladder cancer cells [3] were treated with indicated concentrations (0.1-1.0 mM) of AICAR, MTT survival assay results demonstrated that AICAR, at 0.25-1.0 mM, significantly inhibited GBC-SD cell survival (Fig. **1a**). AICAR displayed dose- and time-dependent response in suppressing GBC-SD cell survival (Fig. **1a**). Further, treatment of AICAR (0.25-1.0 mM) significantly decreased the number of viable GBC-SD cell colonies (Fig. **1b**). On the other hand, the number of trypan blue positive ("dead") GBC-SD cells was significantly increased following AICAR (0.25-1.0 mM) treatment (Fig. **1c**). These results suggest that AICAR was cytotoxic when added to GBC-SD cells.

Next, we tested AICAR's activity in other gallbladder cancer cells. As demonstrated, ACIAR treatment similarly decreased the MTT viability OD of two other established gallbladder cancer cell lines: QBC939 (Fig. **1d**) and Mz-ChA-1 (Fig. **1e**). The potential effect of AICAR in primary cells was also tested. Using the method described [3], we established two lines of primary human gallbladder cancer cells. MTT assay results in Fig. **1f** showed that AICAR (1.0 mM) also inhibited survival of patient-derived primary cancer cells. Intriguingly, same ACIAR treatment was non-cytotoxic to the gallbladder epithelial cells (Fig. **1f**, also two lines). These results together suggest that AICAR induces profound cytotoxicity only to the gallbladder cancer cells.

3.2. AICAR provokes apoptosis in human gallbladder cancer cells

Next, using the methods described previously [3], we examined the potential effect of AICAR on gallbladder cancer cell apoptosis. As demonstrated, AICAR treatment in GBC-SD cells dose-dependently provoked cell apoptosis (Fig. **2a-d**). As the caspase-12 activity (Fig. **2a**), TUNEL percentage (Fig. **2b**) as well as the ssDNA ELISA OD (Fig. **2c**) and ratio of Annexin V positive cells (Fig. **2d**) were all significantly increased following AICAR (0.25-1.0 mM) treatment in GBC-SD cells. Notably, GBC-SD cell apoptosis by AICAR (1.0 mM) was largely inhibited by the caspase-12 inhibitor z-ATAD-fmk ("ATAD") or the pan caspase inhibitor z-VAD-fmk ("VAD") (Fig. **2a-d**). Importantly, these two caspase/apoptosis inhibitors also significantly attenuated AICAR (1.0 mM)-induced GBC-SD cell death (Fig. **2e** and **f**). In the primary gallbladder cancer cells, treatment with AICAR (1.0 mM) similarly induced potent apoptosis activation (Fig. **2g**). AICAR, however, failed to induced apoptosis in gallbladder epithelial cells (Fig. **2g**). Further studies showed that the above caspase blockers also attenuated AICAR (1.0 mM)-induced primary cancer cell death (Fig. **2h**). These results suggest that AICAR provokes caspase-dependent apoptotic death in gallbladder cancer cells.

3.3. AICAR-induced GBC-SD cell death is independent of AMPK activation

AICAR induced AMPK activation in GBC-SD cells, as AMPKα Thr-172 phosphorylation ("p-AMPKα") was significantly increased in AICAR (0.25-1.0 mM)-treated cells (Fig. **3a**). To study its role in AICAR-induced cancer cell death, genetic strategies [22] were applied to interfere AMPK activation. As demonstrated, exogenous expression of AMPKα-shRNA or the dominant negative mutation AMPKα (T172A, "dn-AMPKα") [22,23] almost blocked AICAR-induced AMPK activation (Fig. **3b**). Intriguingly, in AMPK-silenced or mutated GBC-SD cells, AICAR-induced cytotoxicity was not reduced, but actually slightly increased (Fig. **3c** and **d**).

