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MiR-34a/SIRT1 Axis Modulating Therapeutic Effect of Olaparib on Pancreatic Cancer through Suppressing PARP1 and EMT

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ABSTRACT This study analyzed functions of miR-34a and SIRT1 in modulating efficacy of Olaparib in pancreatic cancer in vitro. RNA expressions of miR-34a and SIRT1 were evaluated through RT-qPCR in HPDE6-C7, SW1990, PANC-1 and MIA PaCa-2 cells. MiR-34a was promoted in cancer cell lines while SIRT1 was decreased. Luciferase reporter assay verified bindings between miR-34a and SIRT1. Moreover, E-cadherin was promoted by miR-34a mimics and SIRT1 suppression while N-cadherin and Vimentin were both downregulated. Additionally, PARP1 protein expression was increased after miR-34a promotion and SIRT1 downregulation. Besides that, PARP1 protein levels and EMT were significantly inhibited after treated by Olaparib (0, 0.5, 1 and 1.5µM). In Olaparib-treated cancer cells, SIRT1, PARP1 and EMT were all distinctly decreased after SIRT1 suppression and overexpression of miR-34a induced much lower level of SIRT1.

INTRODUCTION

Pancreatic cancer is a fatal malignant cancer that commonly occurred in males and aged people (Goral 2015) and adenocarcinoma takes about 90 percent among all kinds of pancreatic cancers. With the development of economy and improved life standard, incidence rate and mortality of pancreatic cancer are gradually increased including China (Lin et al. 2015). Though smoking, diabetes, obesity, dietary factor and family history were recognized as risk factors in pancreatic cancer, pathogenesis of this malignancy is still short of comprehension (Andersen et al. 2017; Ezzati et al. 2005; Hamada et al. 2019; Lu et al. 2017; Pothuraju et al. 2018). As one of the most common malignances, overall survival of pancreatic cancer patients is about 5 percent and there are tiny differences among developed countries and developing countries (Sant et al. 2003). According to reports, 5 percent to 10 percent of pancreatic cancer occurs because of heredity (Raimondi et al. 2009). Hence, it is necessary to figure out mechanisms of pancreatic cancer to get better treatment of this threat. With development of gene technology, BRCA2 mutation, a gene related to DNA repair, has been determined to take the highest percentage of genetic pancreatic cancer (Couch et al. 2007; Vincent et al. 2011), revealing that DNA repair is an important step during occurrence of pancreatic cancer. Based on previous studies, Poly (ADP-ribose) polymerase (PARP) 1/2 identifies

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DNA damages and accelerates DNA repair (Wang et al. 2006; Wang et al. 2019), which helps maintain abilities of cancer cells. Under this circumstance, PARP inhibitors attract great concerns for killing pancreatic cancer cells through PARP suppression (Zhu et al. 2020).

MicroRNAs are small endogenous RNAs that can regulate apoptosis, cell growth and differentiation through adjusting expressions of mRNAs at post-transcriptional level (Zhu et al. 2020). In pancreatic cancer, functions of miRNAs were detected as well (Bi et al. 2020). MiR-34a was determined to have correlation with good prognosis of patients suffered from pancreatic cancer which could suppress progressions of tumors (Ji et al. 2009; Tang et al. 2017). Moreover, miR-34a has been reported to downregulate level of PARP1 in BEAS-2B cells (Wu et al. 2019). Besides that, miR-34a has also been proven to suppress PARP1 in human mesenchymal stem cells in vitro (Ghasemzadeh et al. 2018). However, correlation between those two genes in pancreatic cancer has not been mentioned.

