Int J Clin Exp Pathol 2020;13(10):2495-2501
www.ijcep.com /ISSN:1936-2625/IJCEP0114890

Original Article

Effect of the SIRT3-AMPK/PPAR pathway on invasion and migration of cervical cancer cells

Jin-Bao Xiao[1*], Jun-Qi Ma[1*], Kailiman Yakefu[1], Mikrban Tursun[2]

[1]Department of Gynecology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, China; [2]Department of Pathology of Medical University of Xinjiang, Urumqi, China. [*]Equal contributors.

Received May 24, 2020; Accepted June 29, 2020; Epub October 1, 2020; Published October 15, 2020

Abstract: SIRT3 is a mitochondrial deacetylation protein that can promote the invasion and migration of cancer cells. We explored the effects of SIRT3 regulation of the AMPK/PPAR signaling pathway on triglycerides and the invasion and metastasis of cervical cancer cells. Immunohistochemical methods were used to detect SIRT3. The expression of AMPK and PPAR proteins in different cervical lesions was analyzed in combination with clinicopathological parameters. qRT-PCR and western blotting were used to determine the expression levels of SIRT3 in the C33a and SiHa cervical cancer cell lines. To observe the effects of altering SIRT3 levels by lentivirus transfection and the consequent changes in AMPK and PPAR protein expression, oil red O staining was used to determine intracellular triglycerides, and scratch assays and Transwell chamber experiments were performed to evaluate cervical cancer cell migration and invasion. Our data indicate that SIRT3, AMPK, and PPAR protein expression levels show an increasing trend with cervical lesion severity and are related to the degree of lymph node metastasis and differentiation; moreover, increased expression of SIRT3 can promote the expression of AMPK and PPAR proteins, is beneficial to the formation of intracellular neutral fat, and enhances the ability of cells to metastasize and invade. Our results suggest that SIRT3 activates AMPK/PPAR signaling pathways involved in cancer lipid metabolism and promotes metastasis and cell invasion.

Keywords: SIRT3, AMPK, PPAR, cervical cancer, lipid metabolism

Introduction

Cervical cancer is one of the most common malignant tumors among women. Globally, cervical cancer occurs mainly in developing and underdeveloped countries, with a mortality rate of 85% [1]. Although the mortality and morbidity of cervical cancer has decreased significantly in recent decades, fewer than 40% of patients have a survival time of more than five years due to the early occurrence of cervical cancer metastasis [2]. In recent years, research has suggested that the reprogramming of tumor cell energy metabolism provides a powerful basis for the unlimited proliferation and metastatic progression of cancer cells.

Silent information regulator 2-related enzyme 3 (SIRT3) is the most important deacetylase in mitochondria. It is involved in the regulation of the mitochondrial tricarboxylic acid cycle, energy conversion, and oxidative stress and is an important "energy regulator" in the body [3]. Since many researchers generally accept that cancer is a metabolic disease and that mitochondria are at the center of energy metabolism, SIRT3 must be related to tumor metabolism. Current studies have confirmed that cell metabolism, including sugar, fatty acid, and amino acid metabolism, is altered in cancer cells [4-6]. Metabolic abnormalities often affect the ability of tumors to increase in size, invade, and metastasize. At the same time, AMPK and its signaling network also participate in the normal energy metabolism of the body, counteract stress responses, and promote survival [7]. In liver cancer cells, regulating the AMPK/peroxisome proliferator-activated receptor (PPAR) pathway can correct abnormal glucose metabolism [8], and inhibition of AMPK/PPAR pathway activity can reduce the impact of abnormal lipid metabolism [9]. At the same time, increased SIRT3 can cause oxidative stress and

promote the proliferation of ovarian cancer cells [10]. It was concluded that activation of the protein kinase AMPK/PPAR pathway can promote the reprogramming of multiple metabolic modes. This study explores whether SIRT3 regulates the AMPK/PPAR pathway and whether it affects the triglyceride content of cervical cancer cells, to provide a theoretical basis for the treatment of cervical cancer.