These above results suggest activation of AMPK may not be required for AICAR-induced GBC-SD cell death. To further support this notion, we applied two other AMPK activators: A769662 [24] and Compound 13 [16]. Although the two induced comparable AMPK activation as AICAR in GBC-SD cells (Fig. **3e**, upper panel), they failed to induce significant GBC-SD cell

apoptosis (Fig. **3e**, lower panel) and cell death (Fig. **3f**). Similarly, exogenous expression of a constitutively-activate AMPKα (T172D, "ca-AMPKα") induced profound AMPK activation (Fig. **3g**, upper panel), but failed to provoke GBC-SD cell apoptosis and death (Fig. **3g**, lower panel and Fig. **3h**). Notably, the ca-AMPKα protein band was very close to regular AMPKα band (Fig. **3g**, lower panel, "star" labeled). Together, these results indicate that AICAR-induced GBC-SD cell death is independent of AMPK activation.

3.4. ER stress activation mediates AICAR-induced GBC-SD cell death

As discussed in our previous study [3], caspase-12 activation is a characteristic marker of ER stress activation. As shown in Fig. **4a**, AICAR treatment induced CHOP expression, PERK and IRE1 phosphorylations in GBC-SD cells, indicating significant ER stress activation. Significantly, co-treatment with ER stress inhibitor salubrinal (Sal) [15] dramatically attenuated AICAR-induced GBC-SD cell death (Fig. **4b**) and apoptosis (Fig. **4c**). These results imply that ER stress activation mediates AICAR-induced cytotoxicity in GBC-SD cells. shRNA strategy was then applied to stably knockdown CHOP. In line with our previous results [3], the two different lentiviral shRNAs efficiently downregulated CHOP in GBC-SD cells (Fig. **4d**). Accordingly, AICAR-induced lethality was largely attenuated in CHOP-silenced GBC-SD cells (Fig. **4e** and **f**). In another words, GBC-SD cells with CHOP shRNAs were protected from AICAR (Fig. **4e** and **f**). Notably, CHOP knockdown didn't affect AICAR-induced AMPK activation (Fig. **4d**). Neither did AMPKα mutation or silence affect AICAR-induced CHOP expression (data not shown). Therefore, AICAR-induced ER stress activation is independent of AMPK activation.

4. Discussions

In this study, we showed that AICAR, the known AMPK agonist, induced profound cytotoxicity and apoptosis in gallbladder cancer cell lines (Mz-ChA-1, QBC939 and GBC-SD) and primary gallbladder cancer cells. For the mechanism study, we proposed that activation of ER stress, independent of AMPK, could be the key mechanism responsible for AICAR-induced cytotoxicity in gallbladder cancer cells.

Existing evidences have shown that sustained or severe ER stress can induce cell apoptosis.

For example, CHOP is often upregulated following ER stress, which eventually leads to cell apoptosis [25,26]. Besides CHOP, other ER stress-associated proteins could also promote cell apoptosis. For instance, under ER stress, phosphorylated IRE1 and PERK could recruit several adaptor proteins to cleave and activate caspase-12, the latter is known as a key mediator of ER stress-induced apoptosis [17,18,27,28]. Here, we showed that AICAR treatment induced caspase-12 activation, C/EBP homologous protein (CHOP) expression and IRE1/PERK phosphorylations in gallbladder cancer cells. Contrarily, salubrinal (the ER stress inhibitor), z-ATAD-fmk (the caspase-12 inhibitor) as well as CHOP shRNAs significantly attenuated AICAR-induced gallbladder cancer cell death and apoptosis. Therefore, ER stress activation is required for AICAR-mediated cytotoxicity in gallbladder cancer cells. Intriguingly, AICAR treatment failed to induce a profound ER stress activation in the non-cancerous epithelial cells (data not shown), which could explain why these cells were not killed by AICAR. The detailed mechanisms warrant further investigations.

Intriguingly, the results of the study indicated that AICAR-provoked gallbladder cancer cell death was independent on AMPK activation. Inhibition of AMPK, via shRNA knockdown or dominant negative mutation (T172A) of AMPKα, failed to rescue GBC-SD cells from AICAR. Two other AMPK activators, including A769662 and Compound 13, had no effect on gallbladder cancer cell survival and apoptosis. Meanwhile, forced-activation of AMPK by exogenous expression of ca-AMPKα (T172D) failed to induce significant GBC-SD cell death. On the contrary, a stronger cytotoxicity by AICAR was observed in AMPKα1-silenced or -mutated GBC-SD cells, suggesting that AMPK activation may actually be pro-survival in the situation. Thus, our results indicate that AMPK activation is not required for AICAR's cytotoxicity against gallbladder cancer cells, which are in line with findings of other group [10].

