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PKMYT1 aggravates the progression of ovarian cancer by targeting SIRT3

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Abstract. – OBJECTIVE: This experiment aims to elucidate the role of PKMYT1 in the malignant progression of ovarian cancer (OC) and its underlying mechanism.

PATIENTS AND METHODS: Expression pattern of PKMYT1 in 43 paired OC tissues and adjacent normal ones was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The potential relationship between PKMYT1 level and clinical data of OC patients was analyzed. PKMYT1 level in OC patients either with distant metastasis or not was examined. Through Cell Counting Kit (CCK-8) and transwell assay, influences of PKMYT1 on proliferative and metastatic abilities in 3AO and CAOV3 cells were assessed. At last, the role of PKMYT1/SIRT3 regulatory loop in the progression of OC was identified.

RESULTS: PKMYT1 was upregulated in OC tissues relative to controls. OC patients accompanied with distant metastasis had higher abundance of PKMYT1. High level of PKMYT1 predicted worse prognosis in OC patients. Knockdown of PKMYT1 attenuated proliferative, migratory, and invasive abilities in OC cells. Moreover, SIRT3 was downregulated in OC tissues, which was negatively correlated to PKMYT1. Silencing of SIRT3 could abolish the regulatory effect of PKMYT1 on proliferative and metastatic abilities in OC.

CONCLUSIONS: Upregulated PKMYT1 in OC is closely linked to distant metastasis and poor prognosis. PKMYT1 accelerates the malignant progression of OC via negatively regulating SIRT3.

Key Words:
PKMYT1, SIRT3, Ovarian cancer, Malignant progression.

Introduction

Ovarian cancer (OC) is one of the most deadly gynecological malignancies[1-3]. The detective rate of advanced OC is as high as approximately 70% due to the lack of typical symptoms and effective screening methods for early-stage OC[4,5]. Although traditional surgery and cisplatin- or paclitaxel-based chemotherapy have been effectively applied for OC treatment, drug resistance and tumor recurrence are the major reasons for treatment failure. The 5-year survival of OC is only about 30%[6,7]. Therefore, it is urgent to develop novel therapeutic strategies for OC. Notably, tissue heterogeneity and gene mutations are considered as factors influencing tumor-associated pathways[8-11].

Metastasis is a complex process in which tumor cells detach from the primary loci and transfer to distant organs. Locally invasive tumor cells gradually infiltrate into the circulatory system. Extravasation of tumor cells from blood vessels contributes to the formation of micrometastases in the distant organs. Proliferative tumor cells eventually result in the formation of tumor metastases[12,13]. Rate-limiting steps during metastatic process and epigenetic changes responsible for metastasis are urgent to be clarified[14,15]. Moreover, investigations on metastasis-associated genes are beneficial to provide a theoretical basis for inhibiting the progression of malignant tumors[15].

The Wee1 family phosphorylates residues of Ser, Thr, and Tyr. They are bispecific protein kinases that negatively regulate cell cycle progression by phosphorylating inactivated cyclin-dependent kinases[16,17]. To date, three related Wee1 gene products have been identified in mammals, including Wee1a, Wee1b, and PKMYT1[17,18]. In recent years, the Wee1 pathway in somatic cells and tumor diseases has been well concerned[17,18]. It is reported[19] that upregulation of PKMYT1 in non-small cell lung cancer (NSCLC) enhances tumor proliferation by activating the Notch pathway.

SIRT3 is a mitochondrial protein with a great research potential. It is closely linked to energy metabolism, cell senescence, and tumor progres-

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sion. Hence, SIRT3 could be utilized as therapeutic targets for many human diseases, especially aging-related diseases and tumors[20,21]. In this paper, we mainly discussed the role of PKMYT1/SIRT3 regulatory loop in the progression of OC.

Patients and Methods

Patients and PCa Samples

Paired OC tissues and adjacent normal ones were surgically resected from 43 OC patients. None of them received preoperative anti-tumor treatment. Clinical data and follow-up data of enrolled patients were recorded. Tumor staging was assessed based on the guideline proposed by UICC. Patients and their families have been fully informed. This study was approved by the Ethics Committee of Jinan Second Maternal and Child Health Hospital and conducted in accordance with the Declaration of Helsinki.

Cell Culture

Ovarian epithelial cells HOSEPiCs and OC cells (PEO1, A2780, SKOV3, OVCAR3, 3AO, and CAOV3) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C.