Materials and methods

Patients

The cervical cancers of 99 patients who had not previously received a clinical gynecological diagnosis were pathologically confirmed according to the International Federation of Obstetricians and Gynecologists (FIGO) and staged. These patients were seen between 2014 and 2018 at the Department of Obstetrics and Gynecology of the First Affiliated Hospital of Xinjiang Medical University and retrospectively included in this study. The patient group comprised 44 cases of cervical cancer, 36 cases of cervical intraepithelial neoplasia (CIN), and 19 cases of cervicitis. Informed consent was obtained from patients and control subjects. This study was approved by our ethics committee.

Immunohistochemistry

An environmentally friendly dewaxing solution was used for tissue dewaxing, and the procedure was carried out according to the instructions provided in the Zhongshan Jinqiao SP immunohistochemistry (IHC) kit. DAB was used as the chromogen. The results were obtained in a double-blind manner, and the score was determined according to previous literature reports.

Cell lines and cell culture

The cervical cancer cell lines used were C33a (HPV negative) and SiHa (HPV16 positive). The two cell lines were purchased from Wuhan Punosai Life Technology Co., Ltd., and both had SRP inspection qualification reports. Cells were cultured in complete medium according to a DMEM:fetal bovine serum (FBS):PS ratio of 9:1:0.1. The cell culture reagents were stored at 4°C for future use, and cells were passaged at a ratio of 1:3 when the cells reached a density of 85%.

Reagents

MEM, the double antibody (PS), and FBS were purchased from HyClone Corporation in the USA. shSIRT3 lentivirus and SIRT3 overexpression lentivirus were designed and constructed by Shanghai Jikai Gene Corporation. Other reagents were obtained as follows: reversed Recording kit (Thermo, USA); Quantitative fluorescence kit (Takara company in Japan); and BCA protein quantification kit (Thermo, USA). The Sirt3 rabbit anti-human antibody, AMPK rabbit anti-human antibody and PPAR rabbit anti-human antibody were purchased from Abcam Company. GADPH rabbit anti-human antibody was purchased from Wuhan Proteintech Company, and Transwells and Matrigel were purchased from Corning in the USA.

Lentiviral transfection

A total of 1×10^4 cells were seeded into 6-well plates, and a MOI = 30 was selected. The corresponding reagents were added according to the instructions provided by Jikai Gene Corporation, and transfection was performed for 16~18 h. The transfection supernatant was discarded, and the expression of fluorescence was observed under a fluorescence inverted microscope 72 h later.

Quantitative RT-PCR

Total RNA from cervical cancer cell lines was extracted using TRIzol reagent. A cell pellet containing 5×10^5 cells was lysed on ice with 1 ml TRIzol. The lysate was centrifuged, and chloroform was added. The supernatant was removed, and an equal volume of isopropanol was added to the pellet, which was then dissolved and centrifuged. To obtain the RNA, an appropriate amount of DEPC water was added to dissolve the resulting mixture, and the concentration and purity of the RNA was checked. According to the instructions of the reverse transcription kit and fluorescent quantitative kit, the collected RNA was reverse transcribed and then subjected to relative quantification according to the following method: $2^{-\Delta\Delta CT}$.

Western blot

A cell pellet containing 1×10^6 cells was collected, and 50~100 μL of different cell lysis working solutions were added to the pellet (cell

Table 1. Correlation analysis between SIRT3, AMPK, and PPAR protein expression levels

SIRT3	AMPK		r value	*p*-value	PPAR		r value	*p*-value
	+	-			+	-		
+	6	62	0.016	$P < 0.001$	5	59	0.015	$P < 0.001$
-	10	2			8	8		

lysate:PMSF = 100:1). The mixture was placed on ice and subjected to protein quantitation with the BCA protein quantification kit to determine the protein concentration. After denaturation of proteins, the lysate was subjected to SDS-PAGE, and wet transfer at constant pressure (100 V, 1.5 h~2 h) was performed. The membrane was blocked with 5% skim milk for 2 h, incubated with the indicated primary antibody at 4°C overnight, incubated with the appropriate secondary antibody the next day for 2 h, and developed using the AP method.

Oil red O staining

A total of 1×10^4 cells/well were seeded into a 6-well plate, fixed with 4% paraformaldehyde the next day, and stained with oil red O dye. Five fields of view were examined under a microscope to evaluate the intracellular triglyceride content.