Sirtuin 1 is a protein deacetylase which can target histone and non-histone proteins (Alves-Fernandes et al. 2019). Besides that, SIRT1 has been confirmed to participate in DNA damage response, which can accelerate DNA repair and prevent DNA damage (Alves-Fernandes et al. 2019). SIRT1 was proven to be targeted by miR-34a in INS-1 Beta cells (Kong et al. 2019), which has been reported to accelerate the EMT in pancreatic cancer cells (Deng et al. 2014). Besides that, SIRT1 was found to be an oncogene and upregulated PARP in colon cancer (Lucena-Cacace et al. 2018). Nevertheless, its regulation to PARP in pancreatic cancer was seldom detected.

Olaparib is an PARP inhibitor formerly named AZD2281, which was used to treat recurrent ovarian cancer and patients with BRCA mutation (Bochum et al. 2018). Olaparib increased overall rate of survivors in pancreatic cancer (Golan et al. 2019). Based on these studies, the researchers wondered that correlation between miR-34a and SIRT1 were also existed in pancreatic cancer cells and their correlation modulated Olaparib to regulate progression of pancreatic cancer cells by mediating PART1.

Objectives

The study aimed at measuring functions of upregulated miR-34a, suppressed SIRT1, Olaparib treatment and their correlations in mediating EMT and PARP1 expressions in pancreatic cancer cells.

METHODOLOGY

Experimental

Main Reagents

RPMI-1640 medium (Gibco™, USA), FBS (Gibco, USA), Olaparib (Selleck Chemicals, USA), Lipofectamine 3000 (Invitrogen, USA), opti-MEM medium (Gibco, USA), TRIzol (Invitrogen, USA), High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA), All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, USA), PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, USA), pmirGLO (Promega, USA), RIPA Lysis and Extraction Buffer (Thermo Scientific™, USA), BCA protein assay kit (Beyotime, Shanghai, China), anti-PAPR1 (1:1000; ab32138, Abcam, UK), GAPDH (1:2000; ab181602), Goat Anti-Mouse IgG H&L (HRP) (1:800; ab205719), ECL Substrate Kit (High Sensitivity) (ab133406), iBright FL1500 (Invitrogen, USA).

Cell Culture

HPDE6-C7, human normal pancreatic ductal epithelial cell line and pancreatic cancer cell lines SW1990, PANC-1 and MIA PaCa-2 were all obtained from ATCC (USA). After cells were unfrozen in water at 37^0C, RPMI-1640 medium (Gibco™, USA) appended with 10% FBS (Gibco, USA) were applied to culture cells at 37^0C, 5 percent CO_2 in a humidity incubator. Later, Olaparib (0μM, 0.5μM, 1μM and 1.5μM) were added into SW1990 and PANC-1 cells to make preparations for following experiments.

Cell Transfection In Vitro

MiR-34a mimics and small interfering RNA of SIRT1 were provided by GenePharma (Shanghai, China) and controlled sequences were named siNC and NC mimics. SW1990 and PANC-1 cells in log phase were chosen for transfection and cells were first planted onto 6-well plates. Followed by manufacturer's instructions of Lipofectamine 3000 (Invitrogen, USA), NC mimics, miR-34a mimics, siNC, siSIRT1 and miR-34a mim-

ics with siSIRT1 were transfected into SW1990 and PANC-1 cells, respectively after confluences of cells reached 80 percent. After transfections for 24h, the opti-MEM medium (Gibco, USA) was substituted by RPMI-1640 medium with 10 percent FBS. Relative expressions of miR-34a and SIRT1 were detected by RT-qPCR.