Transfection

Cells were inoculated in a 6-well plate and transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) when 70% of them were fused. After 48 h, the transfected cells were harvested for functional experiments. Transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Counting Kit (CCK-8)

Cells were inoculated in a 96-well plate (2×10^3 cells/well). At day 1, 2, 3, and 4, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Transwell Assay

Cells were inoculated in a 24-well plate with 5.0×10^5/ml. 200 μL of suspension was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, penetrated cells in the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample (magnification 20×).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), purified by DNase I treatment, and reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), with β-actin and U6 as internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. The following primers were used. PKMYT1: forward: 5'-CATGCTAGAACGCTGGCAC-3', reverse: 5'-GCACCTTGAAGACCTCTCCG-3'; SIRT3: forward: 5'-GGCGGCAGGGACGATTATTA-3', reverse: 5'-CCCGAATCAGCTCAGCTACA-3'; β-actin: forward: 5'-CAGAGCTCCTCGTCTTGCC-3', reverse: 5'-GTCGCCACCATGAGAGAC-3'.

Western Blot

Cellular protein extracted was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked in 5% skim milk for 1 h, followed by incubation with specific primary antibody overnight at 4°C, and then with secondary antibody for 2 h at room temperature. After washing with 1×TBST for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Statistical Analysis

SPSS 22.0 (IBM, Armonk, NY USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Kaplan-Meier curves were introduced for survival analysis, followed by Log-rank test for comparing differences between two curves. Spearman correlation test was performed to assess the relationship between levels of PKMYT1 and SIRT3 in OC tissues. $p< 0.05$ was considered statistically significant.

Results

Up-regulated PKMYT1 in OC

Compared with HOSEPiCs cells, PKMYT1 was up-regulated in OC cells (Figure 1A). Survival analysis revealed that OC patients with high expression of PKMYT1 had worse survival (Figure 1B). Identically, PKMYT1 was highly expressed in OC tissues compared with that of adjacent normal ones (Figure 1C). In particular, higher abundance of PKMYT1 was observed in OC patients accompanied with distant metastasis compared with that in non-metastatic OC patients (Figure 1D).

