

Enhancing the sensitivity of ovarian cancer cells to olaparib via microRNA-20b-mediated cyclin D1 targeting

Qian Zhong^{1,2} , Ying Xiong^{1,2}, Chen Ling^{1,2}, Yanping Qian^{1,2}, Xia Zhao^{1,2} and Hanshuo Yang³

¹Department of Gynecology and Obstetrics, West China Second University Hospital of Sichuan University, Chengdu, P.R. China; ²Key Laboratory of Birth Defects and Related Diseases of Women and Children, Sichuan University, Ministry of Education, Chengdu, P.R. China; ³State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, P.R. China

Corresponding authors: Qian Zhong. Email: cdcd91761@163.com; Hanshuo Yang. Email: yhansh@126.com

Impact statement

Although PARP inhibitors have shown significant clinical efficacy in the treatment of ovarian cancer, they are effective only in tumors with homologous recombination (HR) repair defect. Our previous study reported that cyclin D1 silencing interfered with RAD51 accumulation, enhanced the therapeutic efficacy of olaparib against BRCA1 wild-type ovarian cancer. Here, we found that miR-20b targets the cyclin D1 gene and induces G0/G1 cell cycle arrest in ovarian cancer cells. MiR-20b also interfered with RAD51 expression, thereby affecting HR efficacy (indicated by decreased γ -H2AX expression) and consequently sensitizing ovarian cancer cells to olaparib. Analysis of TCGA dataset showed that increased miR-20b expression was significantly associated with longer progression-free survival of ovarian cancer patients. This study suggests the possibility to predict the prognoses of patients with ovarian cancer using miRNAs and indicates that PARP inhibition may be useful to treat BRCA wild-type ovarian cancer when miRNA-based drug delivery systems are developed.

Abstract

We previously reported that cyclin D1 silencing interferes with RAD51 accumulation and increases the sensitivity of BRCA1 wild-type ovarian cancer cells to olaparib. However, the mechanisms associated with cyclin D1 overexpression in ovarian cancer are not fully understood. TargetScan predicted the potential binding sites for microRNA-20b (miR-20b) and the 3'-untranslated region of cyclin D1 mRNA; thus, we used luciferase reporter assay to verify those binding sites. The Kaplan-Meier method and log-rank test were used to examine the relationship between miR-20b and progression-free survival of ovarian cancer patients in The Cancer Genome Atlas ($n = 367$) dataset. *In vitro* experiments were performed to evaluate the effects of miR-20b on cyclin D1 expression, cell cycle and response to olaparib. A peritoneal cavity metastasis model of ovarian cancer was established to determine the effect of miR-20b on the sensitivity of olaparib. Immunohistochemistry was performed to evaluate molecular mechanisms. In this work, we demonstrated that miR-20b down-regulates cyclin D1, increases the sensitivity of ovarian cancer cells to olaparib, reduces the expression of RAD51, and induces cell cycle arrest in G0/G1 phase. Ovarian cancer patients with higher expression of miR-20b had significantly longer progression-free survival. These results indicate that miR-20b may be a potential clinical indicator for the sensitivity of ovarian cancer to olaparib and the survival of ovarian cancer patients. Our findings suggest that miR-20b may have therapeutic value in combination with olaparib treatment for ovarian cancer.

Keywords: Ovarian cancer, prognosis, microRNA-20b, olaparib, cyclin D1

Experimental Biology and Medicine 2021; 246: 1297–1306. DOI: 10.1177/1535370221994077

Introduction

Epithelial ovarian cancer has the highest mortality rate among all gynecological tumors. Although the initial response to treatment (surgery plus platinum-based chemotherapy) is usually effective, drug resistance is still a major obstacle to successful treatment.¹ Recently, the development of molecular targeted therapy based on DNA damage repair has opened a new chapter in ovarian cancer therapy. The FDA-approved poly (ADP-ribose) polymerase inhibitors (PARPi) are representative agents

for DNA repair-targeted treatment and are currently used as monotherapy for advanced ovarian cancer with germline BRCA1/2 mutations.² Although PARPis are highly effective, they can only treat tumors with homologous recombination (HR) defects. A large number of tumors with de novo or acquired HR proficiency do not respond to PARPi, which narrows the use of these drugs.^{3–5} Therefore, the development of strategies to block HR repair may enhance the anti-tumor effect of PARPis and expand their application in the treatment of ovarian

cancer.⁶⁻¹⁰ Our previous work demonstrated that silencing of cyclin D1 enhanced the therapeutic efficacy of the PARPi olaparib against BRCA1 wild-type ovarian cancer cells via interference with the accumulation of RAD51 and arresting the cell cycle in G0/G1 phase. In addition, overexpression of cyclin D1 reduced the sensitivity of ovarian cancer cells to olaparib.¹¹ However, the mechanisms associated with cyclin D1 overexpression in ovarian cancer have not been fully studied.

