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Original Article

Retinoic acid aliphatic amide inhibits the AMPK-HIF-1α pathway in human ovarian cancer

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Abstract: Ovarian carcinoma the commonly observed gynecological cancers has a high mortality rate. In the present study effect of retinoic acid aliphatic amide (RACA) in ovarian cancer cells was investigated using proliferation, migration and invasion assays. Western blot was used to examine the Bcl-2, cleaved caspase 3, p-ERK, MMP-2, p-FAK, P-P38, p-AMPKα and HIF-1α protein expression. $CoCl_2$ was used to induce HIF-1α expression in SKOV3ip. 1 and HEY-A8 cells. The results revealed that RACA treatment prompted cell proliferation, invasion and migration but inhibited apoptosis of SKOV3ip. 1 and HEY-A8 cells. RACA treatment also induced upregulation of Bcl-2 and MMP-2, activation of p-P38, p-ERK and p-FAK, inhibition of cleaved caspase 3. RACA treatment also caused upregulatation of HIF-1α in ovarian cells with the activation of p-AMPKα. Upregulation of HIF-1α expression in $CoCl_2$-treated cancer cells resulted in decrease in SDHB. Thus RACA plays a key role in cell proliferation, invasion, migration and apoptosis of human ovarian carcinoma through AMPK-HIF-1α pathway.

Keywords: Tumor progression, HIF-1α, AMPK, gene silence, ovarian carcinoma, apoptosis

Introduction

Ovarian carcinoma, the most leading cause of deaths among gynecological malignancies has an overall 5-year survival rate of around 40% [1, 2]. It is usually detected in the advanced stages [3]. The current treatment strategies include surgical resection in addition to platinum-based chemotherapy. The use of platinum-based drugs and surgical intervention has been shown to induce very adverse side effects which hinder the application of this treatment strategy [3, 4].

In hypoxia-response pathway, hypoxia-inducible factor-1 (HIF-1) acts as a well known mediator and comprises of α- and β-subunit [5]. During hypoxic conditions, HIF-1α on accumulation induces activation of target genes involved in angiogenesis, energy metabolism, adaptive survival or apoptosis [6, 7]. In ovarian cancer HIF-1α is highly expressed and plays a vital role in proliferation [8], invasion and metastasis [9, 10] of tumours. The cellular energy homeostasis and metabolic stresses like hypoxia and respiratory impairment are maintained by AMP-activated protein kinase (AMPK). Tumorigenesis is regulated by AMPK [11]. In tumours with reduced AMPK signaling HIF-1α induces the growth of tumours [12].

Retinoic acid (RA) has been used in the prevention and treatment of dermatological diseases from long back [13, 14]. For the treatment of cancer, retinoic acid and other retinoids have proved to be promising candidates [15]. It is reported that retinoic acids affect in vitro proliferation, differentiation and apoptosis of normal as well as abnormal cells of several cancers. Retinoic acid usually affects colon cancer [16], prostate cancer [17], lung cancer [18] and leukemia [19]. All *trans*-retinoic acid (ATRA) and 9-*cis* RA also influence the morphological differentiation, proliferation and gene expression of neuroblastoma [20] and astrocytoma cells [21]. ATRA [22, 23] and 13-*cis* RA [24] have

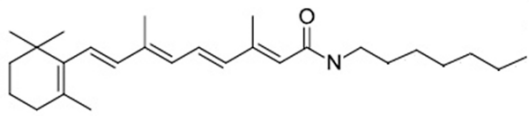

Figure 1. Structure of retinoic acid aliphatic amide (RACA).

been used to treat recurrent malignant cerebral gliomas. In addition retinoids also have anti-proliferation, anti-migration and anti-invasive activity against human malignant gliomas [25, 26]. This suggests that retinoids are suitable anticancer agents for inhibition of tumour progression. In the present study effect of retinoic acid aliphatic amide (RACA, **Figure 1**) in the ovarian cancer cells was investigated. The results demonstrate that RACA treatment results in increased tumour cell proliferation, invasion, migration and decreased apoptosis. Furthermore, HIF-1α and p-AMPKα were found to be upregulated in RACA treated cells. Also, SDHB was downregulated by hypoxia mimetic $CoCl_2$ in human ovarian cancer cells.