RT-qPCR

Based on the specification of TRIzol (Invitrogen, USA), total RNA was separated. Thereafter, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA) was applied for creating cDNA through reverse transcription of SIRT1 RNA and reverse transcription of miR-34a was performed through All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, USA). PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, USA) was used for detecting miR-34a and SIRT1 in Applied Biosystems™ 7500 Real-Time PCR System (Applied Biosystems, USA). Conditions of PCR were listed: predenaturation at 95^{0}C, 5min; denaturation at 95^{0}C, 30s; annealing at 60^{0}C, 30s and extension at 72^{0}C, 30s, 40 cycles. U 6 was applied for normalizing of miR-34a expressions and GAPDH was internal control of SIRT1. Relative expressions were calculated by $2^{-\Delta\Delta Ct}$ methods. Sequences of primers were displayed as follows: miR-34a, F, 5'- CCAGCTGTGAGTGTTTCTTTG-3' and R, 5'-CAGCACTTCTAGGGCAGTAT-3'; U 6, F, 5'- CGAGCUGGUAAAGAAUUUATT-3' and R, 5'- UAAAUUCUUUACCAGCUCGTT-3' (Kong et al. 2019); SIRT1, F, 5'- GCCTCACATGCAAGCTCTAGTGAC-3' and R, 5'- TTCGAGGATCTGTGCCAATCATAA-3', GAPDH, F, 5'-CAGAACATCATCCCTGCCTCTAC-3' and R, 5'-TTGAAGTCAGAGGAGACCACCTG-3' (Nishigaki et al. 2020), E-cadherin, F, 5'- CAAATCCAACAAAGACAAAGAAGGC-3' and R, 5'- ACACAGCGTGAGAGAAGAGAGT-3'; N-cadherin, F, 5'- CATCATCATCCTGCTTATCCTTGT-3' and R, 5'-GGTCTTCTTCTCCTCCACCTTCT-3' and Vimentin, F, 5'- TCGTGATGCTGAGAAGTTTCG-3' and R, 5'-TCTGGATTCACTCCCTCTGGT-3'. Results were obtained from three independent replicates .

Luciferase Reporter Assay

To confirm bindings between SIRT1 and miR-34a, wild type and mutant type of SIRT1 sequences (SIRT1-wt/mut) were amplified and then inserted into pmirGLO (Promega, USA), respectively. Meanwhile, SW1990 and PANC-1 cells were planted into 12-well plates. Wild type of SIRT1 with mimics of NC or miR-34a and mutant type of SIRT1 with mimcs of NC or miR-34a were co-transfected into cells using Lipofectamine 3000 after cell confluence reached 70 percent. Luciferase activities in cells were tested using Dual luciferase reporter assay system (Promega, USA). This experiment was run in a triplicate.

Western Blot

RIPA Lysis and Extraction Buffer (Thermo Scientific™, USA) was applied to extract total protein from SW1990 cells and then total protein were quantified through BCA protein assay kit from Beyotime (Shanghai, China). Later, protein was isolated by 10 percent SDS-PAGE and shifted onto PVDF membranes. Then, 5 percent skimmed milk powder was for blocking membranes and then cultured with primary antibodies, anti-PAPR1 (1:1000; ab32138, Abcam, UK) and GAPDH (1:2000; ab181602) at 4^{0}C overnight. Thereafter, Goat Anti-Mouse IgG H&L (HRP) (1: 800; ab205719) was applied for culturing with PVDF membranes at 25^{0}C after incubated with primary antibodies and rinsed by PBS three times. ECL Substrate Kit (High Sensitivity) (ab133406) was used to developing and iBright FL1500 (Invitrogen, USA) was for imaging. Gray values of proteins were measured and GAPDH was treated as internal interference. The experiments were repeated three times.

Statistical Analysis

Experiments were all run in a triplicate. Data were displayed as mean±SD and analyzed by GraphPad Prism7 (USA) and SPSS 19.0 (IBM, USA). Student's t test was used to examine comparisons between two groups one-way ANOVA was applied to analyze comparisons of EMT and PARP1 in miR-34a mimics and SIRT1 suppression. $P<0.05$ was treated as having statistical significance.

