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AICAR 抑制高脂食物促进的脂肪肝和肝癌发生

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题名页

AICAR 抑制高脂食物促进的脂肪肝和肝癌发生

AICAR prevents HFD-promoted hepatosteatosis and inhibits liver tumorigenesis

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厦门大学博士后研究工作报告

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摘要

肝细胞癌的发展与进程与代谢综合症紧密相关。其中，代谢综合症包括肝脏的脂肪变性与肥胖。腺苷酸活化蛋白激酶（AMPK）是细胞内主要的能量感应器，在营养刺激下，可以通过抑制合成代谢和促进分解代谢调节细胞的代谢平衡。因此，AMPK 的激动剂可以被视为潜在的治疗药物治疗脂肪肝和肝癌。在这，我们通过化学药物和高脂饲料诱导小鼠肝细胞癌，并给小鼠注射 AICAR。我们发现，AICAR 可以抑制肝脏的脂肪合成以及白细胞介素-6（IL-6）的产生，抑制肝脏的脂肪变性，最终减少肝脏的肿瘤发生。AICAR 还可以抑制 IL-6 的炎症信号通路及其下游的 STAT3 的磷酸化激活。但是，短期的 AICAR 用药，对小鼠晚期肝癌的生长没有明显的抑制作用。这些结果提示我们，代谢调控分子 AMPK 可以作为一个很有前景的肝癌治疗靶点，长期的 AICAR 用药对于肥胖引起的肝癌防治是有用的。

关键词： AICAR, AMP, AMPK, 脂肪肝, 肝细胞癌

英文摘要

The development and progression of hepatocellular carcinoma (HCC) are closely associated with metabolic syndrome, including steatosis and obesity. AMP-activated protein kinase (AMPK), a master sensor of energy, regulates metabolic homeostasis by suppressing anabolism and accelerating catabolism in response to nutritional stress. AMPK agonists could therefore be considered as potential therapeutic drugs for treating hepatosteatosis and HCC. Here, by using a mouse model with high fat diet-induced steatosis and chemically induced HCC, we found that AICAR inhibits lipid synthesis and interleukin-6 (IL-6) production in the liver, and prevents steatosis, ultimately resulting in reduced tumorigenesis. AICAR also abrogates IL-6 signaling and STAT3 activation. However, short-term AICAR administration had no significant therapeutic benefit in advanced HCC. These results suggest that the metabolic regulator AMPK is a promising therapeutic target to treat HCC, and that long-term AICAR administration is useful for prevention of obesity-related liver cancer.

Keywords: AICAR, AMP, AMPK, hepatosteatosis, HCC

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Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is closely linked to risk factors such as preexisting alcoholic liver disease, nonalcoholic steatohepatitis, and infection with hepatitis B and C viruses (Naugler et al., 2007; Grivennikov et al., 2010). These factors are all associated with inflammation, which plays a critical role in tumorigenesis. In nonalcoholic steatohepatitis, a condition in which fat accumulates in the liver, the common underlying factor is obesity, rather than alcohol abuse. Obesity has been examined as a potential risk factor for HCC. Indeed, epidemiological studies indicate that excess body weight and obesity may increase cancer risk (Bianchini et al., 2002, Calle and Kaaks, 2004). In men who were free of cancer at enrollment, individuals with high body mass index experience a large, 4.5-fold increase in risk of death from liver cancer during 16 years of follow-up (Calle et al., 2003). Obesity promotes HCC development by enhancing production of the cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), which cause hepatic inflammation, activate the oncogenic transcription factor STAT3 and trigger tumorigenesis (Park et al., 2010),. Thus, there has long been a view that prevention of obesity and associated metabolic diseases may also decrease the risk of liver cancer.