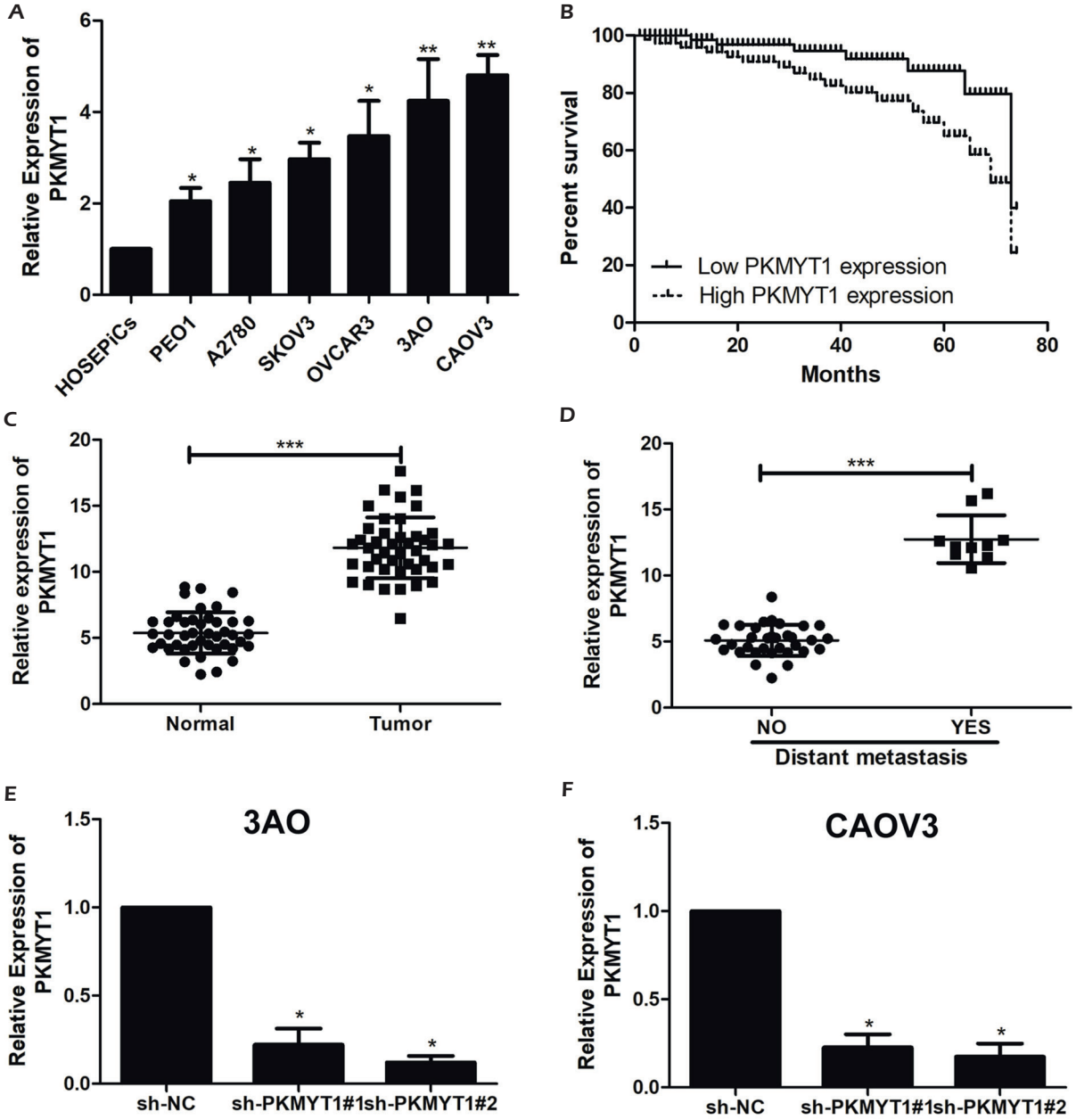

Figure 1. Downregulated PKMYT1 in OC. **A,** PKMYT1 levels in HOSEPiCs cells and OC cells (PEO1, A2780, SKOV3, OVCAR3, 3AO, and CAOV3). **B,** Survival in OC patients expressing high or low level of PKMYT1. **C,** PKMYT1 levels in OC tissues and adjacent normal tissues. **D,** PKMYT1 levels in OC patients either with distant metastasis or not. **E, F,** PKMYT1 levels in 3AO (**E**) and CAOV3 (**F**) cells transfected with sh-NC, sh-PKMYT1#1 or sh-PKMYT1#2.

