

ORIGINAL ARTICLE

Effect of AMPK/Akt/mTOR pathway on cytokine-induced killer cells immunotherapy on colorectal cancer cells

Jianfeng Shi, Jinfeng Wu, Haibin Ni, Xiaobo Zhan, Lu Zhang, Zhe Zhang

Department of General Surgery, Tongde Hospital of Zhejiang Province, Hangzhou, China.

Summary

Purpose: To investigate the effect of cytokine-induced killer cells (CIKs) immunotherapy on colorectal cancer cells and the possible mechanisms using co-culture model of CIKs and human colorectal cancer cells.

Methods: CIKs were induced by INF- γ , CD3 and recombinant human IL-2 culture system in vitro. A co-culture model of CIKs and SW480 colorectal cancer cell line was established. The proliferation, invasion and apoptosis of SW480 cells were detected by CCK-8 (cell counting kit-8), transwell assay and Hoechst staining, respectively. The contents of IL-2, IL-10, insulin-like growth factor (IGF)- β and vascular endothelial growth factor (VEGF) in the culture medium was detected by ELISA (enzyme-linked immunosorbent assay). The expression levels of AMPK/Akt/mTOR signaling-related proteins were detected by Western blot.

Results: CCK-8 assay showed that the proliferative ability of co-cultured CIK+SW480 cells was remarkably lower than

that of SW480 cells ($p < 0.05$). Transwell assay showed that the number of invasive cells in co-cultured CIK+SW480 cells was less than that of SW480 cells ($p < 0.05$). Hoechst staining showed that the apoptosis rate of co-cultured CIK+SW480 cells was higher compared with that of SW480 cells ($p < 0.05$). Increased levels of IL-2 and IL-10 and decreased levels of IGF- β and VEGF were found in the co-culture medium by ELISA ($p < 0.05$). Western blot results indicated that p-AMPK and p-Akt were upregulated, whereas FoxM1 and p-mTOR were downregulated in co-cultured CIK+SW480 cells compared with SW480 cells ($p < 0.05$).

Conclusions: CIKs inhibit the proliferation and invasion of colorectal cancer cells through downregulating FoxM1 and mTOR via AMPK/Akt/mTOR pathway.

Key words: CIKs, colorectal cancer, AMPK/Akt/mTOR pathway, immunotherapy

Introduction

Tremendous changes in lifestyle and dietary habits, as well as deteriorating environment, all contribute to the increased prevalence of malignancies [1]. Cancer-related death ranks second in global mortality [2]. Colorectal cancer is a gastrointestinal system cancer, which is manifested as insidious onset, rapid progression, and short life expectancy [3,4]. The current treatment of colorectal cancer is still dominated by surgery resection, radiotherapy and chemotherapy. Although chemotherapy for colorectal cancer can prolong the overall survival of patients, its bone marrow toxicity would lead to

immune suppression that can seriously affect clinical outcomes [5]. As a novel therapeutic approach for colorectal cancer, biological therapy has been well recognized in the clinical setting. Biological therapy mobilizes natural defense in the body or provides natural substances to obtain anti-tumor effect [6-8].

CIKs are nonspecific cytotoxic cells produced by peripheral blood mononuclear cells (PBMCs) after being stimulated with cytokines, such as CD3, IFN- γ and IL-2 [9]. CIKs are mainly composed of CD3⁺CD56⁺ T cells, which have a strong killing

Correspondence to: Zhe Zhang, MM. Department of General Surgery, Tongde Hospital of Zhejiang Province, No. 234, Gucui Rd, Hangzhou, 310012 Zhejiang, China. Hangzhou, China.
Tel: +86 0571-89972114, E-mail: sjfzz433@163.com
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ability for malignant tumor cells. Functionally, CIKs exert their anti-tumor effects via MHC (major histocompatibility complex) restricted and MHC non-restricted tumoricidal ways. The tumoricidal capacity induced by CIKs has the characteristics of rapid proliferation, broad killing spectrum, and high killing activity [10,11]. It can directly kill tumor cells without damaging the immune function of the body. Meanwhile, CIKs can also regulate and enhance the immune function, which is the latest method of adoptive immunotherapy for tumors.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a highly expressed serine/threonine protein kinase complex. AMPK is consisted of an α -catalytic subunit, a β -regulatory subunit, and a γ -regulatory subunit.