RESULTS

MiR-34a Downregulated in Pancreatic Cancer Cells and Inhibited EMT and PARP1

RNA expressions of miR-34a were measured in HPDE6-C7, MIA PaCa-2, SW1990 and PANC-

1, revealing that miR-34a RNA expression were significantly lower in cancer cells than HPDE6-C7 cells (Fig. 1A, **P<0.05). Thereafter, RNA level of miR-34a was dramatically promoted after up-regulated transfection in SW1990 and PANC-1 cells (Fig. 1B, C, **P<0.05). Meanwhile, factors of EMT were detected through RT-qPCR showing that E-cadherin expression was much higher after miR-34a overexpression than the control group while expressions of N-cadherin and Vimentin were obviously reduced (Fig. 1D, E, **P<0.05). Protein level of PARP1 was checked later, revealing that miR-34a overexpression sharply downregulated protein expression of PARP1 in SW1990 and PANC-1 cells (Fig, 1F, G, H, **P<0.05).

SIRT1, Directly Targeted by miR-34a, was Promoted in Pancreatic Cancer Cells and Promoted EMT and PARP1 Expression

Based on StarBase (http://www.sysu.edu.cn/), putative binding sites between SIRT1 and miR-34a were acquired (Fig. 2A). According to this, The bindings were analyzed through luciferase reporter assay, showing that miR-34a directly bound wild type of SIRT1 in PANC-1 and