In eukaryotic cells, AMP-activated protein kinase (AMPK) is a sensor of energy and nutrient status, and is a master regulator of metabolic homeostasis by switching off anabolic pathways, including synthesis of fatty acids, triglycerides, cholesterol, and

proteins (Hardie, 2014; Carling et al., 2012; Hardie et al, 2012; Zhang et al, 2013). Therefore, AMPK is a good pharmacological target to treat metabolic diseases such as obesity, type 2 diabetes and cardiovascular disease (Steinberg and Kemp, 2009). At the same time, AMPK is also a potential cancer therapeutic (Hardie and Alessi, 2013), because its activation causes cell cycle arrest, presumably by suppressing most of the metabolic changes that occur in rapidly proliferating cells. However, whether AMPK should be activated or inhibited during cancer therapy remains an open question, and requires further investigation (Liang et al., 2013). On the one hand, AMPK activation during energy stress has been shown to enhance survival of cancer cells (Jeon et al., 2012). On the other, AMPK has been found to negatively regulate aerobic glycolysis in cancer cells, and to suppress tumor growth *in vivo* (Faubert et al., 2013).

AICAR (5-aminoimidazole-4-carboxamideriboside) is a membrane-permeable prodrug that activates AMPK (Hardie, 2014). In cells, AICAR is metabolized to the nucleotide ZMP, an AMP analog with similar effects on the AMPK complex (Corton et al., 1995), including allosteric activation, T172 phosphorylation, and protection from dephosphorylation. Here, we chemically triggered HCC in diet-induced obese mice, and treated them with AICAR. We found that long-term AICAR treatment prevented hepatic steatosis, decreased IL-6 production, and inhibited HCC development. Further, AICAR suppressed phosphorylation of the oncogenic transcription factor STAT3. These results suggest that AMPK is a promising target to treat HCC, and that long-term AICAR administration prevents obesity-related liver

cancer.

Materials and Methods

Animals and chemical induction of liver tumors

To induce HCC, C57BL/6 mice received a single intraperitoneal (i.p.) injection of diethylnitrosamine (DEN) (25 mg/kg, Sigma) at 2 weeks of age. Starting at 6 weeks of age and continuing until sacrifice, mice were fed high-fat diet (HFD, 60% fat, 20% carbohydrate, and 20% protein based on caloric content; D12492, Research Diets, New Brunswick, NJ, USA) and received once every other day i.p. injections of PBS or AICAR (350 mg/kg, Toronto Research Chemicals Inc.). Tumors in the liver were counted and measured with a caliper, and liver tissues were promptly collected and frozen for biochemical, histological and immunochemical analyses. Mouse studies were conducted in the Laboratory Animal Center at Xiamen University.

Histological, immunohistochemical, and immunofluorescence assay

Large lobes were fixed in 10% formalin for 24-48 h. Paraffin-embedded liver tissues were stained with hematoxylin-eosin (H&E) , Mason stain and anti-IL-6 antibody (Abcam). Frozen tissue sections were stained with Oil Red O to visualize lipids.

Determination of cytokines and ALT in serum

IL-6, TNF-α and alanine aminotransferase (ALT) in serum were determined using

kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Measurement of triacylglyceride

Serum and liver triacylglyceride (TAG) contents were measured using Labassay triglyceride reagent (290-63701, Wako Pure Chemical Industries, Ltd.).

Western blotting

Liver samples were homogenized in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, and protease inhibitor cocktail). Equal amounts of liver homogenate (25 μg of total protein) were subjected to Western blotting. Blots were probed with antibodies against phosphorylated AMPK, AMPK, phosphorylated STAT3 (Cell signaling), β-actin (Sigma), STAT3 (Proteintech) and IL-6 (Abcam).

RNA isolation and real-time PCR

Total RNA was extracted with Trizol reagent (Invitrogen), and used as template to prepare cDNA using M-MLV RTase system (Takara). Gene expression was quantified using SYBR Green Real-Time PCR kit (Toyobo) and the StepOnePlus™ Real-Time PCR System from Applied Biosystems.

Statistical Analysis

Student's *t* test was used to test statistical significance between groups. Data are presented as mean ± SEM.