Functionally, AMPK responds to various external stimuli of cells and maintains the balance of intracellular supply and demand of energy, thereby regulating cell metabolism [10,11]. Energy deficiency leads to AMPK activation, thereafter causing a series of anabolic pathways that consume

abundant ATPs. Multiple ATP-producing catabolic pathways are activated to maintain the balance of energy metabolism in the body [14]. Among these pathways, mTOR pathway responds to various intracellular and extracellular stimuli and affects physiological functions. AMPK regulates the phosphorylation of mTOR after receiving intracellular nutrition signals, which in turn regulates protein synthesis, cell growth and metabolism through the mTOR pathway [15,16]. For example, AMPK regulates proliferation of breast cancer by regulating s6rp via inhibiting mTOR pathway [17]. It is reported that AMPK/mTOR pathway is activated in multiple tumors, such as bladder cancer, breast cancer, hepatocellular carcinoma, non-small cell lung cancer, colorectal cancer, etc. [18-22]. The above studies all suggested that AMPK/mTOR pathway plays a crucial role in the progression of various tumors.

In this study, CIKs were induced from human PBMCs using culture system of INF- γ , CD3 and recombinant human IL-2 *in vitro* [1]. The specific

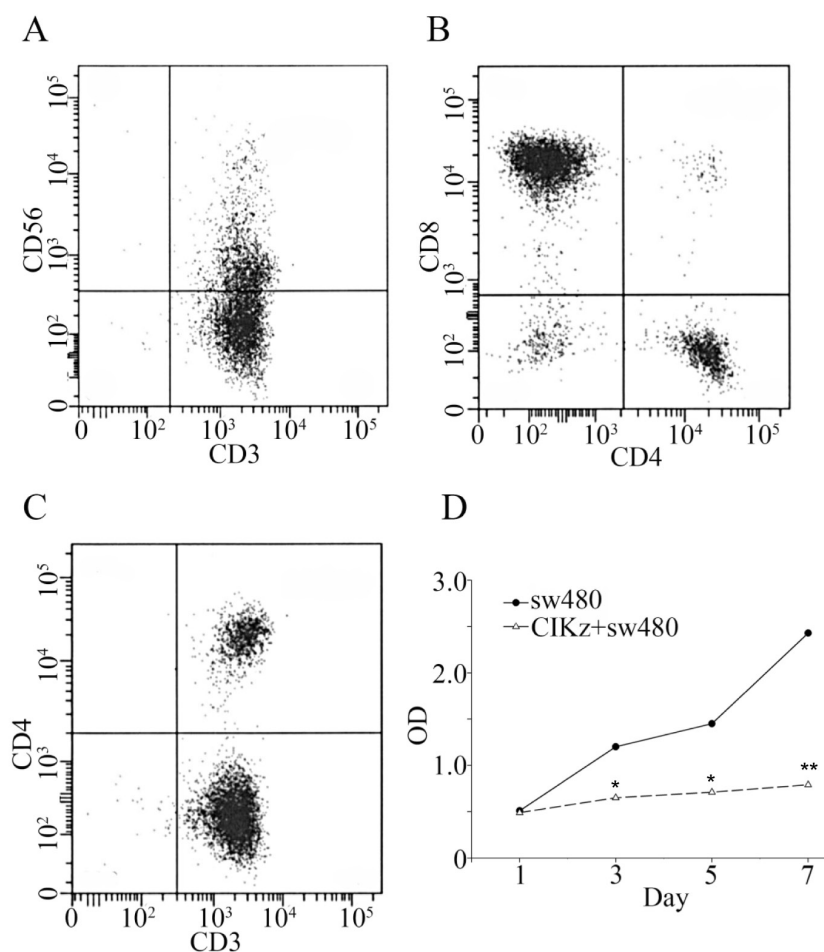


Figure 1. CIKs identification and the effect on cell proliferation. **(A)** FCM results of CD3⁺CD56⁺ in CIKs. **(B)** FCM results of CD4⁺CD8⁺ in CIKs. **(C)** FCM results of CD4⁺CD3⁺ in CIKs. **(D)** Cell proliferation in CIK+SW480 cells and SW480 cells. *p<0.05, **p<0.01 compared with SW480 cells.

effects of AMPK/Akt/mTOR pathway on regulating CIKs-induced proliferation and invasion of SW480 cells were detected. Our results provide an experimental basis for the clinical treatment of colorectal cancer.

Methods

Cell culture