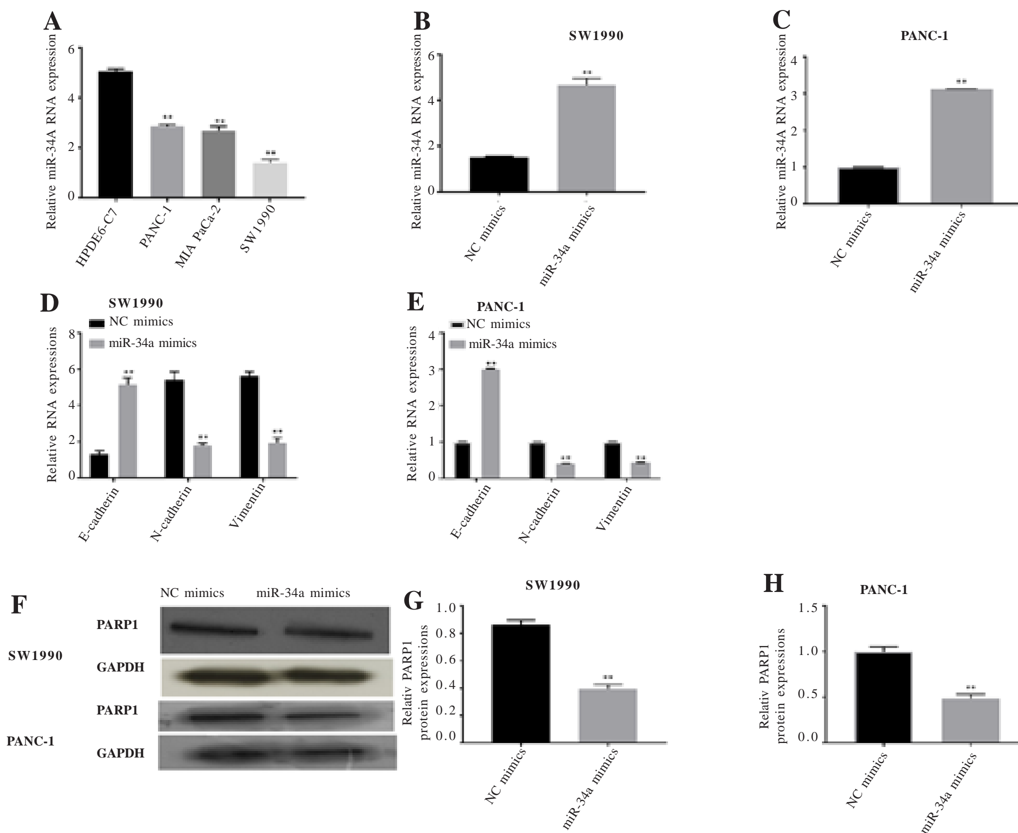

Fig. 1. MiR-34a downregulated in pancreatic cancer cellss and inhibited EMT and PARP1 A: Expression of miR-34a in PANC-1, SW1990, MIA PaCa-2 and HPDE6-C7 cell lines were evaluated with RT-qPCR, **P<0.05 versus HPDE6-C7 cells. B, C: RNA level of upregulated miR-34a in PANC-1 and SW1990 cells were checked through RT-qPCR, **P<0.05 versus NC mimics group. D, E: E-cadherin, N-cadherin and Vimentin expressions were exmained through RT-qPCR with miR-34a upregulation, **P<0.05 compared to NC mimics group. F, G, H: Western blot was for measuring PARP1 protein level after miR-34a was upregulated, **P<0.05 versus NC mimics group. Each experiment was run in a triplicate.

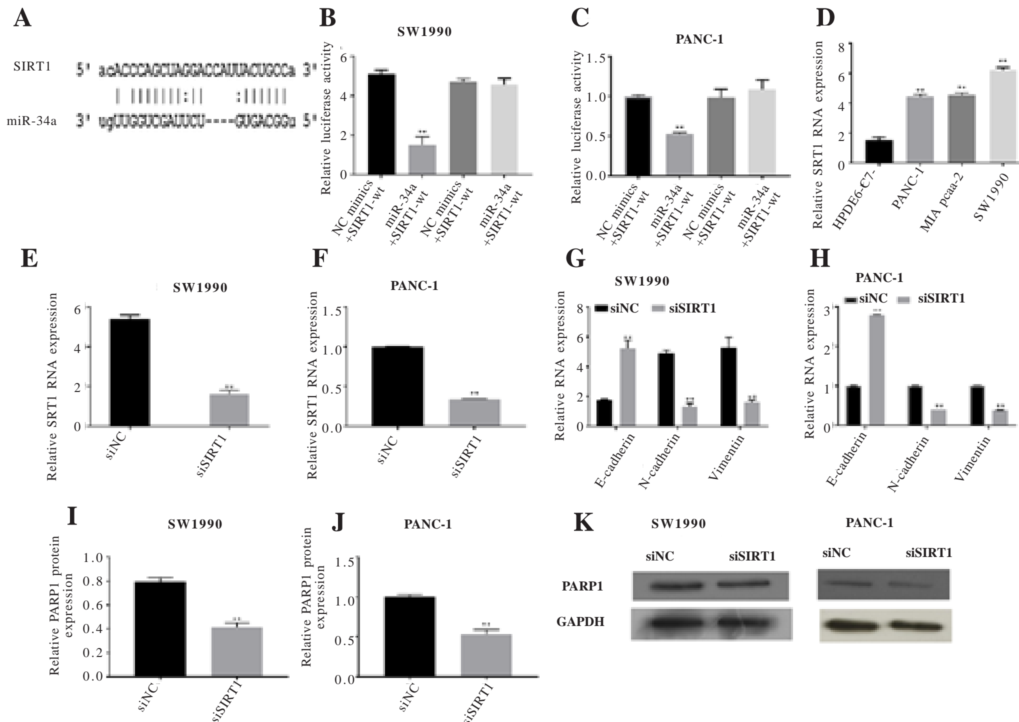

Fig. 2. SIRT1, directly targeted gene of miR-34a, was upregulated in pancreatic cancer cells with promoting EMT and PARP1 expression A: Starbase was applied to provide putative binding sites between miR-34a and SIRT1. B, C: Bindings between miR-34a and SIRT1 were measured through luciferase reporter assay, **P<0.05. D: RNA expression of SIRT1 in MIA PaCa-2, HPDE6-C7,SW1990 and PANC-1 cell lines were validated by RT-qPCR, **P<0.05 compared to HPDE6-C7 cells. E, F: suppressed SIRT1 expression was measured with RT-qPCR in PANC-1 and SW1990 cells, **P<0.05 in comparison with siNC group. G, H: E-cadherin, N-cadherin and Vimentin levels were examined through RT-qPCR, **P<0.05 in comparison with siNC group. I, J, K: PAPR1 protein expression after SIRT1 suppression was measured by western blot, **P<0.05 in comparison with siNC group. All experiments were run in a triplicate.