Results

AICAR inhibits DEN-induced liver tumorigenesis

To examine whether AICAR could reduce HCC risk promoted by high-fat diet, we gave 2-week-old male mice a single intraperitoneal (I. P.) injection of DEN (25 mg/kg), 4 weeks later fed the mice with HFD and administrated AICAR (350 mg/kg) to them once every other day (Figure S1, protocol 1). Nuclear magnetic resonance (NMR) imaging showed that AICAR-treated mice developed fewer HCC nodules at eight months than control, PBS-treated mice (Figure 1A). At 10 months of age, mice that received AICAR developed strikingly fewer HCCs per liver (Figure 1B, left panel). Furthermore, the number of tumors, as well as the size of the largest tumors, was dramatically reduced (Figure 1B). In addition, relative liver weight decreased significantly with AICAR administration (Figure 1B). Relative liver weight is the weight of the liver normalized to body weight. Lastly, AICAR mice gained less weight (Figure 1C). Thus, AICAR suppresses DEN-induced hepatocarcinogenesis.

Short-term AICAR treatment exhibits no significant effect in advanced HCC

To test whether AICAR has therapeutic benefit in advanced HCC, we administered the prodrug at 32 weeks, at which point mice had already developed HCC nodules

(Figure S2, protocol 2). The short-term treatment lasted for the next two months. Tumor size did not reduce notably after a month of treatment (Figure 2A). Finally, we found that AICAR administration over two months did not significantly decrease the number of tumors, the weight of the largest tumor, and the relative weight of the liver. Therefore, short-term administration of AICAR does not confer therapeutic benefits in late-stage HCC.

AICAR prevents HFD-induced hepatosteatosis

Using histological analysis, we investigated whether AICAR could prevent HFD-induced hepatosteatosis, which is known to accelerate liver tumorigenesis (Park, 2010). H&E staining showed further marked ballooning in the liver of control mice (Figure 3A). Consistent with this result, Oil Red staining indicated that AICAR reduced the volume of lipid droplets in the liver (Figure 3B). In addition, TAG levels in both the liver and serum decreased with AICAR administration (Figure 3C and 3D). Finally, Mason staining showed decreased abundance of collagen fibers in AICAR-treated mice (Figure 3E), indicating that liver fibrosis has been suppressed.

Furthermore, mRNA levels of citrate cleavage enzyme (ACL) and fatty acid synthase (FAS), which are both involved in lipid synthesis, decreased in AICAR treated mice (Figure 3F). However, AICAR did not affect mRNA levels of CD36, carnitine palmitoyltransferase (CPT1), and adipose triacylglyceride lipase (ATGL), which are involved in lipid uptake, oxidation, and lipolysis respectively (Figure S3). These data

indicate that AICAR suppressed hepatosteatosis which could be attributed to attenuated lipogenesis.

AICAR suppresses IL-6 production and STAT3 phosphorylation

Obesity enhances production of the inflammatory cytokines IL-6 and TNF-α, which are both critical to obesity-promoted HCC onset and development (Park et al., 2010). To test whether the effects of AICAR are related to the activity of these cytokines, we determined the levels of IL-6 and TNF-α in the serum. IL-6 levels significantly decreased in AICAR mice (Figure 4A). Consistent with this result, AICAR also lowered the abundance of IL-6 mRNA and protein in the liver (Figure 4B-C). On the other hand, levels of TNF-α also decreased, although not to a statistically significant extent (Figure 4A). Using ALT as marker, we also found AICAR administration to protect the liver from injury (Figure 4D).

As has been seen, short-term AICAR treatment does not affect the progression of late-stage HCC (Figure 2), suggesting that AICAR inhibits liver tumorigenesis through a chronic, long-term process. Thus, we compared IL-6 abundance in mice at three months and six months (Figure 4E). At three months, control and AICAR mice have similar body weight (Figure 1C), hepatic lipid content (Figure S4A, left panel) and liver IL-6 levels (Figure 4E, left panel). However, at six months of age, AICAR-treated mice have lower body weight (Figure 1C), and have reduced levels of lipids (Figure S4A, right panel) and IL-6 in the liver (Figure 4E, right panel). These

results suggest that AICAR reduced lipid accumulation in liver via a chronic process that ultimately results in suppressed IL-6 production.