Human colorectal cancer cell line SW480 was taken out from liquid nitrogen and immediately melted at 37°C water bath. Cells were centrifuged at 500 r/min for 5 min. After discarding the supernatant, cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) containing 10% FBS (fetal bovine serum) and maintained in a 5% CO₂ incubator at 37°C. Culture medium was replaced every 2 days.

CIKs were extracted from PBMCs and cultured in RPMI-1640 containing 10% FBS. After cell density was adjusted to 1×10^6 /mL, CIKs were incubated with 1000 μ /mL INF- γ , 50 ng/mL CD3 and 500 μ /mL IL-2 for 24 hrs. Fresh medium containing 500 μ /mL IL-2 was replaced every 3 days.

CIKs identification

After CIKs culture for 14 days, 5 mL of suspension was collected and centrifuged at 1000 r/min for 5 min at room temperature. CIKs were resuspended in 100 μ L of PBS (phosphate buffer saline) and incubated with CD3, CD4, CD8 and CD56 in the dark at 4°C. Thirty min later,

CIKs were identified using flow cytometry (FCM). Dual-positive rate of CD3⁺ CD56⁺ over 20% was considered as successful isolation of CIKs.

Co-culture of CIKs and SW480 cells

SW480 cells were first seeded in the 6-well plates. CIKs were collected and resuspended at a density of 5×10^9 /mL. Forty μ L of CIKs suspension were added in each well and co-cultured with SW480 cells.

CCK-8 (cell counting kit-8) assay

Co-cultured SW480 cells and CIKs seeded into 6-well plates were used for detecting cell proliferation. A hundred μ L of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) were added in each well after cell culture for 6, 24, 48, 72 and 96 hrs, respectively. The absorbance at 450 nm of each sample was measure by a microplate reader (Bio-Rad, Hercules, CA, USA).

ELISA (enzyme-linked immuno sorbent assay)

Culture medium was collected and contents of IL-2, IL-10, TGF- β and VEGF were detected based on the instructions of ELISA kit (R&D Systems, Minneapolis, MN, USA). The absorbance at 450 nm of each sample was measure after termination of color developing for 15 min.

Hoechst assay

Cells were centrifuged, collected in a 1.5 mL tube and fixed with 0.5 ml of fixation solution for 10 min.

