RESEARCH ARTICLE

SIRT7 Exhibits Oncogenic Potential in Human Ovarian Cancer Cells

Hong-Ling Wang[1,2], Ren-Quan Lu[1,2], Su-Hong Xie[1], Hui Zheng[1], Xue-Mei Wen[1,2], Xiang Gao[1], Lin Guo[1,2]*

Abstract

Background: Sirtuin7 (SIRT7) is a type of nicotinamide adenine dinucleotide oxidized form (NAD+)-dependent deacetylase and the least understood member of the sirtuins family; it is implicated in various processes, such as aging, DNA damage repair and cell signaling transduction. There is some evidence that SIRT7 may function as a tumor trigger for human malignancy. Here, we aimed to explore the biological function of SIRT7 in ovarian carcinoma cells and its potential mechanism. Materials and Methods: Expression of SIRT7 in ovarian cancer cell lines was detected by western blotting. Transduced cell lines with SIRT7 knockdown or overexpression were constructed. Cell viability, cologenic, apoptosis-associated and motility assays were performed to elucidate the biological function of SIRT7 in ovarian cancer cells. Results: SIRT7 demonstrated a higher level in ovarian cancer cell lines compared with normal cells. On the one hand, down-regulation of SIRT7 significantly reduced ovarian cancer cell growth, repressed colony formation and increased cancer cell apoptosis; on the other hand, up-regulation promoted the migration of cancer cells. Additionally, repression of SIRT7 also induced change in apoptosis-related molecules and subunits of the NF-κB family. Conclusions: In the present study, our data indicated that SIRT7 might play a role of oncogene in ovarian malignancy and be a potential therapeutic target.

Keywords: SIRT7 - ovarian cancer cells- deacetylase - NF-κB

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Introduction

Sirtuins, belonging to class III histone deacetylase (HDAC), comprise seven members (SIRT1-7) and are characteristic of deacetylating histones and non-histone proteins. They are implicated in interactions between various kinds of proteins and are believed to be an indispensible part of the genetic regulatory network, such as mitosis, cell signal transduction and DNA damage repair (Blander et al., 2004; North et al., 2004). Furthermore, sirtuins have some associations with several pathological conditions, including malignant tumor, cardiovascular disease and diabetes (Vakhrusheva et al., 2008). However, their biological roles in cancer prompted our interests to explore. Some members of the sirtuins family play the role of oncogene by promoting the growth of tumor cells and preventing their apoptosis, whereas others have the opposite function (Bosch-Presegue et al., 2011).

Sirtuin 7(SIRT7), a new member of the sirtuins family, is mainly localized in the nucleus and is characteristic of its locus specific H3K18 deacetylase activity (Barber et al., 2012). As a new member, its biological properties are not well understood. A new breakthrough suggested that SIRT7 can interact with chromatin remodeling complex and modulate the activation of RNA polymerase I transcription (Ford et al., 2006; Tsai et al., 2012). Another study proposed that SIRT7 can promote cell survival by suppressing ER stress in a myc-dependent mannner (Shin et al., 2013). In addition, evidence has suggested that SIRT7 was related to maintenance of cellular transformation in malignancies, such as breast, liver, bladder and colorectal cancer; it has an elevated level in cancerous tissues compared with the noncancerous and its levels are associated with tumor progression (Ashraf et al., 2006; Han et al., 2013; Kim et al., 2013; Yu et al., 2014). On the contrary, a comprehensive research on the expression level of SIRT7 in head and neck squamous cell carcinoma showed a significant down-regulation (Lai et al., 2013). This inconsistent results aroused our interests to unravel the biological function of SIRT7 in ovarian tumorigenesis.

Ovarian cancer is one of the most common malignant diseases and its morbidity is second only to cervical cancer and carcinoma of uterine corpus (Jemal et al., 2003). As a result of its concealed properties, it is difficult to be early diagnosed. A number of patients are confirmed as late-stage ovarian cancer at the time of first diagnosis and the 5-year survival rate is far from satisfactory (Chen et al., 2014). Thus understanding the underlying mechanism that drive ovarian carcinogenesis is crucial for early

*[1]Department of Clinical Laboratory, Fudan University Shanghai Cancer Center, [2]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China *For correspondence: guolin500@hotmail.com*

diagnosis of ovarian cancer and identification of effective therapeutics.

