Original Article
Regulation of Bmi-1 expression by AMPK and its effect on the proliferation of osteosarcoma cells

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Abstract: Adenosine monophosphate-activated protein kinase (AMPK) is modulated by AMP/ATP ratio and is the "detector" for cellular energy. Its activation in various tumor cells can inhibit cell proliferation and migration. Study has found the over-expression of B cell specific murine leukemia viral integration site-1 (Bmi-1). This study thus investigated the regulation of AMPK on Bmi-1 in human osteosarcoma cell line 143B, and the role of AMPK and Bmi-1 on tumor migration and metastasis. 143B cells were cultured and activated for AMPK by different concentrations of AICAR (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L). MTT assay was used to detect the proliferation activity of cells after AICAR intervention. Western blotting was employed to test protein levels of AMPK and Bmi in 143B cells. Cellular mRNA expression of Bmi was quantified by RT-PCR. Different dosages of AICAR significantly inhibited cell proliferation at all time points. 0.5 mmol/L ACAIR had the most significant inhibitory effect (P<0.05). AMPK was activated in 143B cells 1 hour after intervention, with reaching a peak level at 6 hours, followed by gradual decrease until 24 hours. The expression of Bmi was also decreased after AICAR treatment, with the most significant effect in 0.5 mmol/L group (P<0.05). AMPK can inhibit proliferation and metastasis of osteosarcoma cells via suppressing Bmi-1 expression.

Keywords: Adenosine monophosphate-activated protein kinase, B cell specific murine leukemia viral integration site-1, osteosarcoma

Introduction

Osteosarcoma is one common malignant bone tumor in children and teenagers. Due to its high malignancy and frequent metastasis, patients' survival rate is relatively low with unfavorable prognosis. Various treatment plans have been developed recently including surgery, next-generation chemo- or radio-therapy, all of which improved survival rate. However, due to severe adverse effects, lots of patients cannot complete the whole treatment period, leading to early reoccurrence and metastasis of tumors, and amputation of patients with unfavorable prognosis [1]. Adenosine monophosphate-activated protein kinase (AMPK), also names as AMP-dependent protein kinase, is one highly conserved serine/threonine protein kinase in all eukaryotic cells. It is widely involved in regulating bio-energy metabolism, and is expressed in all tissues/organs related with body metabolism. When body is at stress conditions such as hypoglycemia, hypoxia, shock or ischemia, the elevated ration of AMP/ATP can activate intra-

cellular AMPK, which can regulate energy metabolism, in addition to mediating transcriptional regulation for affecting cell cycle [2]. Study has indicated the activation of AMPK for inhibiting cell proliferation and migration [3]. In AMPK signaling pathway, various tumor suppressor factors such as LKB1, p53, TSC1 and TSC2 are involved, as they can antagonize growth factor related signaling pathways induced by multiple stimulus, for potential anti-tumor effects [4]. B cell specific murine leukemia viral integration site-1 (Bmi-1) is one polycomb group gene and is highly expressed in various tumors. Those tumor cells with Bmi-1 expression were then called cancer stem cells [5]. In the whole process of pathogenesis, progression and prognosis, Bmi-1 expression has been shown to be enhanced [6]. This study selected human osteosarcoma cell line 143B, which was treated by AICAR to activate AMPK. The expressional profile of Bmi-1 was detected, along with the detection of proliferation and migration of tumor cells, in an attempt to analyze the regulation of AMPK on Bmi-1 and the effect of these two

genes on tumor cell proliferation and metastasis.

Materials and methods

Cell line

Human osteosarcoma cell line 143B was purchased from Baili BioSciences (China).

Reagents and equipment

AICAR and AMPK antibody were purchased from TRC (US) and CST (US), respectively. Bim-1 antibody and anti-rabbit IgG-HRP secondary antibody were purchased from Bioworld (US). DMEM medium, penicillin/streptomycin, and fetal bovine serum (FBS) were products of Gibco (US). Trizol reagent was produced by Invitrogen (US). Florescent quantitative PCR kit was produced by Sangon (China). Cell incubator was a product of Thermo (US). CO_2 and -80°C fridge were products of SANYO (Japan). Inverted microscope was a product of Nikon (US). PCR cycler was provided by Biometra (US). DNA nuclei concentration detector was produced by Eppendorf (Germany). High-speed centrifuge was purchased from Beckman (US).

Cell culture

143B cells were cultured in RPMI1640 medium in a humidified chamber with 5% CO_2 at 37°C.

AICAR treatment

143B cells at log-phase were counted and seeded into culture plate for forming attached growth of cells. After 24-hour culture in 2% FBS, DMEM with 10% FBS was added with AICAR in treatment cells. Parallel control cells were also investigated without AICAR treatment.

MTT assay

Different concentrations of AICAR (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L) were added to test the viability of cells at 24 hour, 48 hour and 72 hour. In brief, 20 μL MTT reagent (5 mg/mL) was added in each well for 4-hour incubation, followed by 150 μL DMSO. The plate was vibrated for 10 min at low speed to dissolve crystals. Absorbance values at 570 nm were measured in each well.