MicroRNAs (miRNAs) repress the expression of target genes by binding to the 3'-untranslated regions (UTR) of the messenger RNA (mRNA). Multiple studies have shown that miRNAs are highly dysregulated in cancer, indicating that miRNAs may be biomarkers for predicting treatment outcome or serve as therapeutic tools.^{12,13} We speculated that cyclin D1 expression in ovarian cancer may be regulated by miRNA.

Materials and methods

Prediction for miRNAs that targeting cyclin D1 in silico

TargetScan was introduced to predict the possible miRNAs that regulate *CCND1* gene (cyclin D1 is encoded by *CCND1* gene).

Luciferase reporter assay

The 3'-UTR of *CCND1* includes two predicted binding sites. Both the wild-type 3'-UTRs and the mutant 3'-UTRs (synthesized by General Biosystems (Anhui) Co., Ltd) were cloned into the pGL3-control plasmid (Promega) at XbaI/XbaI sites directly downstream from the luciferase coding sequence. For the luciferase reporter assay, cells were seeded in 96-well plates 24 h before transfection at a density of 5×10^3 per well. Then, cancer cells were co-transfected with pRL-TK plasmid and miR-20b mimic or control mimic plus the parent luciferase expression vector, wild-type or mutant *CCND1* 3'-UTR plasmids using Lipofectamine 2000 (Thermo Fisher). Forty-eight hours later, the cells were subjected to lysis, and the luciferase activity was tested via the dual-luciferase assay reporter system (Promega).

Cell culture and reagents

The A2780 and SKOV3 ovarian cells and ES2 cells (BRCA1 wildtype)¹⁴ were from Prof. Yuquan Wei. The cells were cultured according to the previous report.^{1,15} Olaparib was purchased from Sigma-Aldrich. MiR-20b mimic, control mimic, miR-20b inhibitor, and the control inhibitor were bought from GenePharma (Shanghai, China). Sequences of miR-20b mimic and inhibitor were as follows: NC mimic sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACAC GUUCGGAGAATT-3'; miR-20b-5p mimic sense: 5'-CAAAGUGCUCUAUGUGCAG GUAG-3', antisense: 5'-ACCGCACUAUGAGCACUUU GUU-3'; NC inhibitor, 5'-CAGUACUUUUGU GUAGU ACAA-3'; miR-20b-5p inhibitor, 5'-CUACCUGCACUA UGAGCACUUUG-3'.

RT-PCR and Western blot analysis

The experiments were done as previously described.¹¹ The following specific RT-PCR primers were used: hsa-miR-20b-5p, 5'-GTCGTATCCAGTGCCTGTCGTGGAGTCGGC AATTGCACTGGATACGACCTACCTG-3'; U6, 5'-GAATT TCGTGTTCATCCTTG-3'. The following qRT-PCR primers were used: hsa-miR-20b-5p forward, 5'-GCCAAAGTG CTCATAGTGCA-3' and reverse, 5'-CAGTGCCTGTCGTGG AGT-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTA AAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGT CAT-3'; *CCND1* forward, 5'-GCGGAGGAGAAACAAACA GAT-3' and reverse, 5'-GAGGGCGGATTGGA AATGA-3'; *GAPDH* forward, 5'-GGTGTGAACCATGAGAAGTATG A-3' and reverse, 5'-GAGTCCT TCCACGATACCAAAG -3'.

Cell viability and cell cycle analysis

Cells incubated with miR-20b mimic, mimic control, miR-20b inhibitor or the control inhibitor were cultured overnight at 37°C. Afterwards, the cells were incubated with different concentrations of olaparib for five days, and the culture media was replaced with fresh media on the third day. MTT reagent (Sigma) was used to evaluate cell viability. Cell cycle study was carried out as previously described.¹¹

Colony-formation assay

SKOV3 and A2780 Cells were harvested 24 h after transfection with miR-20b mimic or control mimic. Transfected cells were seeded in six-well plates (200 cells/well). The medium was changed every three days. After about two weeks, cells were fixed in 4% PFA for 10 min and stained with crystal violet (Beyotime, China) for 10 min to count the colonies. Numbers of colonies were calculated manually and by Image J three times.