In present study, we aimed to explore the biological characteristics of SIRT7 and its mechanism of carcinogenesis in ovarian cancer cell lines. Firstly, we detected that SIRT7 had a higher level in ovarian cancer cell lines compared with the normal ones. Then we studied the effects of SIRT7 down-regulation and up-regulation on cancer cell lines, HO8910 and SKOV3 with respect to cell proliferation, apoptosis and migration.

Materials and Methods

Cell culture

The normal ovarian cell line, HOSEpic and ovarian cancer cell lines, HO8910 and SKOV3 were obtainded from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin /streptomycin in a humidified incubator with 5% carbon dioxide at 37°C. HEK 293T cells were the collection of our own laboratory and cultured in Dulbecco's Modified Eagle Media (DMEM).

Generation of trarnsduced ovarian cancer cells

RNA interference was used to down-regulate the expression of SIRT7. Short hairpin RNA (shRNA) sequence targeting SIRT7 and control (con) sequence non-targeting SIRT7 were purchased from Invitrogen and cloned into pLKO.1 lentiviral vector to construct shRNA-SIRT7/shRNA-con. Target sequence against SIRT7 was CGCCAAATACTTGGTCGTCTA and target sequence against control was CCTAAGGTTAAGTCGCCCTCG. For SIRT7 overexpression, RNA was extracted from ovarian cancer cells and reversely transcripted into cDNA. SIRT7 opening reading frame (ORF) was synthesized by PCR, digested by EcoRI and NotI, and cloned into PCDH-CMV-MCS-EF1-PURO (PCDH-puro) expression vector to generate SIRT7-PCDH. The primers for SIRT7 were F: ATATGAATTCGCCACCATGGCAG CCGGGGGTCTGATC; R: ATAAGGATGCGGCCG CTTAC G TCACTTTCTTCCTTTTTGT.

To get transduced cell lines, lentivirus mediated transduction was employed. HEK 293T cells were used for virus packaging and were seeded into 6-well plate at a confluence of 50%-60%. After 24h, the cells were transfected with recombined plasmids (shRNA-SIRT7/ shRNA-con or SIRT7-PCDH/con-PCDH) together with psPAX2 and pMD2.G using lipofectamine 2000 (Invitrogen) according to maufacturer's specifications. Forty-eight hours after transduction, virus supernatant was collected and transduced into the ovarian cell lines. Forty-eight hours after lentivirus transfection, stable clones were obtained from 2ug/mL puromycin selection and maintained at 1ug/mL puromycin in culture media. Proteins were harvested to assess the down-regulation and up-regulation efficiency. Then the cell lines were used for in-vitro assay.

Western blotting

Whole cell lysates were separated on 10%-12% SDS-PAGE gradient gels and were transferred onto polyvinylidene fluoride membrane (PVDF). After blocking with skimmed milk to decrease non-specific binding, the membrane was incubated with specific primary antibodies at 4°C overnight. Then the protein bands were hybridized with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody and visualized using chemiluminescence detection system(Thermo, USA). GAPDH served as a loading control. The commercial antibodies used in our research were as follows: antibody against SIRT7 (Santa Cruz Biotecnology, sc-365344), GAPDH (Proteintech, 10494-1-AP), Bcl-2, Bax (Cell Signaling Technology, #2872 and #2774), NF-κB/p65, NF-κB/p50 (Abcam, ab32536 and ab7971) .

Cell proliferation assay

To analyze cell proliferation, we performed cell viability and colony formation assays on ovarian cancer cell lines (HO8910 and SKOV3)with SIRT7 knockdown or negative controls. For cell viability analysis, the cell lines were seeded into 96-well plate in triplicate at a density of 1×10^3 per well; the absorbance at 450nm was collected at the same time every day after CCK-8 reagent was added. For colony formation assay, cells were seeded into 6-well plate in triplicate with 1×10^3 cells/well. After 14 days' incubation, we analyzed the cell clones by crystal violet staining. The clones with more than 50 cells were counted.

Flow cytometry

Transduced ovarian cancer cells HO8910 and SKOV3 (shRNA-SIRT7/shRNA-con) were seeded into 6-well plate at a density of 25×10^4 per well. After 48h of incubation, the cells were collected by trypsinization, washed twice with ice-cold PBS, resuspended with 1×Annexin V binding buffer and stained with propidiumiodide (PI) and Annexin V(BD, USA). Cell apoptosis analysis was detected by a flow cytometer.