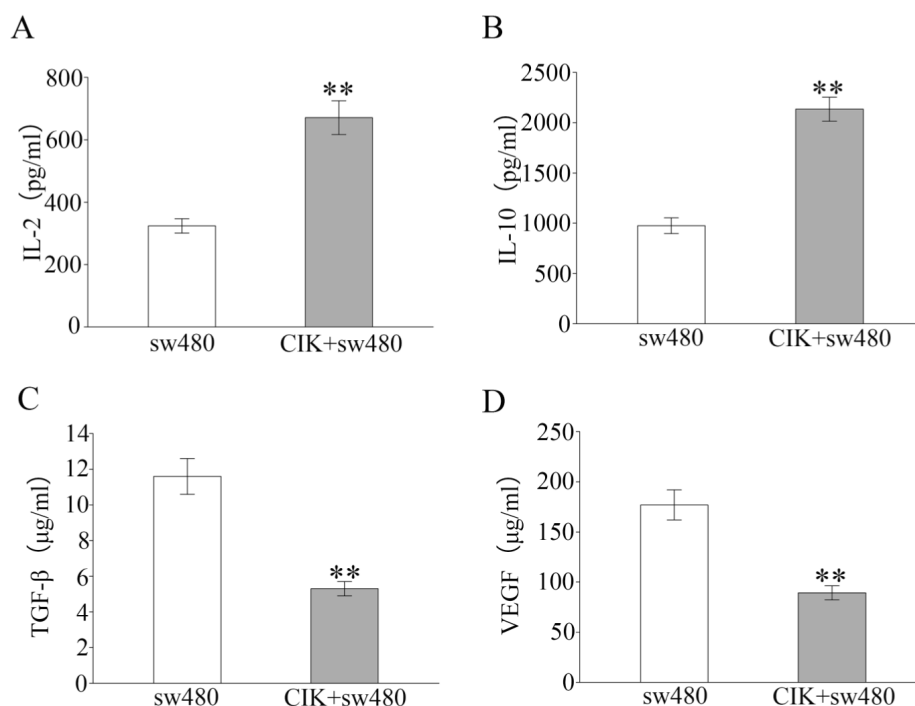


Figure 2. Cytokine contents in culture medium. **(A)** IL-2 content in culture medium of CIK+SW480 cells and SW480 cells. **(B)** IL-10 content in culture medium of CIK+SW480 cells and SW480 cells. **(C)** TGF- β content in culture medium of CIK+SW480 cells and SW480 cells. **(D)** VEGF content in culture medium of CIK+SW480 cells and SW480 cells. **p<0.01 compared with SW480 cells.

After washed with PBS twice, resuspended cells were dropped on the slide, and incubated with 0.5 mL of Hoechst 33258 (Beyotime, Shanghai, China) for 5 min. Excessive liquid from the edge was cleaned and the slide was air-dried. After incubated with a drop of antifade mounting medium, cell apoptosis was observed and captured using an inverted microscope (Nikon, Tokyo, Japan).

Transwell assay

Cells were serum-starved for 12-24 hrs. Cells were digested and resuspended at a density of 1×10^4 /mL. Two hundred μ L of cell suspension were added in the upper chamber and incubated for 48 hrs. Invasive cells were observed using a light microscope (Olympus, Tokyo, Japan).

Western blot

Total protein was extracted from treated cells by RIPA (radioimmunoprecipitation assay) solution (Beyotime, Shanghai, China). Protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA, USA). After the membranes were blocked with skimmed milk, they were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered Saline with Tween 20) and incubated with secondary antibody at room temperature for 1 hr. The protein blot on the membrane was exposed by chemiluminescence.

Statistics

Graphpad Prism 5.0 (La Jolla, CA, USA) was used for homogeneity and variance test. Data that satisfied normality and variance homogeneity were compared using one-way ANOVA or *t*-test. Non-parametric test was performed for data that did not satisfy normality and variance homogeneity. $p < 0.05$ was considered as statistically significant.

Results

Phenotype identification of CIKs

CIKs were extracted from PBMCs and incubated with INF- γ , CD3 and IL-2 for 14 days. Dual-positive rate of CD3⁺CD56⁺ was detected using FCM. FCM results showed that the positive rate of CD3⁺CD56⁺ was 31.7%, which was higher than 20%, indicating the successful isolation of CIKs (Figure 1A-1C).

Effect of CIKs on proliferation of SW480 cells

CCK-8 assay demonstrated that CIKs remarkably inhibited the proliferative abilities of SW480 cells ($p < 0.05$, Figure 1D).