In vivo animal model

Female athymic nude mice (BALB/c, six to eight weeks old) fed in SPF-class laboratory animal room were introduced to develop the peritoneal cavity metastasis model of ovarian cancer. All the animal experiments followed the protocols of the Institutional Animal Care and Use Committee of Sichuan University. SKOV3 ovarian cancer cells were injected intraperitoneally according to the previous report.¹⁵ One week after injection, the mice were randomly divided into four groups ($n = 6$): (1) control group, 100 μ L of 5% glucose solution; (2) control mimic (4 μ g/20 g); (3) miR-20b mimic (4 μ g/20 g); (4) olaparib (50 mg/kg) + control mimic; (5) olaparib + miR-20b mimic. All groups received intra-abdominal injection of drug formulations. Mimic was given every other day and olaparib was given every day for four weeks.¹⁶ Mice were put to death following protocols, and the tumor xenografts were collected when the treatments finished.

Immunohistochemistry study

The immunohistochemistry study was carried out following previous protocols.¹¹ The slices were dewaxed in xylene

and dehydrated with gradient ethanol. The primary antibodies, namely anti-CCND1 (ab134175, Abcam), anti-RAD51 (ab133534, Abcam), and anti- γ -H2AX (ab26350; Abcam), were added into the slides and left overnight at 4°C. After washing with the PBS, the secondary antibody (K1HC-5, Proteintech) was added into the slides. One hour later, the signals were magnified by 3,3'-diaminobenzidine, and the nuclei were counterstained with hematoxylin.

Analysis of clinical data

The expression data of the level-3 miRNA isoform on the basis of miRNA-seq across 367 stage 2–4 ovarian cancers was obtained from The Cancer Genome Atlas (TCGA) data portal on 20 January 2019.

Statistical analysis

The experimental data was reported as the mean \pm SD of three repetitions. The two-sided Student's t test was introduced to compare results from two groups, and one-way analysis of variance method (ANOVA) was introduced to estimate results among multiple groups. * $P < 0.05$ was considered statistically significant. The statistical analyses were done by using SPSS 20.0 and R 2.10.0. The survival curves were analyzed using the log-rank test.

Results

CCND1 is directly targeted by miR-20b

TargetScan 7.2 indicated that the 3'-UTR of the CCND1 mRNA contains two potential miR-20b binding sites (Supplementary Figure S1). The putative binding sites and the flanking sequences are highly conserved across

mammals. To investigate whether miR-20b regulates CCND1, the 3'-UTR of CCND1 mRNA was cloned into the pGL3 luciferase reporter vector. We co-transfected CCND1-3'-UTR-WT and the parent luciferase expression vector into SKOV3 cells (along with miR-20b mimic or the control miR). The co-transfection of CCND1-3'-UTR-WT and miR-20b mimic caused 76% and 77% reduction (two binding sites) in luciferase activity compared with the control miRNA ($P < 0.001$). These results suggested that miR-20b targets CCND1. To determine whether miR-20b regulates CCND1 via the putative binding sites, we generated a mutant construct, CCND1-3'-UTR-MT, in which the putative miR-20b binding sites in the CCND1 3'-UTR were mutated. SKOV3 cells were then transfected with either miR-20b mimic or the control miRNA along with the mutant construct. The results indicated that the deletion of miR-20b and CCND1 binding sites eliminated the influence of miR-20b on the luciferase activity. These results confirmed that miR-20b negatively regulates the CCND1 gene by binding to the 3'-UTR (Figure 1).

MiR-20b reduced cyclin D1 expression and induced cell cycle arrest in ovarian cancer cells

Based on the real-time qPCR results of miR-20b expression in three BRCA1 wild-type ovarian cancer cell lines (Figure 2 (a)), and for consistency with our previous study,¹¹ SKOV3 and A2780 ovarian cancer cells were selected for experiments. Ovarian cancer cells were transfected with control mimic or miR-20b mimic. Real-time qPCR and Western blotting indicated that overexpression of miR-20b led to reduced expression of cyclin D1 mRNA and protein in the two cell lines. Conversely, cells treated with miR-20b inhibitor showed higher expression of CCND1 than cells