Results

SIRT3, AMPK, and PPAR protein expression in cervical cancer lesions

To determine whether the SIRT3, AMPK, and PPAR proteins are dysregulated in cervical cancer lesions, we examined 99 samples by IHC (**Table 2**). The results showed that AMPK and PPAR protein expression was localized to the cytoplasm and that their expression levels varied with the degree of the cervical lesion, showing an increasing trend ($P < 0.001$) in which the expression of AMPK protein became mainly localized to the nucleus; the expression of PPAR was mainly localized to the cytoplasm (**Figure 1**). Combined with the analysis of clinicopathologic parameters, it was found that the expression of AMPK and PPAR was related to lymph node metastasis ($P < 0.001$) and that AMPK protein expression was also related to the degree of differentiation ($P < 0.001$) (**Table 1**). SIRT3 expression was positively correlated with the positive expression of AMPK and PPAR proteins (r = 0.016, r = 0.015) ($P < 0.001$, $P < 0.001$).

mRNA and protein expression of SIRT3, AMPK, and PPAR in two cervical cancer cell lines

The qRT-PCR analysis revealed that the mRNA expression levels of SIRT3, AMPK, and PPAR were higher in C33a cells than in SiHa cells ($P < 0.05$) (**Figure 2**; **Table 3**); furthermore, western blot analysis showed that the protein levels of SIRT3 were consistent with its mRNA levels ($P < 0.05$) (**Figure 2**).

Selection of appropriate cell lines for virus transfection

According to the above results of the two cell lines, the C33a cell line (high background expression of SIRT3) was selected for siSIRT3 lentivirus transfection, and the SiHa cell line (low background SIRT3) was transfected with SIRT3-overexpressing lentivirus. Western blotting analysis was performed to verify SIRT3 protein expression in the two cell lines after transfection. The results showed that transfection of siSIRT3 effectively inhibited SIRT3 protein expression and that SIRT3 overexpression by transfection increased SIRT3 protein expression levels (**Figure 3**).

SIRT3 regulates the AMPK/PPAR signaling pathway

After confirming that SIRT3 expression could be altered effectively, western blotting analysis was performed to detect the expression of the key factor for energy metabolism: AMPK/PPAR. It was found that after SIRT3 expression was inhibited, AMPK/PPAR protein expression decreased ($P < 0.05$), whereas increasing the expression level of SIRT3 increased the expression of AMPK/PPAR ($P < 0.05$) (**Figure 3**).

SIRT3 affects the triglyceride content of cervical cancer cells

In the siSIRT3 group, the intracellular triglyceride content was significantly lower than that of the untransfected and control groups (**Figure 4**).

SIRT3 contributes to the metastasis and invasion of cervical cancer cells

As shown in the figure, in the migration experiment, the number of perforated cells in siSIRT3 group was significantly smaller than that in the

Table 2. Statistical analysis of SIRT3, AMPK, and PPARγ expression and clinicopathologic factors in cervical lesions

Characteristics	N	SIRT3 Positive (%)	*P*	AMPK Positive (%)	*P*	PPARγ Positive (%)	*P*
Chronic cervicitis	19			7 (3.7)	< 0.001	8 (42.1)	0.002
CIN II-III	36			26 (72.2)		27 (75.0)	
CSCC	44			32 (72.7)		32 (72.7)	
Differentiation							
Well	7			5 (71.4)	< 0.001	4 (57.1)	0.114
Low	37			27 (73.0)		28 (75.7)	
L/N metastasis							
Negative	27			16 (59.3)	< 0.001	16 (59.3)	< 0.001
Positive	17			16 (94.1)		16 (94.1)	
FIGO staging							
≤ IB	33			23 (70.0)	0.115	24 (72.7)	0.418
> IIA	11			9 (81.8)		8 (72.7)	
Tumor size							
< 2.5 cm	24			18 (75.0)	0.896	19 (79.2)	0.414
≥ 2.5 cm	20			14 (70.0)		13 (65.0)	