Materials and methods

Chemicals and reagents

Retinoic acid aliphatic amide (RACA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 μM as a stock solution and stored at -20°C. Rabbit antihuman Caspase-3, mouse antihuman Bcl-2, and β-actin were purchased from Cell Signaling (China).

Cell lines and cell culture

SKOV3ip. 1 and HEY-A8 human ovarian adenocarcinoma cancer cell lines were obtained from the Cell Bank Chinese Academy of Science (Shanghai, China). The cells were cultured in RPMI1640 medium (Hyclone) supplemented with 10% foetal bovine serum (Gibco). These cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

RNA extraction and quantitative real-time reverse transcriptase PCR

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used for the RNA extraction from cancer cell lines and reverse transcribed using Prime Script RT reagent Kit (TaKaRa, Shanghai, China). The cDNA was subjected to quantitative real-time reverse transcriptase PCR using a SYBR Green PCR Master Mix Reagent Kit (TaKaRa). ABI 7500 real-time system (Applied Biosystems) was used for real-time PCR and data collection. The primers for *β-actin*, were *5'-TGA CGT GGA CAT CCG CAA AG-3'* (forward) and *5'-CTG GAA GGT GGA CAG CGA GG-3'* (reverse).

ATP assay

The phosphomolybdic acid colorimetric method (Nanjing Jiancheng Bioengineering Institute, China) was employed for the determination of intracellular ATP concentrations. The analysis was performed as per the manufacturer's protocols and BCA kit (Cwbiotech, China) was used for quantification of protein level.

Cell proliferation analysis

Cell counting kit-8 (CCK-8) (Dojindo) was employed for the analysis of SKOV3ip. 1 and HEY-A8 cell proliferation. The cells were seeded at a density of 2.5×10^5 into 96-well plates in the presence or absence of RACA. The mixture of 10 μl CCK8 and 90 μl RPMI1640 was added to each well for 2 h after 1, 2, 3, 4, 5 or 6 day treatment. For each well, absorbance was measured at 450 nm.

Cell invasion and migration assay

After 48 h of RACA treatment, SKOV3ip. 1 and HEY-A8 (6×10^4) cells were placed into the BD matrigel coated upper compartment of the chambers (8 μm pore size, Millipore). The cells were then placed into 24-well plates containing medium with 10% foetal bovine serum. The cells on the upper side of the membrane were cleaned with cotton after incubation for 24 h. The adhering cells on the lower surface after fixing in 4% paraformaldehyde were stained with 0.1% crystal violet. The microscope (Nikon TE300, Tokyo, Japan) was used to count the cells in randomly selected fields on each membrane. All the experiments were performed three times. Similar procedures were used for cell migration in absence of matrigel coating.

Western blot assay

In six well plates 1×10^5 cells were platted per plate for 12 h and then treated with RACA for