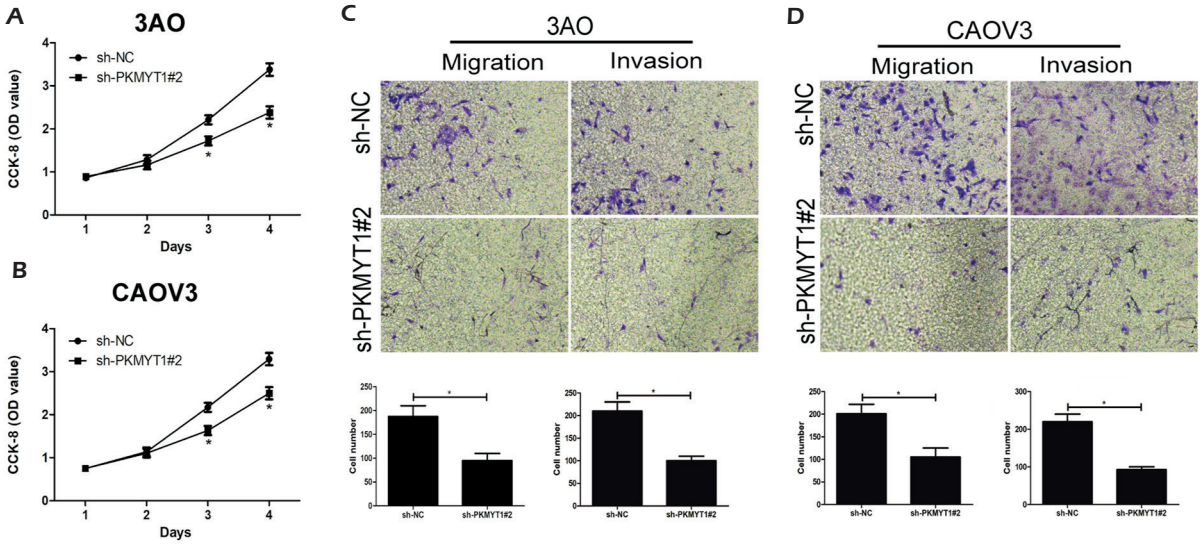

Figure 2. Knockdown of PKMYT1 attenuated proliferative and metastatic abilities in OC. **A-B,** Viabilities in 3AO (**A**) and CAOV3 (**B**) cells transfected with sh-NC or sh-PKMYT1#2. **C-D**, Migration and invasion in 3AO (**C**) and CAOV3 (**D**) cells transfected with sh-NC or sh-PKMYT1#2 (magnification 20×).

Subsequently, potential correlation between PKMYT1 level and clinical data of enrolled OC patients was analyzed. It is shown that PKMYT1 level was positively correlated to distant metastasis, rather than age, tumor staging, and lymphatic metastasis in OC patients (Table I). Therefore, PKMYT1 may be a hallmark for predicting the progression of OC.

Knockdown of PKMYT1 Attenuated Proliferative and Metastatic Abilities in OC

To elucidate the biological role of PKMYT1 in OC, two PKMYT1 shRNAs were constructed. Transfection of either sh-PKMYT1#1 or sh-PKMYT1#2 greatly downregulated PKMYT1 level in 3AO and CAOV3 cells, more significantly in sh-PKMYT1#1 (Figure 1E, 1F). CCK-8 results demonstrated that transfection of sh-PKMYT1#2 markedly reduced viabilities in 3AO and CAOV3 cells (Figure 2A, 2B). Transwell assay revealed decreased numbers of migratory and invasive cells after knockdown of PKMYT1 in OC cells (Figure 2C, 2D).

SIRT3 Was Downregulated in OC

After transfection of sh-PKMYT1#2, both mRNA and protein levels of SIRT3 were upregulated in OC cells (Figure 3A). Compared with adjacent normal tissues, SIRT3 was lowly expressed

Table I. Association of PKMYT1 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	No. of cases	PKMYT1 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.495
<60	16	9	7	
≥60	27	18	9	
T stage				0.280
T1-T2	26	18	8	
T3-T4	17	9	8	
Lymph node metastasis				0.782
No	28	18	10	
Yes	15	9	6	
Distance metastasis				0.014
No	33	24	9	
Yes	10	3	7	

in OC (Figure 3B). Moreover, SIRT3 level was negatively correlated to that of PKMYT1 in OC tissues (Figure 3C). Kaplan-Meier curves illustrated worse survival in OC patients expressing low level of SIRT3 (Figure 3D).

PKMYT1/SIRT3 Regulatory Loop in OC

Based on the above findings, SIRT3 may be responsible during the progression of OC regulated by PKMYT1. We constructed si-SIRT3 and tested its transfection efficacy in OC cells (Figure 4A). Transfection of si-SIRT3 accelerated viability and enhanced numbers of migratory and invasive cells. Of note, co-silence of PKMYT1 and SIRT3 reversed phenotypes in OC cells transfected with sh-PKMYT1#2 only (Figure 4B, 4C). Therefore, SIRT3 was involved in PKMYT1-mediated progression of OC.

Discussion

OC severely affects women's health, and epithelial ovarian tumors are the mostly prevalent subtype[1-3]. The mortality of OC ranks high. It is reported that in 2018, OC-induced deaths account for 5% of the total in the United States[1,2]. More seriously, the incidence of OC is on the rise in recent years[2-5]. Due to anatomical location, hidden onset, and ineffective early diagnosis, about 70% OC patients have been in advanced stage when initially diagnosed[3,5-7]. OC is prone to metastasis into pelvic and abdominal cavity[8-10]. Effective suppression of OC cell metastasis is the therapeutic priority. Clarification of mechanisms underlying metastasis in OC is of significance[11,12].