Additionally, we performed Western blotting to determine the effect of AICAR on the phosphorylation of STAT3, which is a downstream target of IL-6 signaling in the liver. Mice at three or six months of age were sacrificed 1 h after injection with PBS or AICAR. Liver sections were collected promptly, and flash-frozen in liquid nitrogen. Liver homogenates were then subjected to Western blotting (Figure 4F). Notably, AICAR increased the phosphorylation of AMPK at T172 and diminished phosphorylation of STAT3 at Y705 in both the three month- and six month-old livers (Figure 4F). In addition, we found AICAR mitigated DEN effect on phosphorylation of STAT-3 *in vivo* (Figure S4B), by analyzing normal diet-fed mice that were killed 1 h after injection with DEN (100 mg/kg) and AICAR. DEN administration could induce rapid production of IL-6 and phosphorylation of STAT-3 (Naugler et al., 2007). However, AICAR abrogated DEN-induced phosphorylation of STAT-3 (Figure S4B). Hence, AICAR represses DEN-induced cytokine production and signaling, presumably through its AMP-like effects on AMPK, which has previously been shown to suppress IL-6-dependent inflammation in the mouse liver (Nerstedt et al., 2013; Cansby et al., 2014).

Discussion

HFD promotes hepatic steatosis in DEN-treated mice, and thereby enhances the onset and progression of tumorigenesis in liver. Thus, disrupting hepatic steatosis may prevent liver cancer. Indeed, mice deficient in heat shock transcription factor 1 exhibit decreased chronic hepatic steatosis and dramatically reduced tumorigenesis after DEN administration (Jin et al., 2011). Metformin also protects mice from chemically induced liver tumors by inhibiting pathways that drive hepatic lipogenesis (Kavita et al., 2012). However, the protective effect of metformin is believed to be independent of AMPK activation (Kavita et al., 2012). Nevertheless, there is evidence that AMPK agonists represent a class of promising agents that could prevent hepatic steatosis. These compounds would activate AMPK *in vivo* and thus regulate lipid metabolism in the liver. For example, resveratrol, a natural polyphenol in red wine, and S17834, a synthetic polyphenol, potently and persistently stimulate AMPK kinase activity, and inhibit lipid accumulation in the liver (Li et al., 2011). Salicylate, a plant product, could also directly activate AMPK and increase fat utilization in mice (Hawley et al., 2012). These compounds could be considered as candidate anti-cancer drugs for HCC.

In this study, we showed for the first time that AICAR, a membrane-permeable AMPK agonist, prevents lipid accumulation in the liver, and suppresses chemically induced HCC development (Figure 1 and Figure 3). Our results are the first demonstration of the effects of AICAR on primary hepatocellular carcinoma *in vivo,* even though it has already been shown to inhibit cancer cell growth and tumor

development *in vitro* and in xenograft models (Swinnen et al., 2005). However, a short-term AICAR regimen has no obvious effects in advanced HCC (Figure 2). Thus, AICAR may not be suitable to treat late-stage liver cancer.

Further, we demonstrated that AICAR reduced lipid accumulation in liver via a chronic process that ultimately results in suppressed IL-6 production (Figure 4). Obesity-associated chronic elevation in IL-6 is an important contributing factor to liver tumorigenesis (Park et al., 2010). In addition, IL-6 is a risk indicator, and is strongly correlated with poor prognosis in HCC (Porta et al., 2008). Further, IL-6 expression is elevated in HCC progenitor cells, and the progression of these cells to HCC depends on autocrine IL-6 (He et al., 2013). These indicate that IL-6 is a tumor promoting cytokine and AICAR decreased IL-6 levels which may account for HCC suppression.

An additional mechanism through which AICAR might suppress HCC is to enhance cancer cell death. Indeed, HCC in obese mice exhibits reduced apoptotic cell death relative to HCC in lean mice (Park et al., 2010), even though obese mice experience greater liver damage from DEN administration. Thus, AICAR might also, conceivably, prevent cancer development by reversing the ability of HCC cells to survive in the obese liver. Unexpectedly, we found that AICAR reduces apoptotic cell death (Figure 4A) and compensatory proliferation (Figure 4B), even though it promotes apoptosis in many cancer cell lines *in vitro*. The mechanism underlying this effect is unknown, and