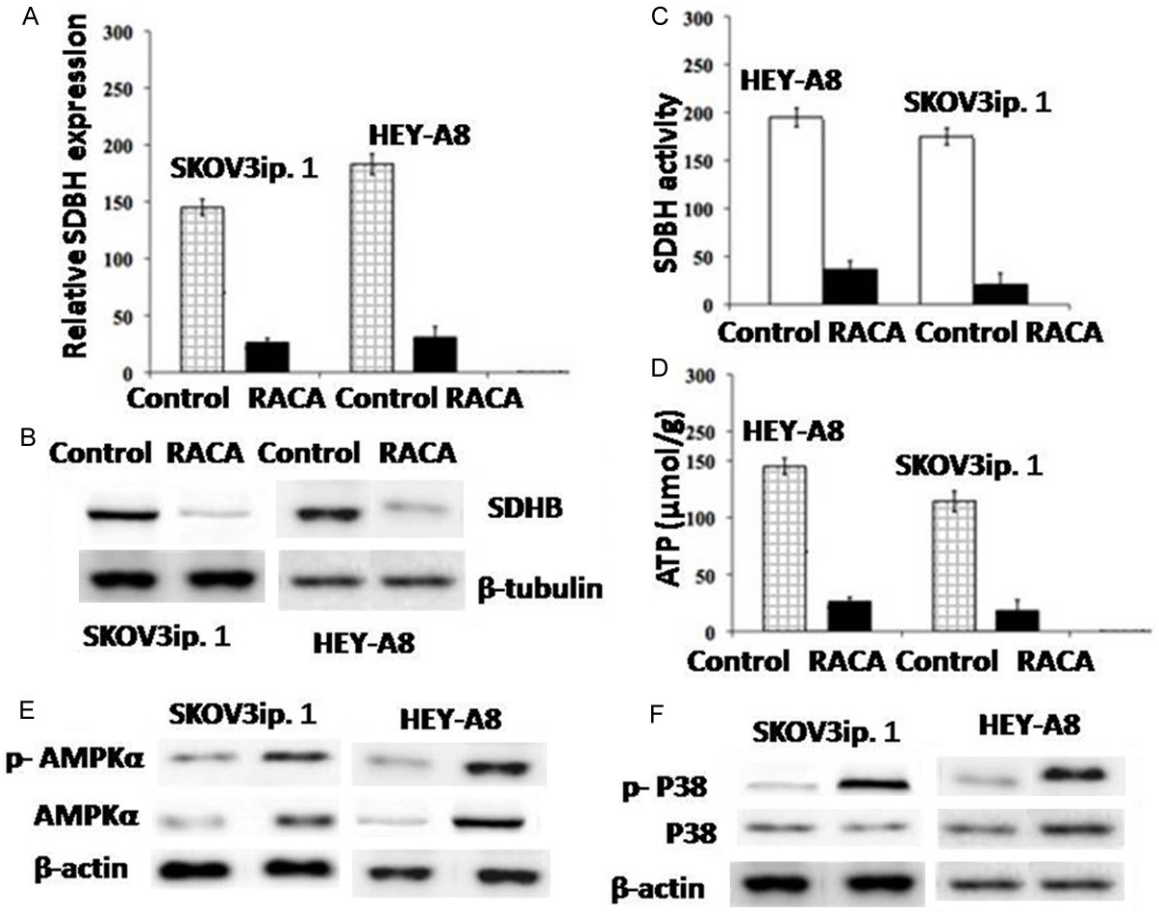

Figure 2. Effect of RACA in SKOV3ip. 1 and HEY-A8 cells. The cells were treated with RACA (A) real-time PCR (24 h) and (B) Western blot (48 h) was performed to detect *SDHB* mRNA and protein levels, respectively. 48 h after RACA treatment, SDH activity (C) and ATP level (D) were examined, p-AMPKα, AMPKα (E) and p-P38, P38 (F) were measured in SKOV3ip. 1 and HEY-A8 cells.

48. The untreated cells were used as control. After 48 h, cells were lysed in lysis buffer (50 mMTris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40, and 5 mM cocktail) and the lysate was centrifuged to remove cell debris. For determination of protein concentration protein Assay System (Bio-Rad, Hercules, CA, USA) was used. The protein were loaded and resolved by electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. The proteins were transferred onto a PVDF membrane using semi-dry method which was then blocked with 5% non-fat dry milk overnight. Incubation of membranes with primary antibodies against β-tubulin (1:4000, Epitomics), β-actin (1:500, Abmart), caspase3 (1:1000, Cell Signalling Technology), Bcl-2 (1:4000, Epitomics), MMP-2 (1:500, Abcam), FAK (1:1000, CST), p-FAK (1:1000, CST), AMPKα (1:1000, CST), p-AMPKα (1:1000, CST), GAPDH (1:4000, Abmart), P38 (1:1000, CST), p-P38 (1:1000, CST), ERK (1:1000, CST), p-ERK (1:1000, CST), HIF-1α (1:1000, Epitomics) was performed overnight at 4°C. The membranes after washing with TBST were incubated with IR Dye 800 CW conjugated goat (polyclonal) anti-Rabbit IgG or anti-Mouse IgG (1:10000) antibodies for 1 h. Odyssey system was used to detect the expression of specific proteins as per the manufacturer's protocol.

Statistical analysis

The data presented are the means ± standard error (SEM). The Student's *t* test was used to compare the variables between groups using Graph Pad Prism 5.0 software (San Diego, CA). The differences were considered statistically significant at $P < 0.05$.