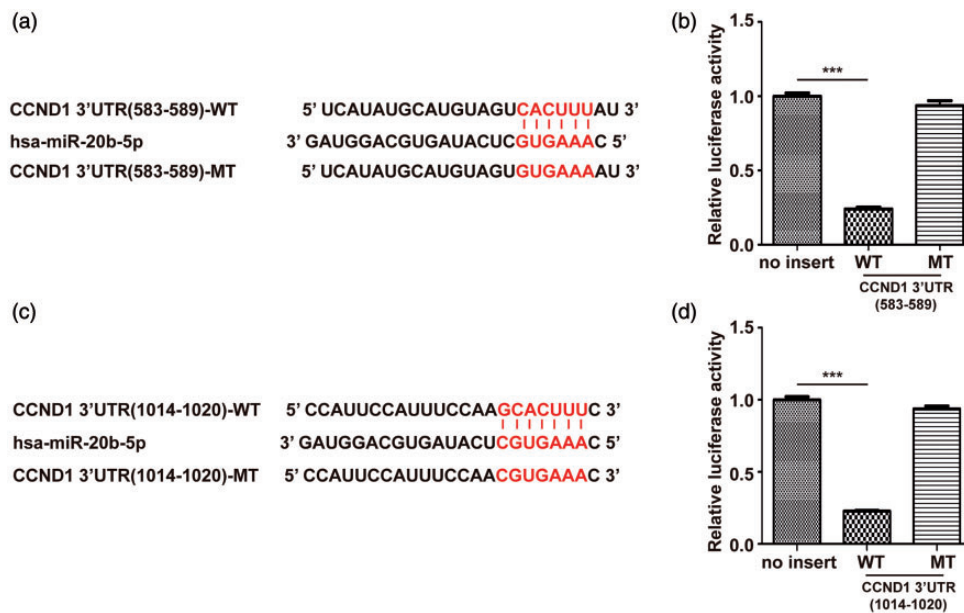


Figure 1. Targeting of cyclin D1 by miR-20b. (a) and (c) TargetScan predicted that two miR-20b binding sites were involved in the CCND1 3'-UTR, which are highly conserved among various species. (b) and (d) The luciferase reporter study indicated that miR-20b directly targeted the CCND1 3'-UTR. The SKOV3 cells were transfected with pRL-TK plasmid and control mimic or miR-20b mimic plus the parent luciferase expression vector, mutant or wild-type CCND1 3'-UTR plasmids. The reporter's luciferase activity was normalized to the internal renilla luciferase activity. Results shown here were mean \pm SD of triple experiments. *** $P < 0.001$. (A color version of this figure is available in the online journal.)