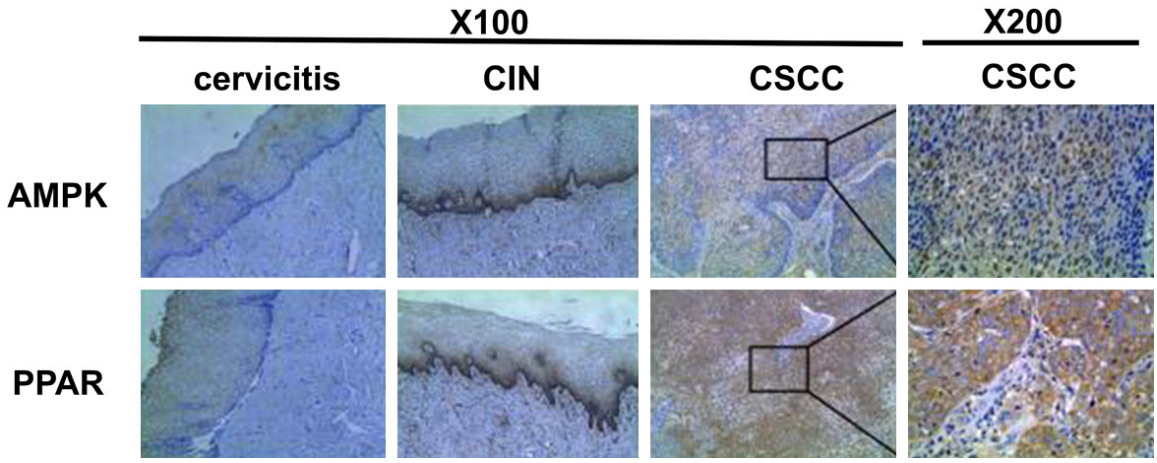

Figure 1. Expression of AMPK and PPAR proteins in different cervical lesions (×100, ×200).

untransfected group and control group (**Figure 5**). Additionally, the Transwell results indicated that fewer cells passed through the Matrigel in the group with alterations to SIRT3 expression than in the untransfected and control groups (**Figure 6**).

Discussion

The biologic characteristics of tumor cells include their rapid proliferation and strong viability, meaning that tumor cells need to reprogram their energy metabolism to adapt to changes in their energy requirements. Therefore, abnormal energy metabolism is a sign of tumorigenesis. The metabolic system of the cell plays a major regulatory role in maintaining tumor cell growth and proliferation. SIRT3, an important mitochondrial deacetylase, has been shown to participate in the metabolism of glucose, fatty acids, amino acids and other substances in a variety of tumor tissues [11]. AMPK/PPAR reduces liver steatosis and dyslipidemia by inhibiting Srebp1, a key factor in energy metabolism [12]; furthermore, the AMPK/PPAR pathway plays a role in inhibiting tumor cell apoptosis in skin cancer [13]. The immunohistochemical results of our study

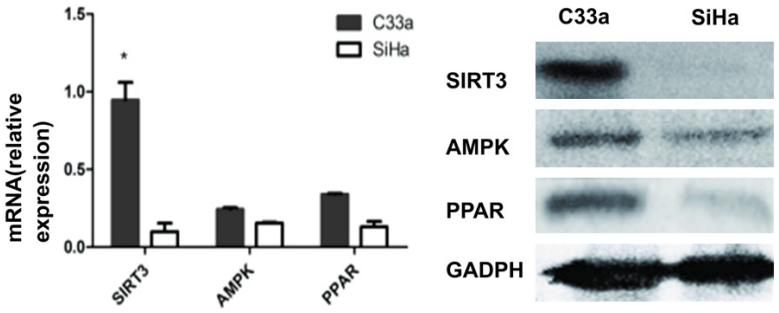

Figure 2. mRNA and protein expression of SIRT3, AMPK, and PPAR.

Table 3. SIRT3, AMPK and PPAR mRNA expression levels

Gene	C33a	SiHa
SIRT3	0.95±0.16	0.1±0.08
AMPK	0.24±0.16	0.15±0.01
PPAR	0.34±0.01	0.13±0.05