SW1990cells (Fig. 2B, C, **P<0.05). Then, expressions of SIRT1 in HPDE6-C7 and cancer cell lines were analyzed through RT-qPCR, which determined that cancer cell lines had much higher expressions of SIRT1 compared to normal HPDE6-C7 cell line (Fig. 2D, **P<0.05). Later, inhibited SITR1 was produced to measure functions of SIRT1 in PANC-1 and SW1990 cells and knockdown of SIRT significantly suppressed its RNA expression (Fig. 2E, F, **P<0.05). Moreover, N-cadherin and Vimentin were both repressed after SIRT1 downregulation while E-cadherin was significantly upregulated (Fig. 2G, H, **P<0.05). Besides that, PARP1 protein expressions were also inhibited by knockdown of SIRT1 (Fig. 2I, J, K, **P<0.05).

MiR-34a Enhanced Therapeutic Effects of Olaparib Through Binding SIRT1

Different concentrations of Olaparib (0, 0.5, 1 and 1.5 μM) was used to measured changes of PARP1 expressions, which showed that PARP1 protein expressions were significantly suppressed dose dependently by increased concentrations of Olaparib (Fig. 3A, B, C, **P<0.05). Moreover, EMT was measured as well, which showed that E-cadherin expression was upregulated after Olaparib treatment while N-cadherin and Vimentin were downregulated (Fig. 3D, E, **P<0.05). Thereafter, correlation between SIRT1 and miR-34a in cancer cells were measured for regulating effects