PKMYT1 is reported[19,22] to be upregulated in many types of malignancies, and its level is cor-

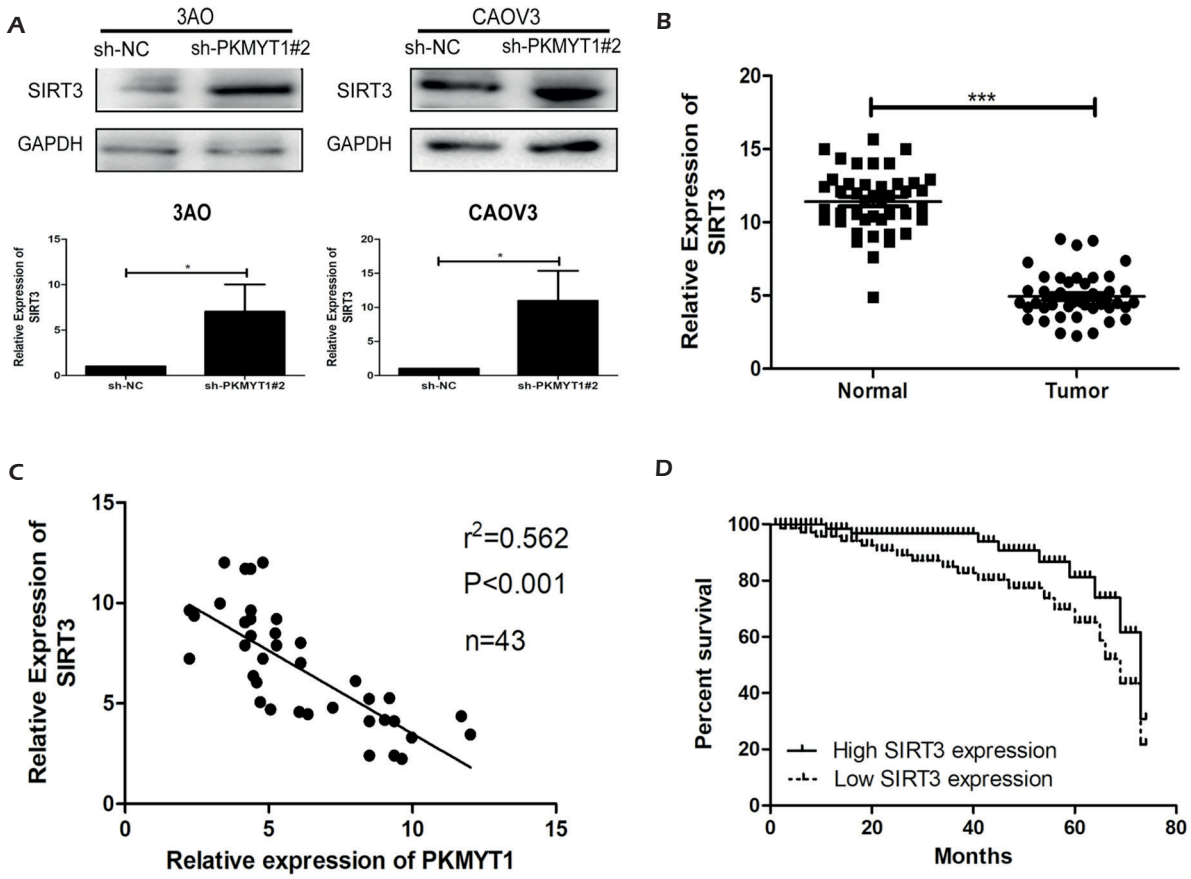

Figure 3. SIRT3 was downregulated in OC. **A,** Relative level of SIRT3 in 3AO and CAOV3 cells transfected with sh-NC or sh-PKMYT1#2. **B,** SIRT3 levels in OC tissues and adjacent normal tissues. **C,** A negative correlation between expression levels of PKMYT1 and SIRT3 in OC tissues. **D,** Survival in OC patients expressing high or low level of SIRT3.