Other studies, however, have shown that forced-activation of AMPK could induce cancer cell death and apoptosis [19,29,30,31,32]. For example, a number of anti-cancer drugs are able to activate AMPK signaling, which leads to in-activation of mTOR (a well-known pro-cancerous signaling) and activation of pro-apoptotic JNK-p53 cascades [19,29,30,31,32]. Thus, it is possible that AMPK-regulated signalings, *i.e.* mTOR and JNK-p53, didn't play a significant role in gallbladder cancer cell survival/apoptosis. Indeed, basal mTOR activation was low in the tested gallbladder cancer cells (data not shown). The other possibility is that activation of AMPK couldn't provoke the pro-apoptotic signalings. Or these signaling pathways

are simply depleted or mutated in the gallbladder cancer cells. As a matter of fact, we didn't detect significant JNK-p53 signaling activation in AICAR-treated gallbladder cancer cells (data not shown). Together, we show that AICAR induces gallbladder cancer cell death via activating ER stress but not AMPK.

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Conflict of interests. The authors declare that they have no conflict of interests.

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Figure legends

Fig. 1. AICAR induces human gallbladder cancer cell death. Established gallbladder cancer cell lines, GBC-SD (**a**-**c**), QBC939 (**d**) and Mz-ChA-1 (**e**), as well as the primary human gallbladder cancer cells (**f**, "Cancer cells", two lines) and the surrounding epithelial cells (**f**, "Epithelial cells", two lines), were either left untreated ("C") or treated with indicated concentrations of AICAR (0.1-1.0 mM), cell were cultured for applied time before survival was evaluated by the MTT assay (**a**, **d**-**f**) or colony formation assay (**b**); Cell death was examined by trypan blue staining assay (**c**). The values were expressed as the means ± SD. For each assay, n=5. The experiments were repeated four times, and similar results were obtained. * $\mathbf{P} < 0.05$ vs. "C" group.

Fig. 2. AICAR provokes human gallbladder cancer cell apoptosis. GBC-SD cells were either left untreated ("C"), treated with AICAR (0.1-1.0 mM) or plus the caspase-12 inhibitor z-ATAD-fmk ("ATAD", 50 μM) and the pan caspase inhibitor z-VAD-fmk ("VAD", 50 μM) for indicated time, relative caspase-12 activity was shown (**a**); Cell apoptosis was examined via the TUNEL assay (**b**), the ssDNA apoptosis ELISA assay (**c**), and the Annexin V FACS assay (**d**); Cell survival and cell death were tested by MTT assay (**e**) and trypan blue staining assay (**f**), respectively. The primary cancer cells or the surrounding epithelial cells, treated with/out z-ATAD-fmk ("ATAD", 50 μM) or z-VAD-fmk ("VAD", 50 μM), were subjected to AICAR treatment (1.0 mM) for indicated time, cell apoptosis was tested by ssDNA ELISA assay (**g**); Cell death was examined by the trypan blue staining assay (**h**). The values were expressed as the means ± SD. For each assay, n=5. The experiments were repeated four times, and similar results were obtained. * $\mathbf{P} < 0.05$ vs. "C" group. $^{\#}$ $\mathbf{P} < 0.05$.

Fig. 3. AICAR-induced GBC-SD cell death is independent of AMPK activation. GBC-SD cells were treated with/out applied concentrations of AICAR, expression of p- and regular AMPKα was tested by Western blot assay (**a**). GBC-SD cells expressing AMPKα shRNA, dominant negative AMPKα (T172A, "dn-AMPKα") or the empty vector ("Vector") were treated with/out AICAR (1.0 mM), listed proteins were tested by Western blots (**b**); Cell

survival (**h**) and apoptosis (**i**) were also tested. GBC-SD cells were treated with/out A-769662 (10 μM), Compound 13 ("C13", 10 μM) or AICAR (1.0 mM), AMPK activation (**e**, upper panel), cell apoptosis (**e**, lower panel) and survival (**f**) were tested. GBC-SD cells expressing constitutively-active AMPKα (T172D, "ca-AMPKα", two clones) or the empty vector ("Vector") were subjected to Western blot assay of AMPK activation (**g**, upper panel); Cells were cultured, cell apoptosis (**g**, lower panel) and survival (**h**) were also tested. AMPKα phosphorylation (vs. regular AMPKα) was quantified (**a**, **b**, **e** and **g**). The values were expressed as the means ± SD. For each assay, n=5. The experiments in this figure were repeated three times, and similar results were obtained. * **P** < 0.05 vs. "C" group. # **P** < 0.05 vs. AICAR only group (**c** and **d**).