Migration assay

The SIRT7-PCDH/con-PCDH Transduced HO8910 and SKOV3 were starved for 2h before being seeded into 24-well plate without matrigel-coated inserts (8-mm pore; BD Falcon). After 24hs' incubation, cells were fixed with 4% paraformaldehyde (PFA) and stained by crystal violet. The images were visualised under microscope.

Wound healing assay

The cell lines (HO8910 and SKOV3) overexpressing SIRT7 or controls were seeded into 6-well plate (4×10^5 per well). After 24h of incubation, they were at a confluence of 100%, scratched by tips, washed by PBS for three times and incubated with RPMI 1640 free of FBS. Then we collected images at 0h, 24h or 36h and recorded the migration distance.

Statistical analysis

Statistical analysis was performed with SPSS software (version 18.0). Data were expressed as means ± standard deviation. Continuous variables between two groups were assessed with Student's two-tailed t-test. $p<0.05$ was

considered statistically significant. Statistical significance was showed as *p<0.05 or **p<0.01. Each experiment was performed independently for three times.

Results

SIRT7 has an elevated level in ovarian cancer cell lines and exhibits its oncogenic property by promoting proliferation of cells

To examine the level of SIRT7 in different ovarian cancer cell lines, together with the human normal ovarian cells, we harvested the proteins of cells, analyzed by western blotting and identified that SIRT7 had a higher level compared with the normal ones (Figure 1A).

As shown above, SIRT7 had a higher level in ovarian cancer cell lines, thus we hypothesized that SIRT7 might have the function of promoting the growth of cells. The method of RNA interference was performed to silence the expression of SIRT7 and to investigate its effect on ovarian cancer cells, HO8910 and SKOV3. The efficiency of knockdown was analyzed by western blotting (Figure 1B). The cell viability assay indicated that silencing of SIRT7 inhibited cancer cell proliferation (**p<0.01 in HO8910 and SKOV3, Figure 1D). The colony formation assay clearly showed that shRNA-con group had a higher colony-forming efficiency than shRNA-SIRT7 (*p<0.05 in HO8910 and **p<0.01 in SKOV3, Figure 1E). This confirmed that depletion of SIRT7 might have growth suppressive effect on ovarian cancer cells.

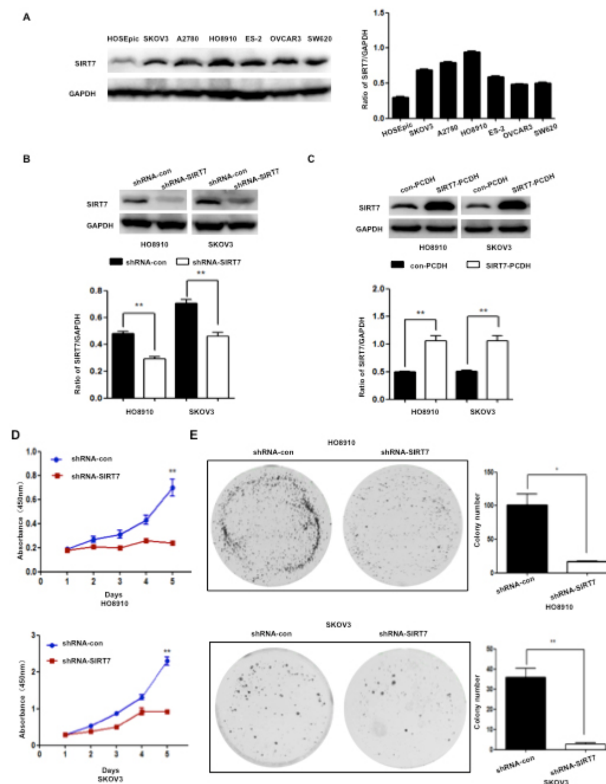

Figure 1. Expression level of SIRT7 in Ovarian Cancer Cell Lines and Effect of SIRT7 Knockdown on Cell Proliferation. (A) Western blotting was used to investigate the SIRT7 expression level in 5 ovarian cancer cell lines. SKOV3 and HO8910 cells lines showed high expression of SIRT7. GAPDH served as an internal control. (B, C) Endogenous SIRT7 level in SKOV3, HO8910 cells after tranduction with corresponding lentivirus (shRNA-con/shRNA-SIRT7 or con-PCDH/SIRT7-PCDH). SIRT7 was down-regulated and up-regulated in these cancer cells lines respectively. GAPDH served as an internal control. (D) Cell viability assay showed that knockdown of SIRT7 significantly reduced ovarian cancer cells proliferation (**, p<0.01 in HO8910 and SKOV3). (E) The colony formation number in shRNA-con groups was significantly more than shRNA-SIRT7 groups in monolayer culture (*, p<0.05 in HO8910 and **, p<0.01 in SKOV3)