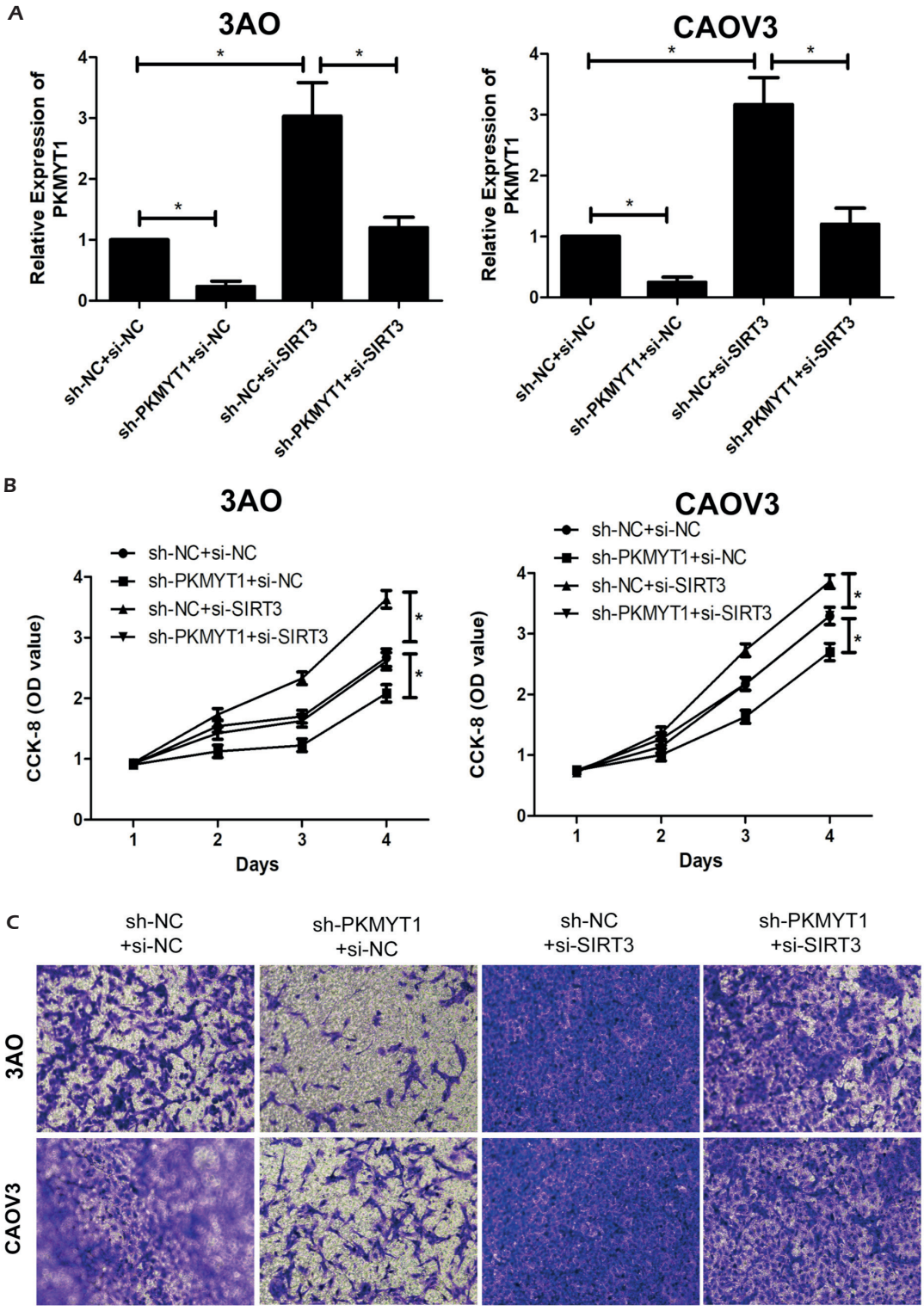

Figure 4. PKMYT1/SIRT3 regulatory loop in OC. 3AO and CAOV3 cells were transfected with sh-NC+si-NC, sh-PKMYT1#2+-si-NC, sh-NC+si-SIRT3 or sh-PKMYT1#2+si-SIRT3. **A,** PKMYT1 level; **B,** Viability; **C,** Migration (magnification 20×).