Fig. 4. ER stress activation mediates AICAR-induced GBC-SD cell death. GBC-SD cells were treated with/out applied concentrations of AICAR, expression of indicated ER stress proteins was tested by Western blots (**a**). GBC-SD cells, pre-treated with/out salubrinal ("Sal", 10 μM) for 1 hour, were treated with AICAR (0.5/1.0 mM) for applied time, cell survival (**b**) and cell apoptosis (**c**) were tested. GBC-SD cells, infected with scramble-shRNA ("SCR shRNA") or CHOP-shRNA (-1/-2), were subjected to Western blot assay of applied proteins (**d**). Above cells were treated with AICAR (1.0 mM), cell survival (**e**) and apoptosis (**f**) were tested. CHOP expression (*vs.* tubulin) and protein phosphorylations were quantified (**a** and **d**). The values were expressed as the means ± SD. For each assay, n=5. The experiments in this figure were repeated three times, and similar results were obtained. * **P** < 0.05 vs. "C" group. # P < 0.05 vs. AICAR only group.

fig. 1

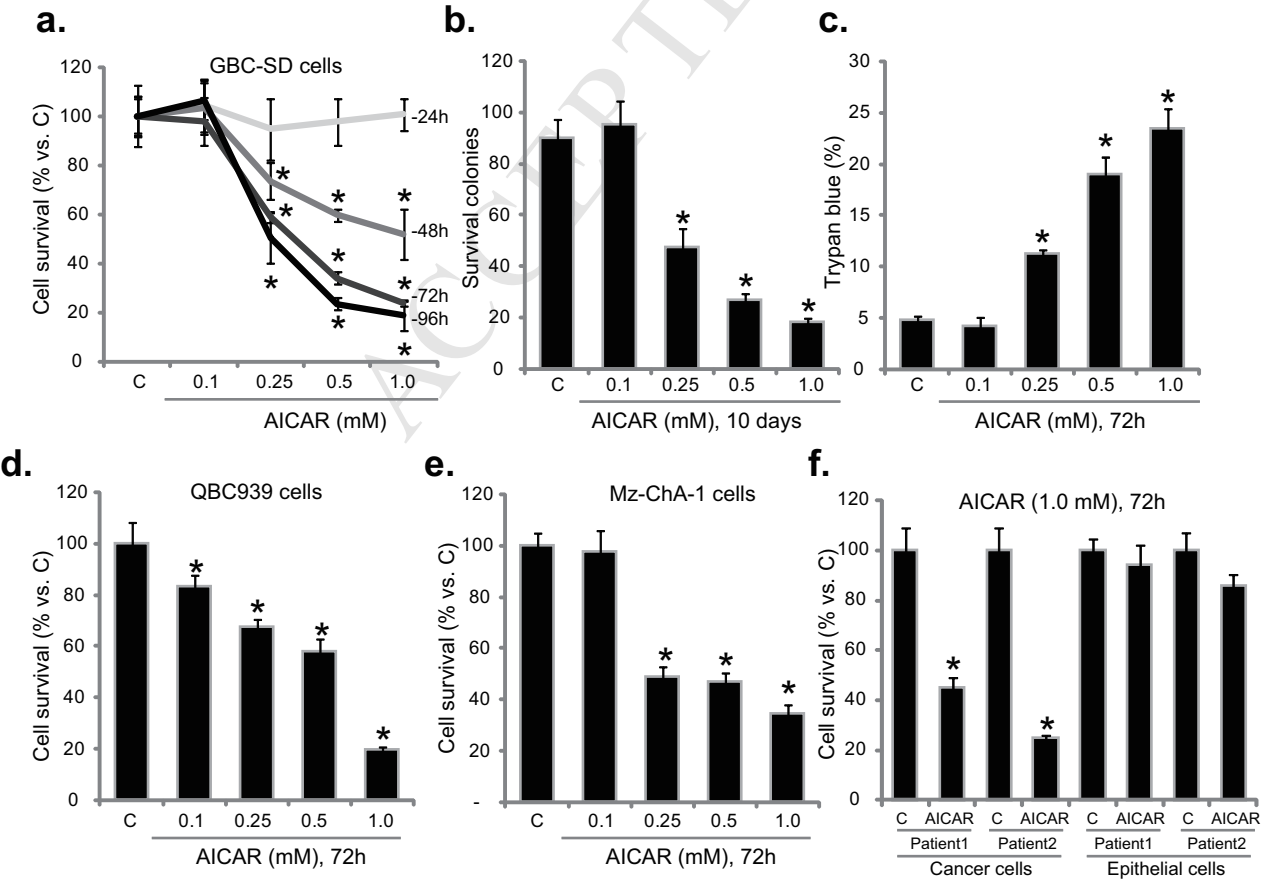


fig. 2

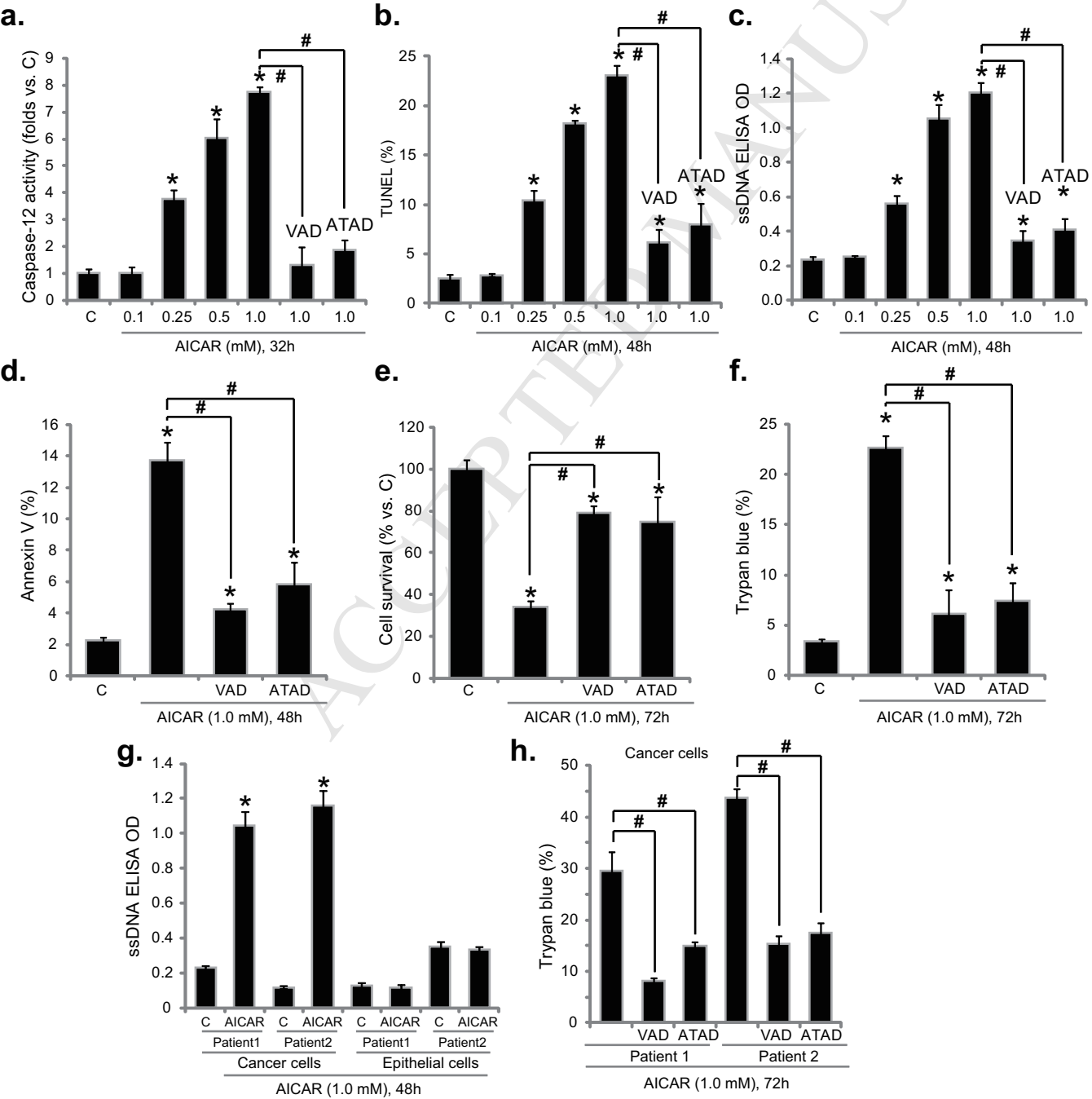


fig.3

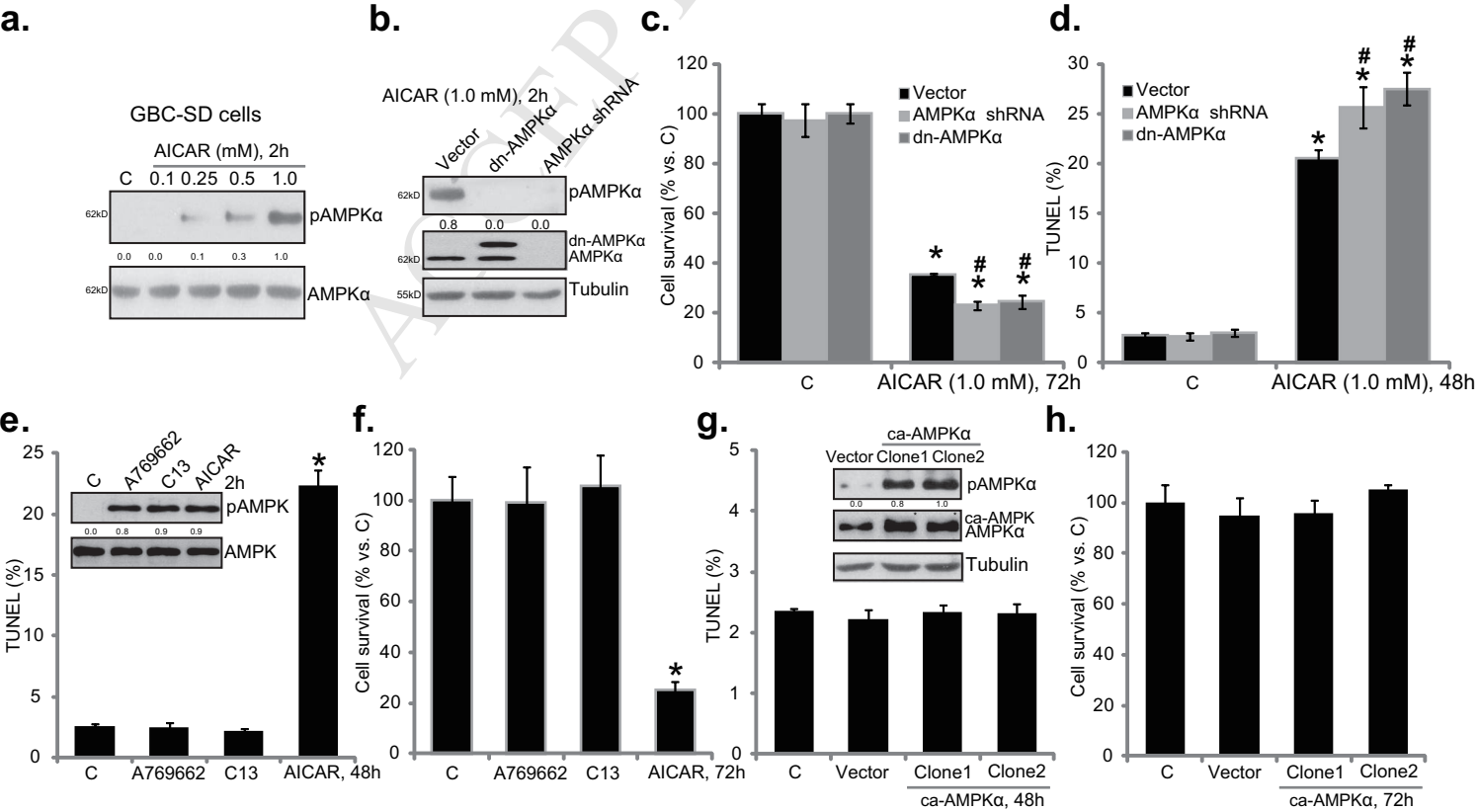
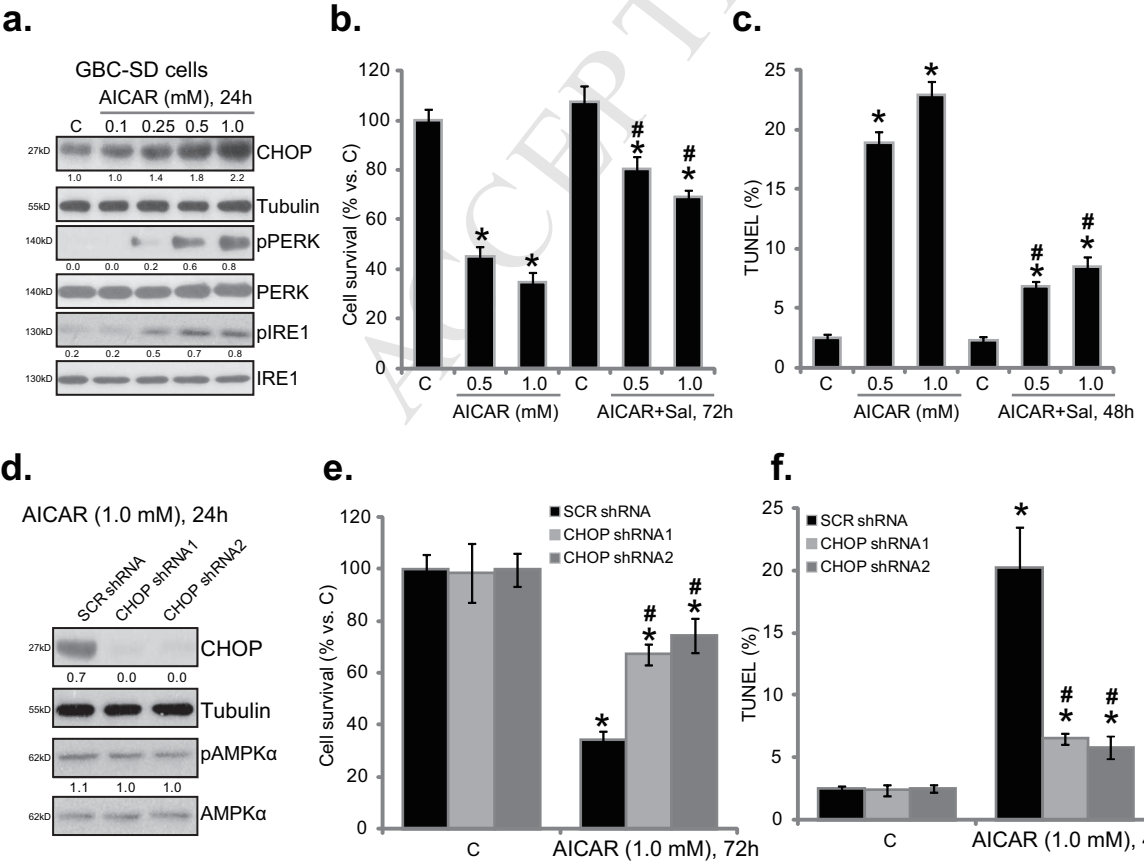


fig. 4



- AICAR induces death of established and primary human gallbladder cancer cells
- AICAR provokes apoptosis in human gallbladder cancer cell
- AICAR-induced GBC-SD cell death is independent of AMPK activation
- ER stress activation mediates AICAR-induced gallbladder cancer cell death
- AICAR-induced ER stress activation is independent of AMPK activation