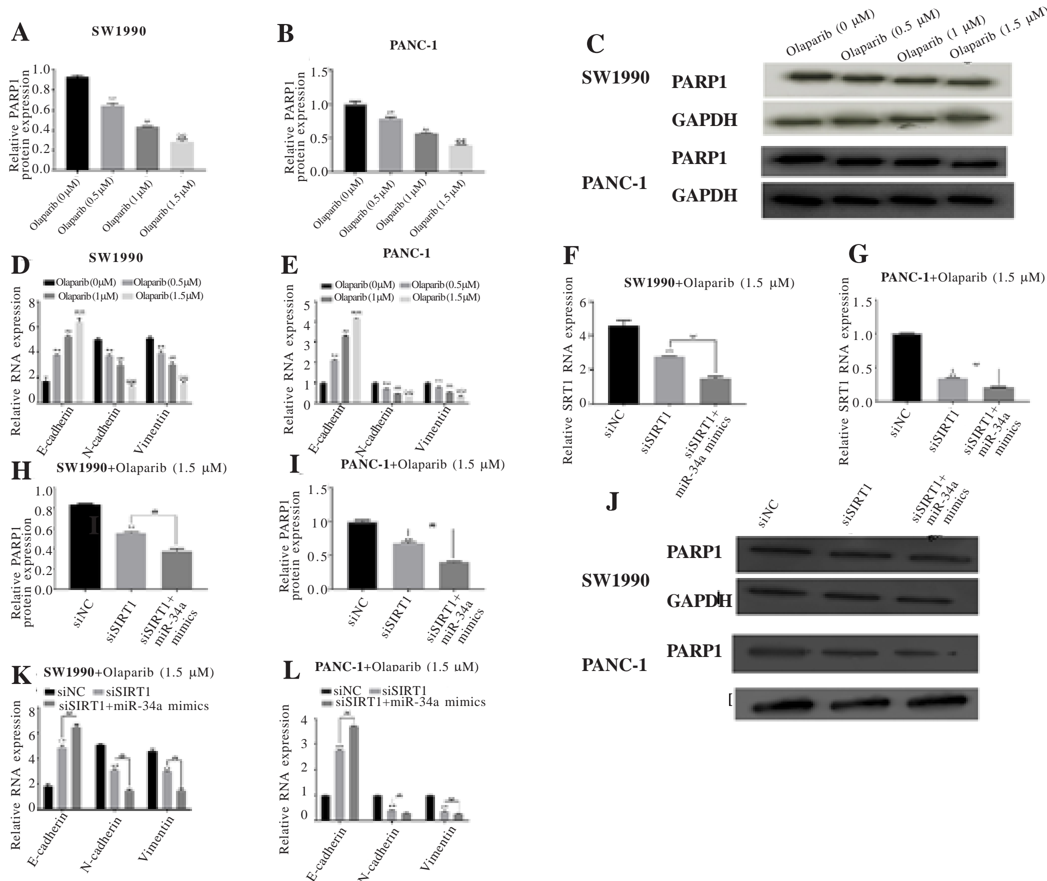

Fig. 3. MiR-34a magnified therapeutic effect of Olaparib through binding SIRT1 A, B, C: PARP1 protein expressions with Olaparib (0, 0.5, 1, 1.5ìM) treatment in SW1990 and PANC-1 cells were exmained using western blot, **P<0.05 versus Olaparib (0ìM) group, ^^P<0.01 versus Olaparib (0.5ìM) group and @@P<0.001 in comparison with Olaparib (1ìM) group. D, E: E-cadherin, N-cadherin and Vimentin in SW1990 and PANC-1 cells after treated by Olaparib (0, 0.5, 1, 1.5ìM) were detected by RT-qPCR, **P<0.05 versue Olaparib (0ìM) group, ^^P<0.01 in comparison with Olaparib (0.5ìM) group and @@P<0.001 versus Olaparib (1ìM) group. F, G: Expressions of SIRT1 after SIRT1 suppression and siSIRT1 with miR-34a mimics in SW1990 and PANC-1 cells treated by 1.5ìM Olaparib were evaluated with RT-qPCR, **P<0.05 versus siNC group and ##P<0.05 versus siSIRT1 group. H, I, J: Western blot was applied to measure protein expressions of PARP after SIRT1 suppression and siSIRT1 with miR-34a overexpression in Olaparib-treated PANC-1 and SW1990 cells, **P<0.05 versus siNC group and ##P<0.05 versus siSIRT1 group. K, L: E-cadherin, N-cadherin and Vimentin after treated with Olaparib (1.5ìM) were detected by RT-qPCR after SIRT1 suppression and siSIRT1 with miR-34a overexpression, **P<0.05 versus siNC group and ##P<0.05 versus siSIRT1 group. Experiments were all repeated three times.

of Olaparib on pancreatic cancer. After SIRT1 was inhibited, expression of SIRT1 was significantly decreased and mimics of miR-34a facilitated the inhibition of SIRT1 (Fig. 3F, G, **P<0.05). Moreover, miR-34a overexpression promoted the downregulation of PARP1 expression after SIRT1 suppression (Fig. 3H, I, J, **P<0.05). E-cadherin was upregulated by SIRT1 knockdown and then enhanced by overexpression of miR-34a while N-cadherin and Vimentin were dramatically downregulated (Fig. 3K, L, **P<0.05).