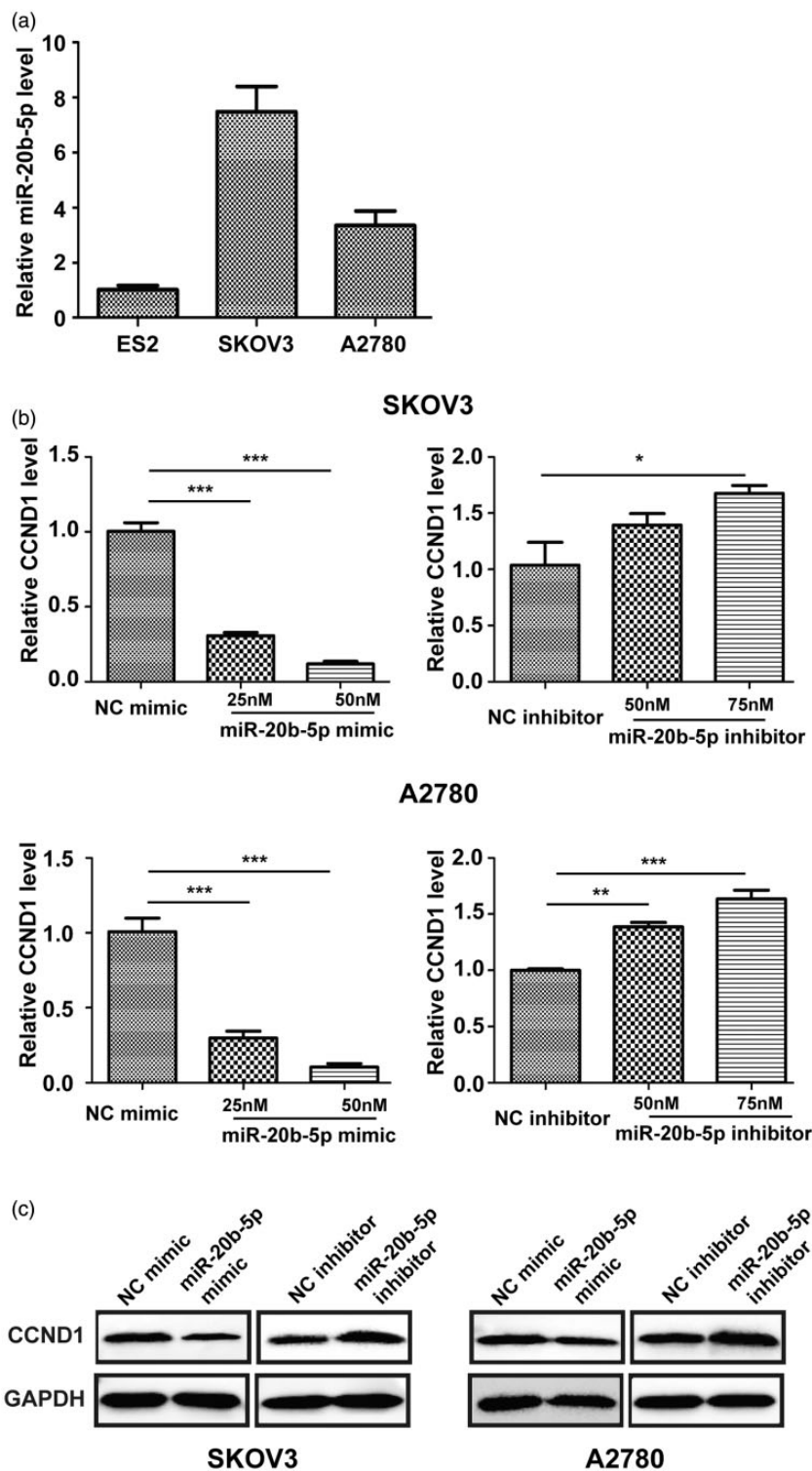


Figure 2. MiR-20b mimic or inhibitor treatment changed CCND1 expression. (a) Different expression level of miR-20b in three cell lines of ovarian cancer. (b) and (c) ovarian cancer A2780 and SKOV3 cells were treated with control mimic, miR-20b mimic, miR-20b inhibitor or the control inhibitor for 48 h; RT-qPCR and Western blot demonstrated the changes of CCND1 level. Results here were mean \pm SD (repeated 3 times). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

transfected with the control inhibitor (Figure 2(b) and (c)). We further found that the miR-20b mimic induced a cell cycle arrest in G0/G1 phase, along with a reduction of S-phase cells. In contrast, the percentage of cells in G0/G1 phase was decreased after treatment with miR-20b inhibitor (Figure 3(a) and (b)). These phenomena were consistent

with our previous study, in which we down-regulated or up-regulated *CCND1*.¹¹ These results suggested that miR-20b regulates the cell cycle through the regulation of *CCND1*. A previous study reported that olaparib-treated cells did not go through apoptosis.¹⁷ Consistent with the previous study, the combination treatment of miR-20b