Knockdown of SIRT7 triggers the apoptosis of ovarian cancer cells

As shown in our research that knockdown of SIRT7 could repress proliferation of ovarian cancer cell lines, so we speculated that SIRT7 might be associated with apoptosis. Then flow cytometry assay was performed on transduced cell lines HO8910 and SKOV3. The data clearly depicted that the apoptotic cells of shRNA-con was markedly less than shRNA-SIRT7 (*p<0.05 in HO8910 and SKOV3 group , Figure 2A and Supplementary 1A). To test whether increased apoptosis in SIRT7 knockdown group was correlated with apoptosis-associated molecules, we examined the level of Bcl-2 and Bax. While the level of Bcl-2 was down-regulated in shRNA-SIRT7 group, Bax had no obvious change (Figure 2B). We also explored the association of SIRT7 with nuclear factor kappa B (NF-ϰB) signaling pathway and found that NF-ϰB/p65 were down-regulated after SIRT7 was silenced, whereas NF-ϰB/p50 did not change significantly (Figure 2).

Overexpression of SIRT7 increases the motility of ovarian cancer cells

Transduced cell lines overexpressing SIRT7 were generated on the basis of PCDH-puro, which did not contain any external sequence and served as a control. The efficiency of overexpression was assessed by western blotting (Figure 1C). The motility assays without matrigel

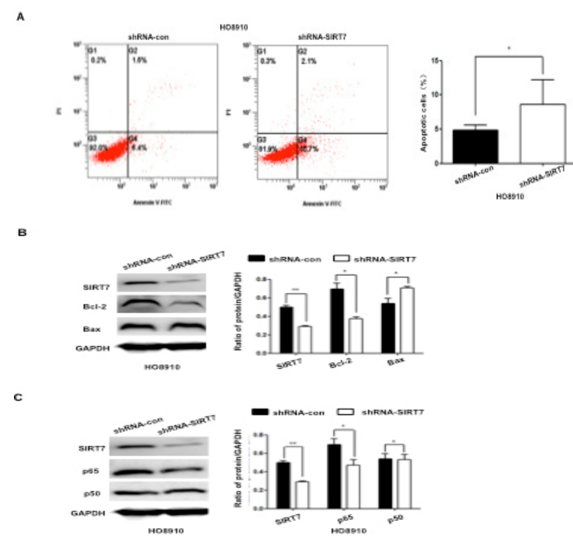

Figure 2. SIRT7 Down-regulation Increases Apoptosis of Ovarian Cancer Cells. (A) The number of apoptotic cells induced by SIRT7 knockdown was more than shRNA-con (*, p<0.05 in HO8910). (B)The expression level of apoptosis-related molecules after endogenous SIRT7 level was silenced in HO8910: Bcl-2 and p65 was downregulated; Bax and p50 did not show obvious change (*p<0.05)