DISCUSSION

PARP1 plays a key role in repairing damaged DNA to put off cell death, which has been detected to promote occurrence of pancreatic cancer (Xu et al. 2019). PARP inhibitor can capture PARP1 protein in DNA lesion, resulting in double-strand breaks (Zhu et al. 2020). Olaparib, a PARP inhibitor, was applied for combination therapy of many kinds of cancer in clinical stage (Lee et al. 2014). However, combination of other drugs could cause apparent toxicity of blood (Rajan et al. 2012). Therefore, how to improve effects of Olaparib on treating pancreatic cancer is a hot spot. During previous studies, miR-34a acts as a tumor inhibitor in pancreatic cancer. According to the study of Yan Chen et al, miR-34a suppressed proliferation and invasiveness of pancreatic cancer cells, which also inhibited the progression of pancreatic cancer in vivo (Chen et al. 2019). Additionally, miR-34a has been demonstrated to suppress EMT in pancreatic cancer cells through downregulating expressions of Snail1 and Notch1 (Tanget al. 2017). MiR-34a was found to express lower in pancreatic cancer cells. Moreover, overexpressed miR-34a upregulated its expression of pancreatic cancer which promoted E-cadherin level and reduced expressions of N-cadherin and Vimentin. Compared to former studies, this research has demonstrated that miR-34a suppressed PARP1 protein expression in pancreatic cancer cells in vitro, which is a breakthrough. Therefore, researchers first got primary knowing that miR-34a could retard progression of pancreatic cancer and block EMT as well as PARP1 expression. However, whether there is connection between miR-34a and Olaparib still need further detections.

Recently, SIRT1 was proven to take part in progression of cancer as an anti-apoptosis biomarker (Saunders et al. 2007). Potential binding sites between SIRT1 with miR-34a were discovered through measurements of StarBase, which was proven to accelerate progression of pancreatic cancer (Gong et al. 2013). The researchers have demonstrated that miR-34a could directly combine with SIRT1 in pancreatic cells. Moreover, SIRT1 was found to express higher in pancreatic cancer cell lines. Suppression of SIRT1 blocked EMT and PARP1 in SW1990 and PANC-1 cells. Therefore, PARP1 downregulation caused by SIRT1 knockdown in pancreatic cancer cells in vitro has been first demonstrated in this study. Thereafter, correlation between miR-34a and SIRT1 were checked for evaluating effects on Olaparib. Olaparib treatment in SW1990 cells significantly inhibited PARP1 expression and EMT. Moreover, siSIRT1 in SW1990 and PANC-1 help to reduce EMT and PARP expressions. Furthermore, after knockdown of SIRT1, overexpression of miR-34a caused a increase of E-cadherin but significantly inhibited N-cadherin, Vimentin and PARP1. Hence, compared with previous studies, this research has demonstrated that SIRT1 inhibition significantly promoted efficacy of Olaparib in PANC-1 and SW1990 cells and miR-34a upregulation magnified effects of Olaparib. According to these measurements, the researchers could determine that miR-34a contributed to treatment effect of Olaparib on pancreatic cancer through binding SIRT1.

CONCLUSION

Therapeutic effect of Olaparib was magnified by miR-34a in pancreatic cancer through targeting SIRT1, which caused suppressed EMT and PARP1. Hence, MiR-34a and SIRT1 might be new biomarkers for improving efficacy of Olaparib in pancreatic cancer. However, in vivo and clinical detections are urgently needed for getting further knowledge.

RECOMMENDATIONS

MiR-34a was downregulated in pancreatic cancer cells while SIRT1 was significantly promoted. SIRT1 was then proven to be targeted by miR-34a in SW1990 and PANC-1 cells. PARP1 and EMT were both distinctly suppressed by miR-34a overexpression or SIRT1 inhibition. Moreover, Olaparib treatment (0, 0.5, 1 and 1.5μM) inhibited PARP1 protein levels dose dependently and SIRT1 knockdown with miR-34a upregulation enhanced effects of Olaparib through blocking PARP1 and EMT.

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ABBREVIATION LIST

MiR-34a: microRNA-34a; PARP: poly-ADP-ribose polymerase; EMT: epithelial-mesenchymal

transition; mRNA: messenger RNA ; SIRT1: Sirtuin 1; FBS: Fetal bovine serum; RT-qPCR: quantitative reverse transcription Polymerase Chain Reaction; ATCC: American Type Culture Collection.

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