Figure continued

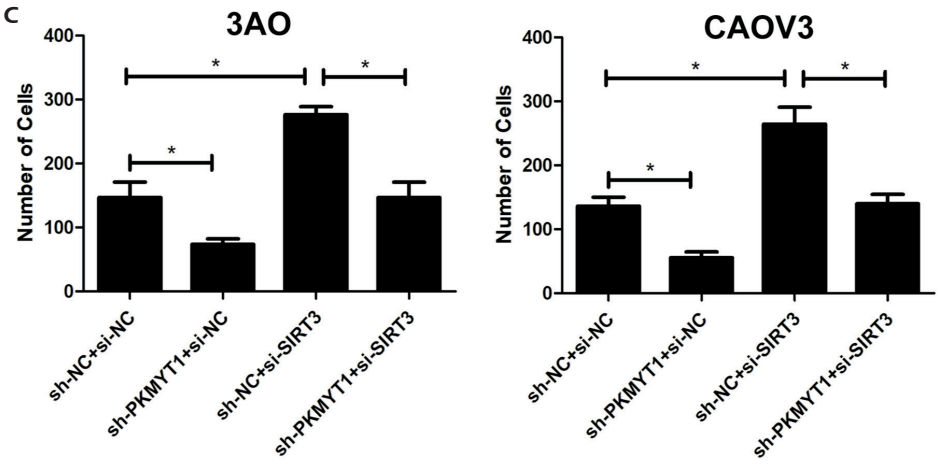

Figure 4 (continued). PKMYT1/SIRT3 regulatory loop in OC. 3AO and CAOV3 cells were transfected with sh-NC+si-NC, sh-PKMYT1#2+si-NC, sh-NC+si-SIRT3 or sh-PKMYT1#2+si-SIRT3. **A,** PKMYT1 level; **B,** Viability; **C,** Migration (magnification 20×).

related to prognosis of tumor patients. PKMYT1 may be a crucial oncogene. Here, we detected PKMYT1 levels in 43 paired OC tissues and adjacent normal ones. Our results uncovered that upregulated PKMYT1 in OC tissues was closely linked to distant metastasis, exerting a carcinogenic role.

RNA interference technology (RNAi) is highly efficient and specific for inhibiting target gene expressions by transfection of a small interfering RNA (siRNA) with 21-23 bp long[23,24]. Transfection of siRNA into cells could markedly downregulate the target gene, thus leading to downstream gene changes[25,26]. Here, PKMYT1 level in 3AO and CAOV3 cells was effectively downregulated by RNAi. Subsequently, functional experiments identified that silence of PKMYT1 attenuated proliferative, migratory, and invasive capacities in OC.

SIRT3, the major deacetylase in mitochondria, plays a crucial role in modulating oxygen reactive species and limiting the oxidative damage in cellular components[20]. In some types of cancer, SIRT3 functions as a tumoral suppressor, as SIRT3 could trigger cell death under stress conditions[20,21]. Notably, SIRT3 level was found to be upregulated in OC cells with PKMYT1 knockdown. A negative correlation between expression levels of PKMYT1 and SIRT3 was observed in OC tissues. SIRT3 was downregulated in OC tissues, which was negatively related to survival in OC patients. Based on the above findings, we speculated that SIRT3 was responsible for PKMYT1-induced aggravation of OC. Rescue experiments demonstrated that silencing of SIRT3 could abolish the regulatory effect of PKMYT1 on proliferative and metastatic abilities in OC. Collectively, PKMYT1/SIRT3 regulatory loop aggravated the malignant progression of OC.

Conclusions

Upregulated PKMYT1 in OC is closely linked to distant metastasis and poor prognosis. PKMYT1 accelerates the malignant progression of OC *via* negatively regulating SIRT3.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Eisenhauer EA. Real-world evidence in the treatment of ovarian cancer. Ann Oncol 2017; 28: i61-i65.
2) Markman M. Pharmaceutical management of ovarian cancer: current status. Drugs 2019; 79: 1231-1239.
3) Webb PM, Jordan SJ. Epidemiology of epithelial ovarian cancer. Best Pract Res Clin Obstet Gynaecol 2017; 41: 3-14.
4) Meng X, Schwarzenbach H, Yang Y, Muller V, Li N, Tian D, Shen Y, Gong Z. Circulating mitochondrial DNA is linked to progression and prognosis of epithelial ovarian cancer. Transl Oncol 2019; 12: 1213-1220.