Western blotting for AMPK level

After treating with 0.5 mmol/L AICR, proteins were extracted at 1 hour, 6 hour, 12 hour and 24 hour. Proteins were separated in 8% SDS-PAGE. After blocking at room temperature for 1 hour, primary antibody dilution (1:200 or 1:500 for β-actin) was added for 30-min incubation. The membrane was then kept in 4°C overnight. On the next day, TBST was used to wash the membrane, with addition of anti-rabbit IgG-HRP dilutions (1:2000) for 1-hour incubation. After TBST washing, chromogenic substrate A and B were added for develop the membrane, which was scanned and analyzed for optical density using Quantity One software.

RT-PCR for Bmi expression

143B cells at log-phase were treated with different concentrations of AICAR (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L) for 24 hours. Cellular mRNA was then extracted and quantified for total RNA concentration (by D260/280 values). 200 ng of RNA were used as template to synthesize cDNA using polyA tail. PCR amplification was then performed using specific primers (Bmi-Forward, 5'-CACCA TGAAG CCTAC AC-TGT GTTTC C-3'; Bmi-Reverse, 5'-TTAAA CCATT CGGCA GCAGC GG-3'; GAPDH-Forward, 5'-GCC-AA GGTCA TCCAT GACAA CTTTG G-3'; GAPDH-Reverse, 5'-GCCTG CTTCA CCACC TTCTT GATGT C-3'). PCR conditions were: 95°C pre-denature for 30 sec, followed by 30 cycles each containing 95°C denature for 30 sec, 58°C annealing for 30 sec, and 72°C elongation for 30 sec. DNA products were separated in 5% agarose gel electrophoresis. Quantity One software was used to analyze the optical density (OD) values of Bmi bands in all groups against internal reference.

Western blotting for Bmi level

143B cells at log phase were treated with 0.5 mmol/L AICR. Proteins were extracted and separated in 8% SDS-PAGE. After blocking at room temperature for 1 hour, primary antibody dilution (1:200 or 1:500 for β-actin) was added for 30-min incubation. The membrane was then kept in 4°C overnight. On the next day, TBST was used to wash the membrane, with addition of secondary antibody (1:2000) for 1-hour incubation. After TBST washing, chromogenic substrate A and B were added for develop the

Table 1. MTT assay for proliferation activity of 143B cells

Time	Experimental group			Control
	0.1 mmol/L	0.5 mmol/L	1.0 mmol/L	
24 h	0.125±0.012	0.996±0.026*	0.882±0.024*	0.126±0.036
48 h	0.151±0.045*	0.102±0.028*,#,&	0.135±0.021*	0.162±0.046
72 h	0.223±0.051*	0.116±0.153*,#,&	0.152±0.037*	0.254±0.025

Note: *, $P<0.05$ compared to control group; #, $P<0.05$ compared to 0.1 mmol/L group; &, $P<0.05$ compared to 1.0 mmol/L group.

Table 2. AMPK level in 143B cells

Experimental group				Control
1 h	6 h	12 h	24 h	
0.652±0.124*	0.987±0.016*	0.892±0.011*	0.623±0.005*	0.114±0.002

Note: *, $P<0.05$ compared to control group.

Figure 1. Western blotting for AMPK level after AICAR treatment.

membrane, which was scanned and analyzed for optical density using Quantity One software.

Statistical analysis

SPSS 17.0 software was used to process all collected data, of which enumeration data were compared by chi-square test, while measurement data were compared by analysis of variance (ANOVA) and were presented as mean ± standard deviation (SD). A statistical significance was defined when $P<0.05$.

Results

Proliferation of 143B cells after AICAR treatment

Different concentrations of AICAR (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L) were used to treat 143B cells for 24, 48 and 72 hours. Results showed significant inhibition of cell proliferation by AICAR at all time points. 0.5 mmol/L AICAR had the most significant inhibitory effects ($P<0.05$, **Table 1**).

AMPK level in 143B cells after AICAR

We further employed 0.5 mmol/L AICAR to treat human osteosarcoma 143B cells, and found

activation of AMPK 1 hour afterwards. AMPK level reached a peak at 6 hour and gradually decreased at 24 hour ($P<0.05$, **Table 2**; **Figure 1**).

Bmi mRNA level in 143B cells

We further employed RT-PCR to quantify Bmi mRNA level in 143B cells at 24 hours after 24 hours of AICAR treatment. Results showed significant depression of Bmi expression at 0.5 mmol/L AICAR ($P<0.05$, **Table 3**).

Bmi protein expression level

AICAR (0.5 mmol/L) was used to treat 143B cells. Protein level of Bmi was tested at 30 min, 1 hour, 6 hour, and 12 hour after treatment. Results showed gradually decreased Bmi protein level in 143B cells with elongated treatment ($P<0.05$, **Table 4**; **Figure 2**).