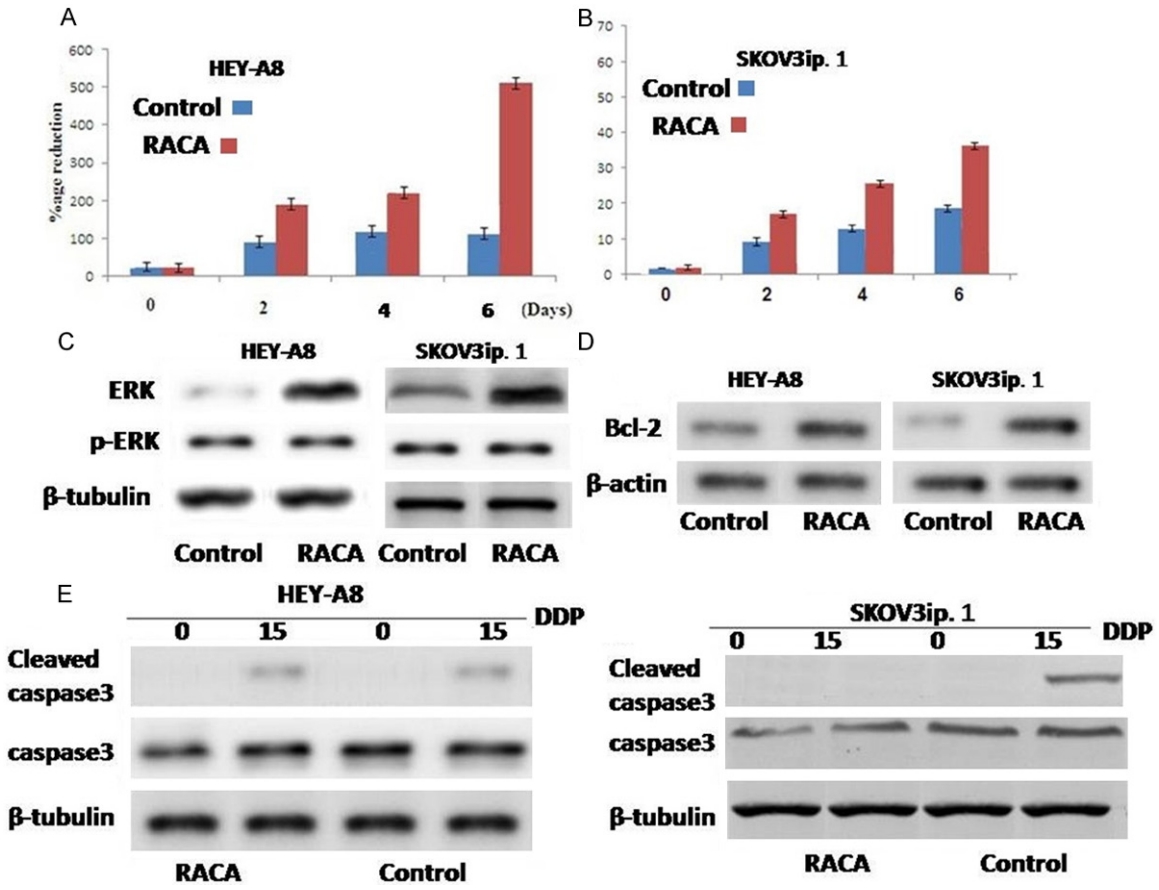

Figure 3. RACA treatment contributed to ovarian cancer cell proliferation in SKOV3ip. 1 and HEY-A8 cells. A, B. Cell proliferation was assessed following RACA treatment for 6 days by CCK8. C. After 48 h of RACA treatment p-ERK and ERK were examined by western blot. D, E. After 48 h of RACA treatment SKOV3ip. 1 and HEY-A8 cells were treated with DDP at indicated concentration for 24 h, cleaved caspase 3, total caspase 3, and Bcl-2 were then analysed by western blot.