Cytokine content in co-culture medium

ELISA results indicated that the content of IL-2 and IL-10 in co-culture medium were much higher

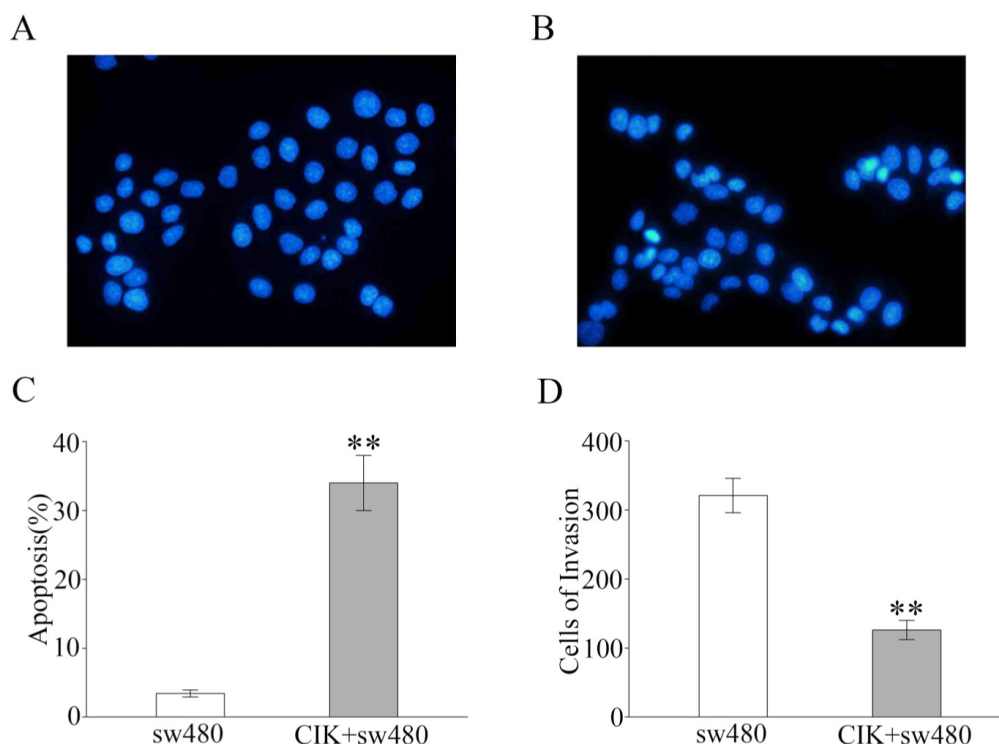


Figure 3. Effects of CIKs on cell apoptosis and invasion. **(A)** Hoechst staining of SW480 cells. **(B)** Hoechst staining of CIK+SW480 cells. **(C)** Apoptotic rate of CIK+SW480 cells and SW480 cells. **(D)** Invasion of CIK+SW480 cells and SW480 cells. ** $p < 0.01$ compared with SW480 cells.

than that of SW480 culture medium ($p < 0.05$, Figure 2A and 2B). On the contrary, TGF- β and VEGF content was decreased in co-culture medium ($p < 0.05$, Figure 2C and 2D).

Effect of CIKs on apoptosis of SW480 cells

To explore the effect of CIKs on regulating apoptosis of colorectal cancer, Hoechst staining was performed. The data showed that uniform fluorescence was seen in SW480 cells without obvious apoptosis (Figure 3A). However, dense and massive fluorescence could be seen in the nucleus or cytoplasm in co-cultured CIK+SW480 cells (Figure 3B). The apoptotic rate of CIK+SW480 cells was also remarkably elevated than that of SW480 cells ($p < 0.05$, Figure 3C).

Effect of CIKs on invasion of SW480 cells

Transwell assay elucidated that after cell culture for 48 hrs, the amount of invasive cells in CIK+SW480 cells was remarkably smaller than that of SW480 cells, indicating that CIKs inhibited cell invasion of colorectal cancer ($p < 0.05$, Figure 3D).