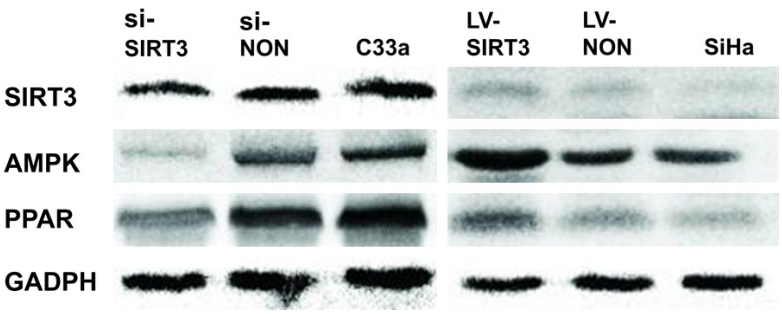

Figure 3. SIRT3, AMPK and PPAR protein expression after silencing or overexpression of SIRT3.

showed that the expression levels of AMPK and PPAR showed an increasing trend with the degree of the cervical tissue lesion ($P < 0.001$). After further analysis of clinical parameters, it was found that increased expression levels of AMPK and PPAR were related to lymph node metastasis ($P < 0.001$) and that the expression level of AMPK was related to the degree of tissue differentiation ($P < 0.001$), suggesting that the AMPK/PPAR pathway has a certain effect on the invasion and metastasis of cervical cancer.

Studies of different tumor tissues show that the fatty acid synthesis ability of tumor tissues with strong invasion ability is generally enhanced [14, 15]. Fatty acids and their polyunsaturated derivatives are important structures that make up cell membranes and affect membrane fluidity and physiologic functions [16]. Thus, for rapidly proliferating and strongly invasive tumor cells, there is high demand for fatty acids, and thus, a large amount of fatty acids needs to be produced to maintain these living conditions. Some researchers have found that in liver cancer cells, magnolol activates the AMPK/PPAR pathway, reduces the expression of acc1 and reduces the occurrence of fatty liver malignancy [12]. PPAR can increase fatty acid metabolism and accelerate the proliferation and metastasis of prostate cancer cells [17]. It has been reported that the poor prognosis and high mortality of cervical cancer are closely related to invasion and early metastasis of tumor cells [18]. Does this indicate a link between this malignant biologic behavior of cervical cancer and abnormal fatty acid metabolism? There are few reports on the regulatory effects of SIRT3 on the AMPK/PPAR pathway. This study tested SIRT3. The background expression of AMPK and PPAR showed that the protein and mRNA expression levels of SIRT3, AMPK, and PPAR in C33a cells were higher than those in SiHa cells ($P < 0.05$). Subsequently, siSIRT3 or LV-SIRT3 overexpression lentivirus was used to alter SIRT3 levels in cells. In this way, the activity and function of SIRT3 in the C33a and SiHa cells were inhibited and enhanced, respectively, and we found that SIRT3 could effectively promote the activation of the AMPK/PPAR pathway. Analysis by oil red O staining showed that after inhibition of SIRT3 expression, the intracellular tri-ester content decreased significantly. The results of the trace test and the Transwell assay confirmed that SIRT3 could promote the migration and invasion of cervical cancer cells to a certain extent. The above experimental results show that SIRT3 can activate the AMPK/PPAR pathway, effectively promote intracellular triglyceride synthesis, and further enhance the ability of cervical cancer cells to invade and migrate.

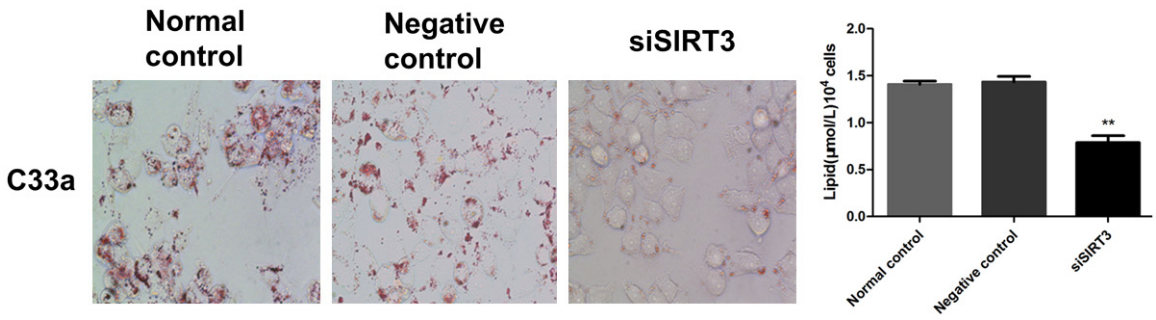

Figure 4. Synthesis of triglycerides in C33a cell group.