Results

The effect of RACA on ATP and AMPK/P38 MAPK in human ovarian cancer cells

The effect of RACA on ATP and AMPK/P38 MAPK was examined using 20 μM RACA following 24 h treatment. RACA treatment leads to a significant decrease in SDHB mRNA level by 92.23% and 94.34% in SKOV3ip. 1 and HEY-A8 cells ($P < 0.001$), respectively (**Figure 2A**). Furthermore, SDHB protein level and activity were also decreased after 48 h RACA treatment (**Figure 2B**, **2C**). ATP was also markedly decreased in RACA treated cells (**Figure 2D**). It is reported that AMPK acts as an energy sensor to modulate metabolic stresses like hypoxia and respiratory impairment [27, 28]. The stimuli that increase AMP or decrease ATP induce activation of AMPK [29]. For investigation of the effect of RACA treatment on AMPK activation, the level of phosphorylated AMPKα (p-AMPKα) was analysed. The results revealed a significant increase in P-AMPKα in RACA treated cells compared to control group (**Figure 2E**). During metabolic stress AMPK/P38 MAPK signalling cascade plays a vital role to stimulate uptake of glucose [30, 31]. The effect of decreased AMPK activity in RACA treated cells on phosphorylated p38 MAPK (p-P38 MAPK) level was also examined. A marked increase in P-P38 MAPK was observed in the RACA treated cells compared to control cells (**Figure 2F**).

RACA treatment inhibited ovarian cancer cell proliferation

To examine the effect of RACA on cell proliferation CCK8 was used. The results revealed a significant decrease in ovarian cancer cell prolif-

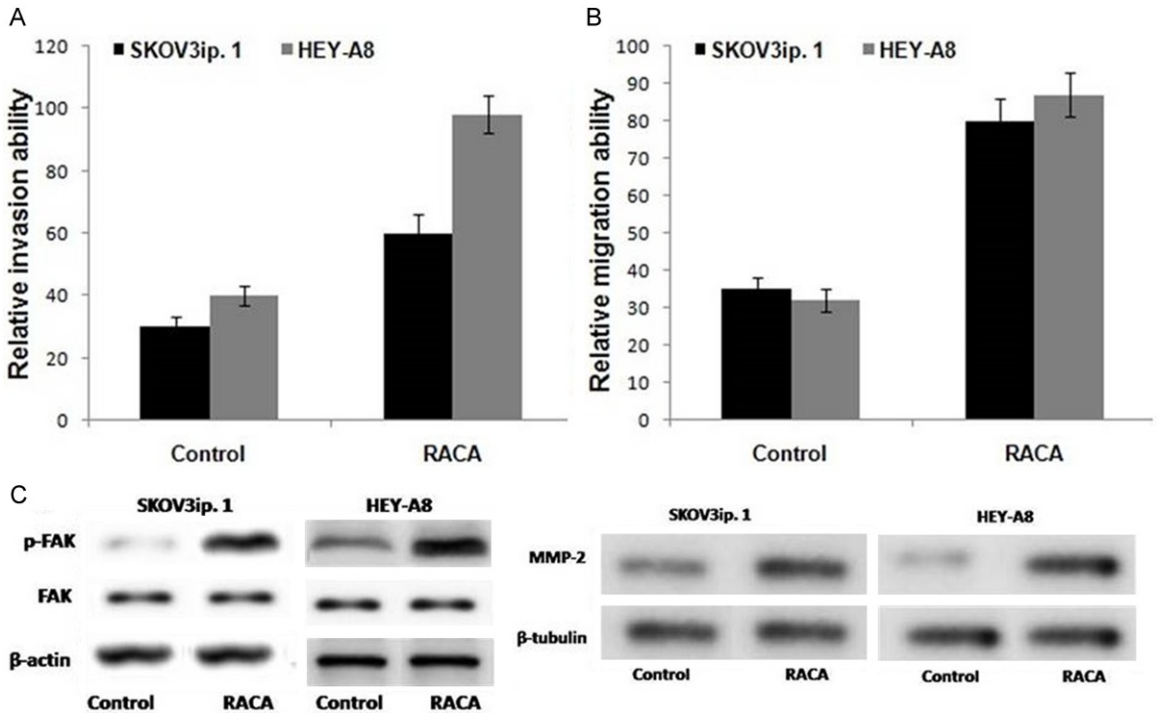

Figure 4. RACA treatment promoted ovarian cancer cell invasion and migration. The transwell assay and the Boyden Chamber test were used to evaluate cell invasion and migration after SKOV3ip. 1 and HEY-A8 cells were treated with RACA for 48 h. The number of invaded and migrated cells was counted using a bright-field microscope (200 ×). Representative images and the relative cell invasion and migration rate were shown in (A) and (B). (C) 48 h after RACA treatment, MMP-2 and p-FAK in RACA treated SKOV3ip. 1 and HEY-A8 cells were analysed by western blot.