CIKs regulating colorectal cancer via AMPK/Akt/mTOR pathway

In order to investigate the underlying mechanism of CIKs in regulating colorectal cancer, protein expressions of some key factors were detected by Western blot. The data elucidated that p-AMPK and p-Akt were upregulated (Figure 4A-4D), whereas FoxM1 and p-mTOR were downregulated in co-

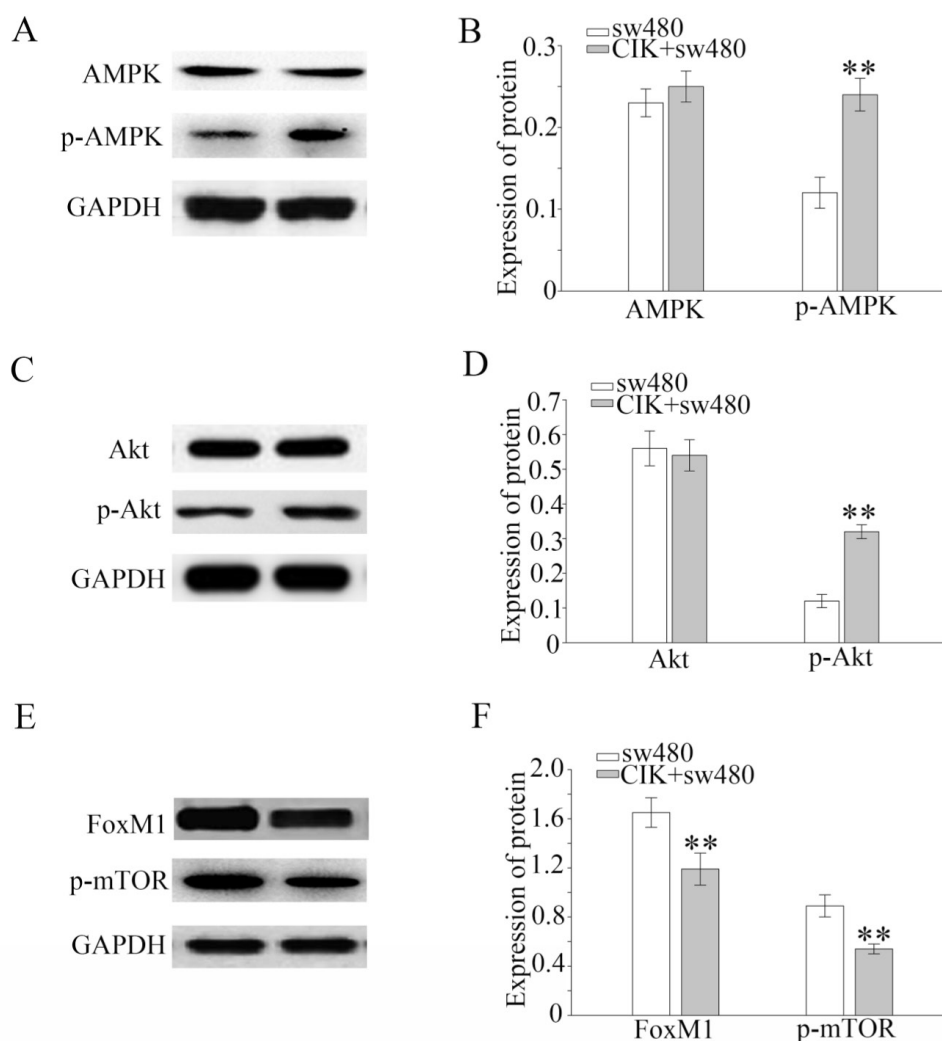


Figure 4. CIKs regulated colorectal cancer via AMPK/Akt/mTOR pathway. **(A)** Protein expressions of AMPK and p-AMPK in CIK+SW480 cells and SW480 cells. **(B)** Comparison of protein expressions of AMPK and p-AMPK in CIK+SW480 cells and SW480 cells. **(C)** Protein expressions of Akt and p-Akt in CIK+SW480 cells and SW480 cells. **(D)** Comparison of protein expressions of Akt and p-Akt in CIK+SW480 cells and SW480 cells. **(E)** Protein expressions of FoxM1 and mTOR in CIK+SW480 cells and SW480 cells. **(F)** Comparison of protein expressions of FoxM1 and mTOR in CIK+SW480 cells and SW480 cells. ** $p < 0.01$ compared with SW480 cells.

cultured CIK+SW480 cells compared with those of SW480 cells (Figure 4E and 4F).