100.0
75.0
50.0
25.0
0

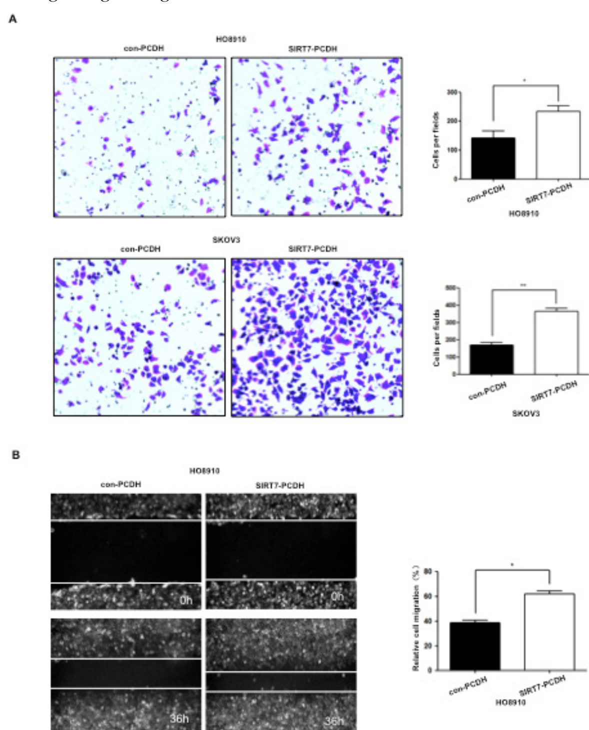

Figure 3. Effect of SIRT7 Overexpression on Motility of Ovarian Cancer Cells. (A) The cell number per field in SIRT7-PCDH were more than con-PCDH (*, p<0.05 in HO8910 and **, p<0.01 in SKOV3), which indicated that SIRT7 overexpression promoted the motility of ovarian cancer cells. (B) Increased motility was seen in cells transduced with SIRT7-PCDH in HO8910 (*p<0.05)

confirmed that SIRT7 overexpression increased cells migration (*p<0.05 in HO8910 and **p<0.01 in SKOV3, Figure 3). Moreover, the results of the wound healing assays showed that the migration distance of cells with SIRT7 ORF was more than its relative controls, which further demonstrated that cells with SIRT7 overexpression had a higher motility (*p<0.05 both in HO8910 and SKOV3 group, Figure 3B).

Discussion

Sirtuins can target many celluar proteins to modulate their state "acetylation-deacetylation", affecting physiological responses that may be instrumental in treating diseases (Lavu et al., 2008). A wide array of reports have shown that sirtuins can exert complex effects on various regulatory molecules and signaling pathways. A classic study on SIRT7 detected that SIRT7 is associated with activation of RNA polymerase I and rDNA transcription (Ford et al., 2006). A new breakthrough in SIRT7 identified that it can interact with p53 by deacetylation, leading to increased apoptosis of cardiomyocytes (Vakhrusheva et al., 2008). Numerous explorations on SIRT7 have shed lights on its biological properties, but its underlying mechanism in human malignancies remain elusive. In recent years, emerging studies have been performed on SIRT7 and provided some potential mechanisms for SIRT7 in promoting cancer development. Researches in hepatocelluar carcinoma and bladder cancer indicated that SIRT7 promoted cancer cell proliferation and was regulated by microRNA, such as miR-125a-5p and miR-125b; a research in colorectal cancer identified that SIRT7 functioned as an oncogene by activation of RAF-MEK-ERK signaling pathway and increased cancer cells motility by epithelial-to-mesenchymal transition (EMT) (Han et al., 2013; Kim et al., 2013; Yu et al., 2014). However, the level of SIRT7 in ovarian malignancies and the corresponding consequences of its aberrant regulation have not been discovered.

In this study, we identified that SIRT7 is specifically overexpressed in ovarian cancer cell lines. Down-regulation of SIRT7 was shown to affect ovarian cancer cells via the following effects including reduced cell growth, increased apoptosis; up-regulation of SIRT7 promoted the motility of cancer cells. For the mechanism, we found that the level of apoptosis-related Bcl-2 was decreased after SIRT7 silenced. Moreover, SIRT7 show much homology to SIRT1 which has been best characterized and can target NF-κB. NF-κB signaling pathway has been reported to be of vital importance in the proliferation, apoptosis and migration of malignant diseases (Alvero, 2010). NF-κB/p65 and NF-κB/p50, main subunits of NF-κB family, may bind with apoptosis-related genes or proteins to modulate their expression once activated. Here, we detected that NF-κB/p65 was down-regulated when SIRT7 was depressed, whereas NF-κB/p50 had no significant change. Then co-immunoprecipitation was performed on the hypothesis that SIRT7 might interact with NF-κB/p65. However, we did not get successful results. Thus we speculated that there might be some proteins mediating the interaction of SIRT7 with NF-κB/p65, which needed to be discovered.

In conclusion, our reports describe SIRT7 as a protumorigenic protein. SIRT7 was up-regulated in cancer cells compared with the normal counterpart. SIRT7-deficiency in human ovarian cancer cells leads to cancer cell growth stardation and increased apoptosis; SIRT7-overexpression promotes the motility of ovarian cancer cells. However, further research need to be conducted on the clinical significance of SIRT7 in ovarian cancer and its precise molecular mechanism.

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