eration on RACA treatment ($P < 0.05$) (**Figure 3A**, **3B**). To control cell proliferation extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein (MAP) kinase pathway plays a vital role [32]. It is well known that ERK1/2 MAP kinase pathway controls cell proliferation [32, 33]. ERK1 and ERK2 are activated in response to virtually all mitogenic factors [32]. ERK signalling is often up regulated in a diverse range of human cancers [34]. It was discovered that the level of p-ERK was increased in the RACA treated cells compared to control (**Figure 3C**), indicating that RACA treatment promoted tumour cell growth through ERK pathway in SKOV3ip. 1 and HEY-A8 cells.

RACA treatment prevented apoptosis in human ovarian cancer cells

It is reported that cisplatinum (DDP, Sigma) is used for the treatment of ovarian cancer. It forms a platinum complex which binds to DNA and cross-links DNA resulting in cell apoptosis, or systematic cell death. In the cells treated with DDP cleaved caspase-3 was detected after 24 h, however its expression was decreased in RACA treated cells (**Figure 3D**). On the other hand the expression of Bcl-2 was significantly enhanced in RACA treated cells (**Figure 3E**). These results clearly indicate that RACA treatment prevents ovarian cancer cell apoptosis.

RACA treatment prompted cell invasion and migration of SKOV3ip. 1 and HEY-A8 Cells

Results from matrigel cellular invasion assay revealed that RACA treatment prompted SKOV3ip. 1 and HEY-A8 cell ($P < 0.01$) invasiveness tendency (**Figure 4A**). However, RACA treatment upregulated migration ability of SKOV3ip. 1 ($P < 0.001$) and HEY-A8 cells ($P < 0.001$) (**Figure 4B**). The focal adhesion kinase (p-FAK), a nonreceptor protein tyrosine kinase on activation plays a crucial role in migration, proliferation, survival and metastasis of the cells. The level of p-FAK overexpression is correlated with enhanced metastasis and reduction in human ovarian cancer survival. MMP-2 contributes to tumour invasion and metastasis by degradation of the extracellular matrix (ECM) proteins. We observed a marked up-regulation