5) Chon HS, Sehovic M, Marchion D, Walko C, Xiong Y, Extermann M. Biologic mechanisms linked to prognosis in ovarian cancer that may be affected by aging. J Cancer 2019; 10: 2604-2618.
6) Markman M. Pharmaceutical management of ovarian cancer: current status. Drugs 2019; 79: 1231-1239.
7) Rinninella E, Fagotti A, Cintoni M, Raoul P, Scaletta G, Quagliozzi L, Miggiano G, Scambia G, Gasbarrini A, Mele MC. Nutritional interventions to improve clinical outcomes in ovarian cancer: a systematic review of randomized controlled trials. Nutrients 2019;11. pii: E1404. doi: 10.3390/nu11061404.
8) Zijlstra M, Timmermans M, Fransen H, van der Aa M, Reyners A, Raijmakers N, van de Poll-Franse L. Treatment patterns and associated factors in patients with advanced epithelial ovarian cancer: a population-based study. Int J Gynecol Cancer 2019; 29: 1032-1037.
9) Amidi E, Mostafa A, Nandy S, Yang G, Middleton W, Siegel C, Zhu Q. Classification of human ovarian cancer using functional, spectral, and imaging features obtained from in vivo photoacoustic imaging. Biomed Opt Express 2019; 10: 2303-2317.
10) Lheureux S, Braunstein M, Oza AM. Epithelial ovarian cancer: evolution of management in the era of precision medicine. CA Cancer J Clin 2019; 69: 280-304.
11) Chen SN, Chang R, Lin LT, Chern CU, Tsai HW, Wen ZH, Li YH, Li CJ, Tsui KH. MicroRNA in ovarian cancer: biology, pathogenesis, and therapeutic opportunities. Int J Environ Res Public Health 2019; 16. pii: E1510. doi: 10.3390/ijerph16091510.
12) Motohara T, Katabuchi H. Ovarian cancer stemness: biological and clinical implications for metastasis and chemotherapy resistance. Cancers (Basel) 2019; 11. pii: E907. doi: 10.3390/cancers11070907.
13) Desai JP, Moustarah F. Cancer, peritoneal metastasis. StatPearls Publishing [Internet], 2019.
14) Moffitt L, Karimnia N, Stephens A, Bilandzic M. Therapeutic targeting of collective invasion in ovarian cancer. Int J Mol Sci 2019; 20. pii: E1466. doi: 10.3390/ijms20061466.
15) Mahalaxmi I, Devi SM, Kaavya J, Arul N, Balachandar V, Santhy KS. New insight into NANOG: A novel therapeutic target for ovarian cancer (OC). Eur J Pharmacol 2019; 852: 51-57.
16) Sturgiss EA, Elmitt N, Haesler E, van Weel C, Douglas KA. Role of the family doctor in the management of adults with obesity: a scoping review. BMJ Open 2018; 8: e19367.
17) Schmidt M, Rohe A, Platzer C, Najjar A, Erdmann F, Sippl W. Regulation of G2/M transition by inhibition of WEE1 and PKMYT1 kinases. Molecules 2017; 22: pii: E2045.
18) Fattaey A, Booher RN. Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14. Prog Cell Cycle Res 1997; 3: 233-240.
19) Sun QS, Luo M, Zhao HM, Sun H. Overexpression of PKMYT1 indicates the poor prognosis and enhances proliferation and tumorigenesis in non-small cell lung cancer via activation of Notch signal pathway. Eur Rev Med Pharmacol Sci 2019; 23: 4210-4219.
20) Schumacker PT. A tumor suppressor SIRTainty. Cancer Cell 2010; 17: 5-6.
21) Chen S, Yang X, Yu M, Wang Z, Liu B, Liu M, Liu L, Ren M, Qi H, Zou J, Vucenik I, Zhu WG, Luo J. SIRT3 regulates cancer cell proliferation through deacetylation of PYCR1 in proline metabolism. Neoplasia 2019; 21: 665-675.
22) Wang XM, Li QY, Ren LL, Liu YM, Wang TS, Mu TC, Fu S, Liu C, Xiao JY. Effects of MCRS1 on proliferation, migration, invasion, and epithelial mesenchymal transition of gastric cancer cells by interacting with Pkmyt1 protein kinase. Cell Signal 2019; 59: 171-181.
23) Whitten MM. Novel RNAi delivery systems in the control of medical and veterinary pests. Curr Opin Insect Sci 2019; 34: 1-6.
24) Mahmoodi CG, Dana H, Gharagouzloo E, Grijalvo S, Eritja R, Logsdon CD, Memari F, Miri SR, Rad MR, Marmari V. Small interfering RNAs (siRNAs) in cancer therapy: a nano-based approach. Int J Nanomedicine 2019; 14: 3111-3128.
25) Chernikov IV, Vlassov VV, Chernolovskaya EL. Current development of siRNA bioconjugates: from research to the clinic. Front Pharmacol 2019; 10: 444.
26) Schuster S, Miesen P, van Rij RP. Antiviral RNAi in insects and mammals: parallels and differences. Viruses; 11. pii: E448. doi: 10.3390/v11050448.