Discussion

Colorectal cancer is a frequent disease, ranking fourth among all malignant tumors. There were approximately 1.4 million new cases of colorectal cancer worldwide in 2012 and is expected that the number of new cases will exceed 2.4 million by 2035 [23]. Studies have shown that males are more frequently affected by colorectal cancer than females [24]. Although researches on colorectal cancer have been conducted for a long time, the survival rate of colorectal cancer is still not satisfactory due to primary treatment failure and tumor recurrence. The 5-year survival rate of colorectal cancer is only about 50%.

AMPK/mTOR pathway is a well-studied pathway. AMPK, as an intracellular energy receptor, can sense intracellular AMP/ATP levels. Decreased intracellular ATP level stimulates AMPK phosphorylation, which in turn leads to mTOR phosphorylation. Subsequently, phosphorylation of s6rp induced by mTOR activation promotes cell proliferation, migration and invasion [26]. Studies have shown that the function of AMPK/mTOR pathway varies a lot in different cells. In papillary thyroid cancer, TMP21 regulates cell growth through inducing cell autophagy via AMPK/mTOR pathway [27]. In non-small cell lung cancer, Yuanhuacine (YC) upregulates AMPK phosphorylation and inhibits mTOR phosphorylation, therefore inhibiting the proliferation, migration and invasion of tumor cells [28]. Kim et al. found that DHA can induce necrosis of non-small cell lung cancer cells by activating AMPK/mTOR pathway [29]. The above results indicated the significant role of AMPK/mTOR pathway in different tumors. Relative studies demonstrated that AMPK/mTOR pathway affects proliferation, migration, invasion, apoptosis, and autophagy in colorectal cancer [30,31].

FoxM1 is a member of the Fox transcription factor family, which participates in the regulation

of cell proliferation, differentiation and organ development [31,32]. A large number of studies have shown that FoxM1 is overexpressed in solid tumors, including pancreatic cancer, breast cancer, prostate cancer, glioma, liver cancer, and gastric cancer, suggesting its potential effect on tumor promotion [33,34]. As a transcription factor, FoxM1 is involved in tumor cell proliferation, invasion, metastasis, EMT (epithelial mesenchymal transition), and angiogenesis. Studies have shown that Akt can regulate FoxM1 expression through Akt/FOXO3a/FoxM1 pathway [35].

CIKs are capable of killing tumor cells through a variety of anti-tumor mechanisms. The biological effects of CIKs on tumor cells and the underlying mechanisms are the focus of our research. In the present study, we found that co-cultured CIKs and SW480 cells remarkably inhibited the proliferation and invasion of SW480 cells. Apoptosis of SW480 cells was inhibited by CIKs. Besides, contents of IL-2 and IL-10 in co-culture medium were elevated, while contents of IGF- β and VEGF were decreased, indicating the inhibitory effect of CIKs on colorectal cancer. Furthermore, Western blot results demonstrated that p-AMPK and p-Akt were upregulated, whereas FoxM1 and p-mTOR were downregulated in co-cultured CIK+SW480 cells compared with those of SW480 cells, suggesting the crucial role of AMPK/Akt/mTOR pathway in the proliferation and invasion of colorectal cancer. Our results provide a theoretical basis for improving clinical outcomes of colorectal cancer patients.

Conclusions

CIKs inhibit the proliferation and invasion of colorectal cancer cells through downregulating FoxM1 and mTOR via AMPK/Akt/mTOR pathway.

Conflict of interests

The authors declare no conflict of interests.

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