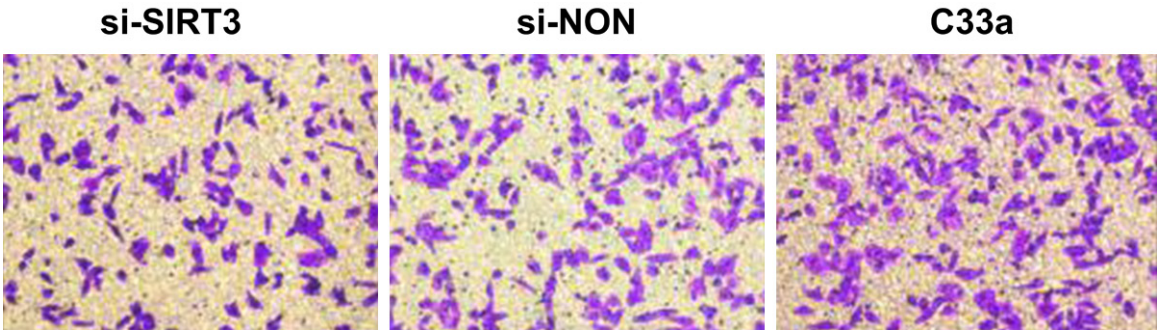

Figure 5. C33a cell migration after silencing of SIRT3 expression.

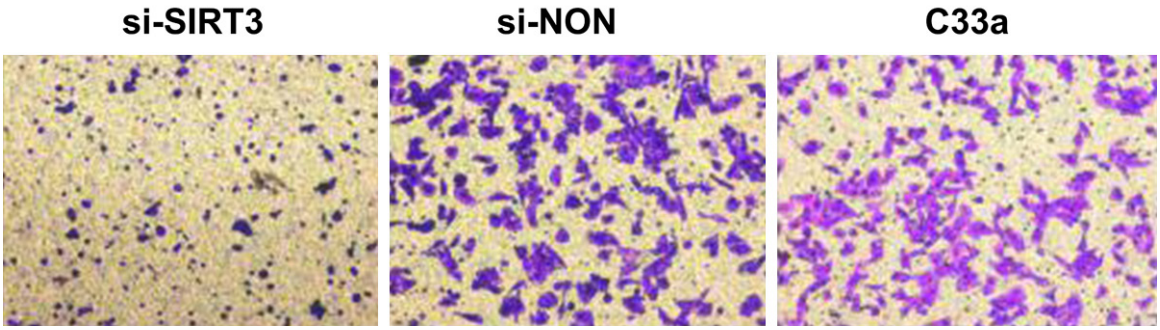

Figure 6. C33a cell invasion after silencing of SIRT3 expression.

Increased synthesis of fatty acids by tumor cells enhances the invasion of cells in vitro and promotes the growth of primary tumors and distant metastases in vivo [19]. The results of our study suggest that inhibiting the activity of SIRT3 in cervical cancer cells and reducing fatty acid synthesis may be new therapeutic approaches for regulating the invasion and metastasis of cervical cancer.

Acknowledgements

This study was supported with Xinjiang Uighur Autonomous Region Fund (2019D01C193).

Disclosure of conflict of interest

None.

Address correspondence to: Mikrban Tursun, Department of Pathology, College of Basic Medicine, Medical University of Xinjiang, Urumqi, China. E-mail: 42654117@qq.com

References

[1] Austad K, Chary A, Xocop SM, Messmer S, King N, Carlson L and Rohloff P. Barriers to cervical cancer screening and the cervical cancer care