Discussion

Osteosarcoma has a relatively high incidence in children and teenagers, occupying about 8.9% of all cancer-related death. Its disability adjusted survival is 10 years longer than common malignant tumors such as gastrointestinal cancer, pulmonary cancer and breast cancer [7]. With the rapid progression of tumor molecular biology, genetic factors have been unrevealed in pathogenesis, progression, prognosis and treatment efficacy [8]. Tumor cells have unique energy metabolism pathway as its glucose-uptake ability is more potent than normal cells, but with impaired metabolic function of glucose. Even under sufficient oxygen supply, tumor cells prefer glycolysis than mitochondrial respiration with higher ATP productivity, a phenomenon called Warburg event that can produce large amounts of lactate and ATP [9]. AMPK exists in the form of hetero-trimer inside the body. It is one serine/threonine protein kinase consisting of α-catalytic subunit and β, γ-regulatory subunits [10]. AMPK has been found to inhibit tumor growth, thus calling metabolic tumor suppressor. When AMPK expression was silenced, intracellular lactate level was increased to facilitate energy metabolism

Table 3. Bmi mRNA level in 143B cells

Experimental group			Control
0.1 mmol/L	0.5 mmol/L	1.0 mmol/L	
0.892±0.124*	0.561±0.006*,#,&	0.733±0.011*	0.925±0.137

Note: *, P<0.05 compared to control group; #, P<0.05 compared to 0.1 mmol/L group; &, P<0.05 compared to 1.0 mmol/L group.

Table 4. Bmi protein level in 143B cells

Experimental group				Control
30 min	1 h	6 h	12 h	
0.502±0.05[a]	0.415±0.031[a,b]	0.162±0.011[a,b,c]	0.056±0.006[a,b,c,d]	0.621±0.076

Note: [a], P<0.05 compared to control group; [b], P<0.05 compared to 30 min group; [c], P<0.05 compared to 1 h group; [d], P<0.05 compared to 6 h group.

Figure 2. Bmi protein expression by Western blotting.

of tumor cells [11]. As one AMPK agonist, AICAR can be transported into cells by adenosine carrier, and formed AMP analog by the phosphorylation of adenosine kinase to exert AMPK function [12]. AMPKY has been found to inhibit the proliferation of smooth muscle of rabbit aorta mainly via affecting cell cycle [13]. In this study, we treated human osteosarcoma cells 143B with different concentrations (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L) of AICAR at different time points (24 hour, 48 hour and 72 hour). Our results showed the inhibition of AICAR on cell proliferation at all time points, especially in 0.5 mmol/L AICAR. AMPK activation occurred 1 hour after treatment, reached a peak level at 6 hour, and gradually decreased at 24 hour. Our results indicated that ACAIR could activate AMPK for elevating its expression level, in order to inhibit cell proliferation. 0.5 mmol/L AICAR had the most potent effect of AMPK activation and a peak-decrease pattern in time manner.

This study used different concentrations to treat 143B cells. After 24 hours, Bmi expression was decreased most significantly in 0.5 mmol/L AICAR group. With elongated time (30 min, 1 hour, 6 hour, and 12 hour), Bmi level was gradually decreased, suggesting the most significant activation of AMPK by 0.5 mmol/L AICAR, which caused the most significant depression of Bmi. With elongated AICAR treatment, the level of Bmi gradually decreased in 143B cells, suggesting that AMPK might inhibit cell proliferation via suppressing Bmi expres-

sion. Previous study found indicating value of BMI-1 gene on malignant tumors, as it is closely related with pathogenesis and progression of cancer [14]. In osteosarcoma, chondrosarcoma and Ewing's sarcoma tissues, Bmi-1 protein level is significantly higher than normal bone or chondrocyte tissues. Therefore BMI-1 might be one target for treating malignant bone tumors, although its mechanism has not been confirmed [15, 16]. Another study in 10 different osteosarcoma cell lines and 42 patients pointed PH3 as tumor-suppressor gene, as those cells with PHC3 positive expression had lower Bmi-1 protein. In those cells with higher Bmi-1 positive expression rate, mRNA level of PHC3 was lowered, thus causing rapid progression of osteosarcoma cells [17]. In gastric carcinoma, BMI-1 gene expression is accompanied with low-differentiation types, thus potentiating proliferation, invasion and metastasis of tumors, and is positively correlated with malignancy of tumors [18]. Survival analysis has found lower survival rate in patients with Bmi-1 positive expression, as compared to those with negative expressions [19]. Using RNA interference to suppress Bmi-1 gene expression also has been found to decrease proliferation, invasion and metastasis ability of gastric tumor cells [20].

In summary, the activation of AMPK in osteosarcoma 143B cells can inhibit Bmi-1 expression. With elongated treatment time, the suppression of Bmi-1 expression is more significant and 143B cell proliferation is inhibited. Our results suggested AMPK might inhibit the proliferation and metastasis of osteosarcoma cells via inhibiting Bmi-1 expression, although its detailed mechanisms still require further studies to substantiate.

Disclosure of conflict of interest

None.

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