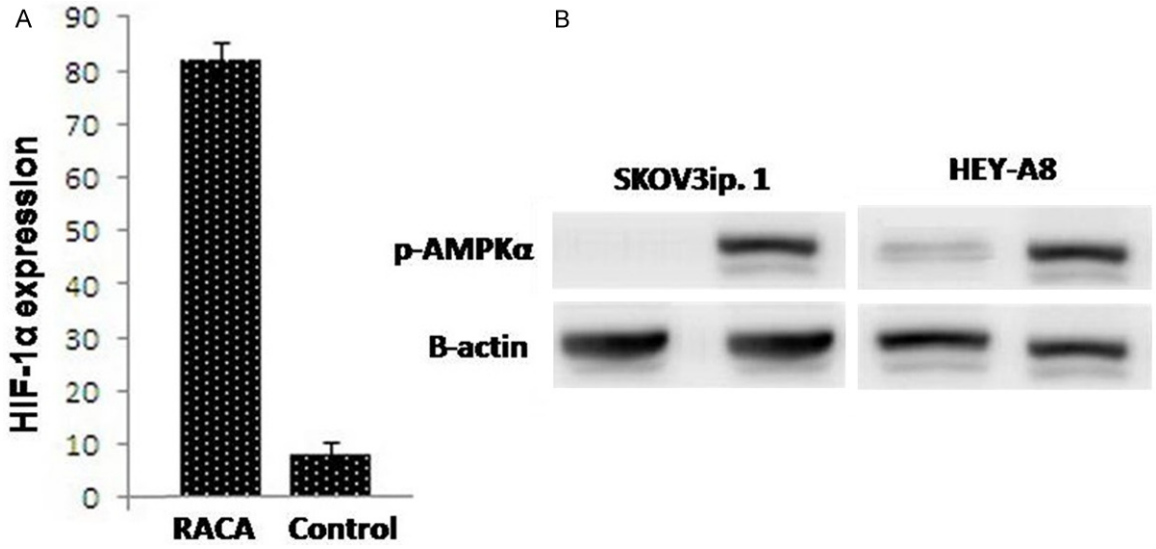

Figure 5. Effect of RACA on HIF-1α expression in ovarian cancer cells. SKOV3ip. 1 and/ or HEY-A8 cells were treated with RACA (A) for 48 h, HIF-1α expression was analyzed by western blot with β-actin or β-tubulin as a loading control. (B) SKOV3ip. 1 and HEY-A8 cells were treated with $CoCl_2$ at indicated concentrations for 24 h, HIF-1α and SDHB were examined by Western blot.

of p-FAK and MMP-2 expression in RACA treated cells (**Figure 4C**).

RACA affected HIF-1α level in ovarian cancer cells

Inhibition of HIF-1α protein synthesis and suppression in its trans-activation activity via AMPK signalling by mitochondrial dysfunction in human hepatoma HepG2 cells is well known. We observed upregulation of HIF-1α in RACA treated SKOV3ip. 1 and HEY-A8 cells (**Figure 5A**). The effect of RACA treatment on energy metabolism in SKOV3ip. 1 cell was examined by monitoring the level of p-AMPKα. Results showed a significant increase in the level of p-AMPKα in RACA treated cells compared to the control (**Figure 5B**). These findings suggest the involvement of AMPK-HIF-1α signalling pathway in RACA induced change in ovarian cancer cell phenotype.

Discussion

The study was designed to investigate the role of RACA in ovarian carcinoma. We found that the ATP level was decreased in RACA treated cancer cells. On the other hand, p-AMPKα and p-P38 MAPK level were increased in the RACA treated cells, suggesting that RACA treatment could activate AMPK pathway in ovarian cancer.

In the present study, RACA treatment increased proliferation in SKOV3ip. 1 and HEY-A8 cells, accompanied with elevated p-ERK level. Bcl-2 is functioned to oppose the apoptosis pathway of programmed cell death [35, 36]. Effector caspases are responsible for initiating the events that lead to the hallmarks of apoptosis. Caspase-9, an essential initiator caspase required for apoptosis through mitochondrial pathway, is activated on the apoptosome complex, which directly resulted in cleave and activate effector caspases, such as caspase-3 [37, 38]. Antiapoptotic Bcl-2 level was increased and cleaved caspase 3 was decreased after treated with or without DDP in RACA treated SKOV3ip. 1 and HEY-A8 cells. FAK and MMPs are associated with metastases [39], secretion and activation of MMP-2 may be responsible for increased motility, invasiveness and metastasis of malignant cells [40]. In addition, increased FAK expression and activity frequently correlate with metastatic disease and poor prognosis [41]. We found that RACA treatment promoted ovarian cancer invasion and migration accompanied with up-regulated expression of MMP-2 and p-FAK.

SDHB is one of the subunits of SDH which takes part in TCA cycle and respiratory chain, AMPK could modulate metabolic stresses such as hypoxia. Study showed mitochondrial dysfunctions could result in reduced HIF-1α protein

synthesis through AMPK-dependent manner in HepG2 cells. Hypoxia is a characteristic of many malignancies arising from various sites [42] and HIF-1α is a hypoxia responsive factor. The expression of HIF-1α was strongly elevated in RACA treated ovarian cancer cells. Moreover, SDHB was decreased in $CoCl_2$-treated cancer cells accompanied by HIF-1α upregulation. HIF-1α also promotes cell migration by regulating MMP-2 [43] and FAK [44], which is in line with our current data. These results showed SDHB might affect cancer cell proliferation, invasion, migration, and apoptosis *via* AMPK-HIF-1α in ovarian carcinoma.

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Disclosure of conflict of interest

None.

Abbreviations

MMP-2, Matrix metalloproteinase-2; FAK, Focal adhesion kinase; Bcl-2, B-cell lymphoma-2; $CoCl_2$, Cobalt chloride; DDP, Cis-platinum; RIPA, Radio immuno-precipitation assay; PMSF, Phenylmethanesulfonyl fluoride; TBST, Tris-buffered saline with Tween-20; NC, Nitrocellulose membrane; PVDF, Polyvinylidene Fluoride.

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