continuum in rural guatemala: a mixed-method analysis. J Glob Oncol 2018; 4: 1-10.
[2] Liu Q, Liu S, Wang X, Zhang J and Liu K. LncRNA MAGI2-AS3 is involved in cervical squamous cell carcinoma development through CDK6 up-regulation. Infect Agent Cancer 2019; 14: 37.
[3] Abdul NS, Nagiah S, Anand K and Chuturgoon AA. Molecular docking and mechanisms of fusaric acid induced mitochondrial sirtuin aberrations in glycolytically and oxidatively poised human hepatocellular carcinoma (HepG2) cells. Toxicon 2020; 173: 48-56.
[4] Yang M, Yang C and Pei Y. Effects of downregulation of SIRT3 expression on proliferation and apoptosis in esophageal squamous cell carcinoma EC9706 cells and its molecular mechanisms. Biomed Mater Eng 2014; 24: 3883-90.
[5] Desouki MM, Doubinskaia I, Gius D and Abdulkadir SA. Decreased mitochondrial SIRT3 expression is a potential molecular biomarker associated with poor outcome in breast cancer. Hum Pathol 2014; 45: 1071-1077.
[6] Torrens-Mas M, Oliver J, Roca P and Sastre-Serra J. SIRT3: oncogene and tumor suppressor in cancer. Cancers (Basel) 2017; 9: 90.
[7] Steinberg GR and Kemp BE. AMPK in health and disease. Physiol Rev 2009; 89: 1025-1078.
[8] Wolozin B, Brown J 3rd, Theisler C and Silberman S. The cellular biochemistry of cholesterol and statins: insights into the pathophysiology and therapy of Alzheimer's disease. CNS Drug Rev 2004; 10: 127-46.
[9] Liu L, Zhang T, Hu J, Ma R, He B, Wang M and Wang Y. Adiponectin/SIRT1 axis induces white adipose browning after vertical sleeve gastrectomy of obese rats with type 2 diabetes. Obes Surg 2020; 30: 1392-1403.
[10] Kim YS, Gupta Vallur P, Jones VM, Worley BL, Shimko S, Shin DH, Crawford LC, Chen CW, Aird KM, Abraham T, Shepherd TG, Warrick JI, Lee NY, Phaeton R, Mythreye K and Hempel N. Context-dependent activation of SIRT3 is necessary for anchorage-independent survival and metastasis of ovarian cancer cells. Oncogene 2020; 39: 1619-1633.
[11] Jing E, Emanuelli B, Hirschey MD, Boucher J, Lee KY, Lombard D, Verdin EM and Kahn CR. Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. Proc Natl Acad Sci U S A 2011; 108: 14608-13.
[12] Tian Y, Feng H, Han L, Wu L, Lv H, Shen B, Li Z, Zhang Q and Liu G. Magnolol alleviates inflammatory responses and lipid accumulation by AMP-activated protein kinase-dependent peroxisome proliferator-activated receptor α activation. Front Immunol 2018; 9: 147.
[13] Junco JJ, Cho J, Mancha A, Malik G, Wei SJ, Kim DJ, Liang H, DiGiovanni J and Slaga TJ. Role of AMPK and PPARα in the anti-skin cancer effects of ursolic acid. Mol Carcinog 2018; 57: 1698-1706.
[14] Kuhajda FP. Fatty acid synthase and cancer: new application of an old pathway. Cancer Res 2006; 66: 5977-80.
[15] Koundouros N and Poulogiannis G. Reprogramming of fatty acid metabolism in cancer. Br J Cancer 2020; 122: 4-22.
[16] Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta 1999; 1451: 1-16.
[17] Li L, Hua L, Fan H, He Y, Xu W, Zhang L, Yang J, Deng F and Zeng F. Interplay of PKD3 with SREBP1 promotes cell growth via upregulating lipogenesis in prostate cancer cells. J Cancer 2019; 10: 6395-6404.
[18] Hong H, Zhu H, Zhao S, Wang K, Zhang N, Tian Y, Li Y, Wang Y, Lv X, Wei T, Liu Y, Fan S, Liu Y, Li Y, Cai A, Jin S, Qin Q and Li H. The novel circCLK3/miR-320a/FoxM1 axis promotes cervical cancer progression. Cell Death Dis 2019; 10: 950.
[19] Okumura T, Ohuchida K, Sada M, Abe T, Endo S, Koikawa K, Iwamoto C, Miura D, Mizuuchi Y, Moriyama T, Nakata K, Miyasaka Y, Manabe T, Ohtsuka T, Nagai E, Mizumoto K, Oda Y, Hashizume M and Nakamura M. Extra-pancreatic invasion induces lipolytic and fibrotic changes in the adipose microenvironment, with released fatty acids enhancing the invasiveness of pancreatic cancer cells. Oncotarget 2017; 8: 18280-18295.