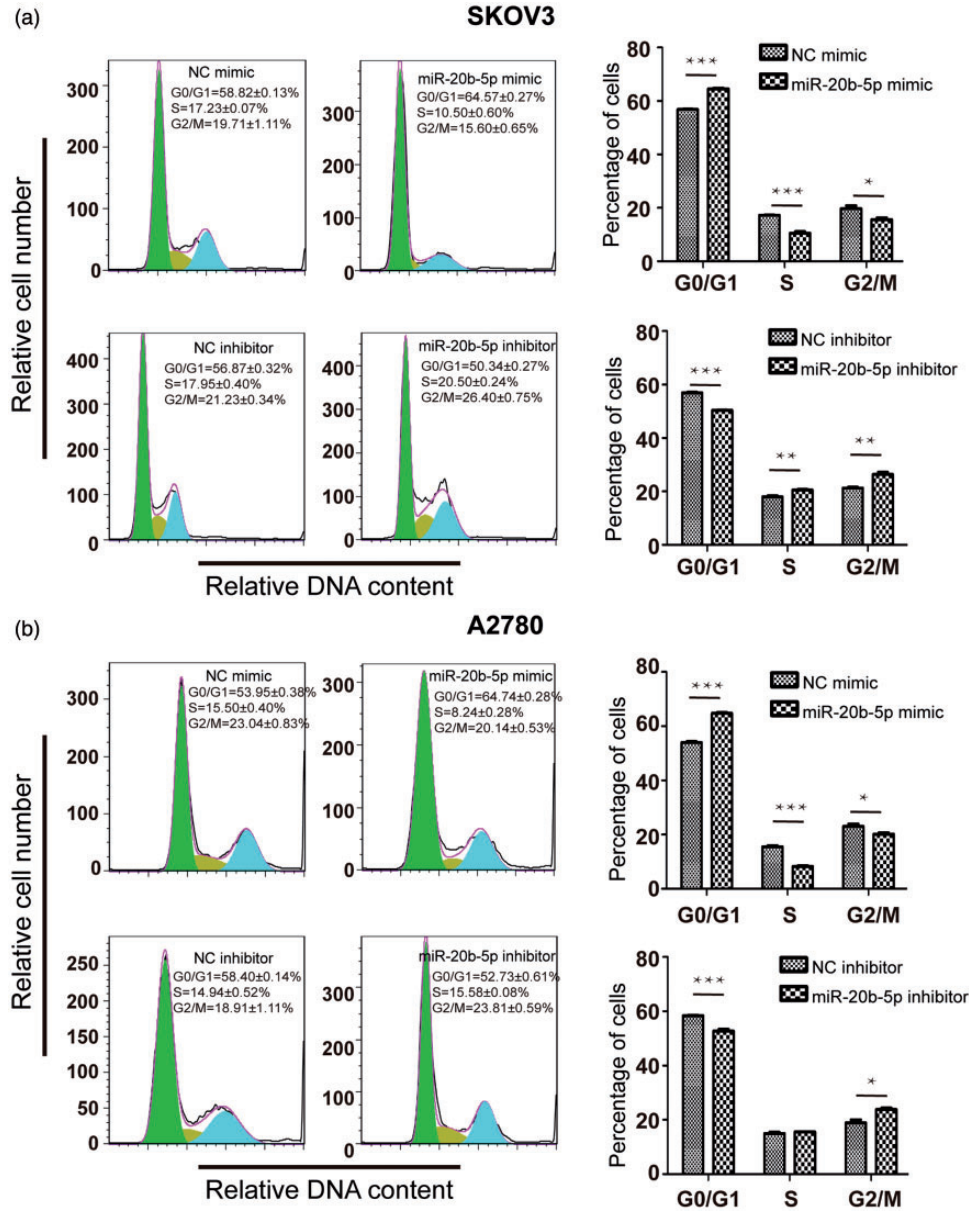


Figure 3. MiR-20b regulated cell cycle of the olaparib-treated cells. (a) Up-regulation or down-regulation of miR-20b by transfecting mimic or inhibitor in SKOV3 cells and 12 h later treated with olaparib at IC₅₀ concentration (15.42 μM). Five days after treatment, cell cycle analysis was conducted by flow cytometry. (b) Up-regulation or down-regulation of miR-20b by transfecting mimic or inhibitor in A2780 cells and 12 h later incubated with olaparib at IC₅₀ concentration (65.06 μM). Five days after treatment, cell cycle analysis was carried out by flow cytometry. Results here were mean ± SD (repeated three times). *P < 0.05, **P < 0.01, and ***P < 0.001. (A color version of this figure is available in the online journal.)

mimic or inhibitor with olaparib had little effect on the number of sub-G1 cells, which suggested that the cells did not undergo apoptosis (data not shown).

MiR-20b increased the sensitivity of ovarian cancer cells to olaparib in vitro

SKOV3 and A2780 ovarian cancer cells were treated with control mimic, miR-20b mimic, control inhibitor or miR-20b inhibitor. After 12h, cells were incubated with olaparib at different concentrations (SKOV3 cells: 0, 4, 8, 16, 32 μM, and A2780 cells: 0, 16, 32, 64, 128 μM) for five days. We found that miR-20b mimic-treated SKOV3 and A2780 cells showed more pronounced response to olaparib than control mimic-treated cells. In contrast, the cells treated with

miR-20b inhibitor showed resistance to olaparib compared with control cells in the two cell lines (Figure 4(a) and (b)). Upregulation of miR-20b markedly inhibited the growth ability of SKOV3 and A2780 cells, as indicated by decreased colony numbers (Supplementary Figure S2).

MiR-20b increased the sensitivity of ovarian cancer cells to olaparib in vivo

To explore the impact of miR-20b on the sensitivity of ovarian cancer cells to olaparib *in vivo*, we studied the efficacy of a combination treatment comprising of miR-20b mimic and olaparib in mice carrying ovarian cancer peritoneal xenografts. An intraperitoneal carcinomatosis model was created in nude mice by intraperitoneal injection of SKOV3 cells.

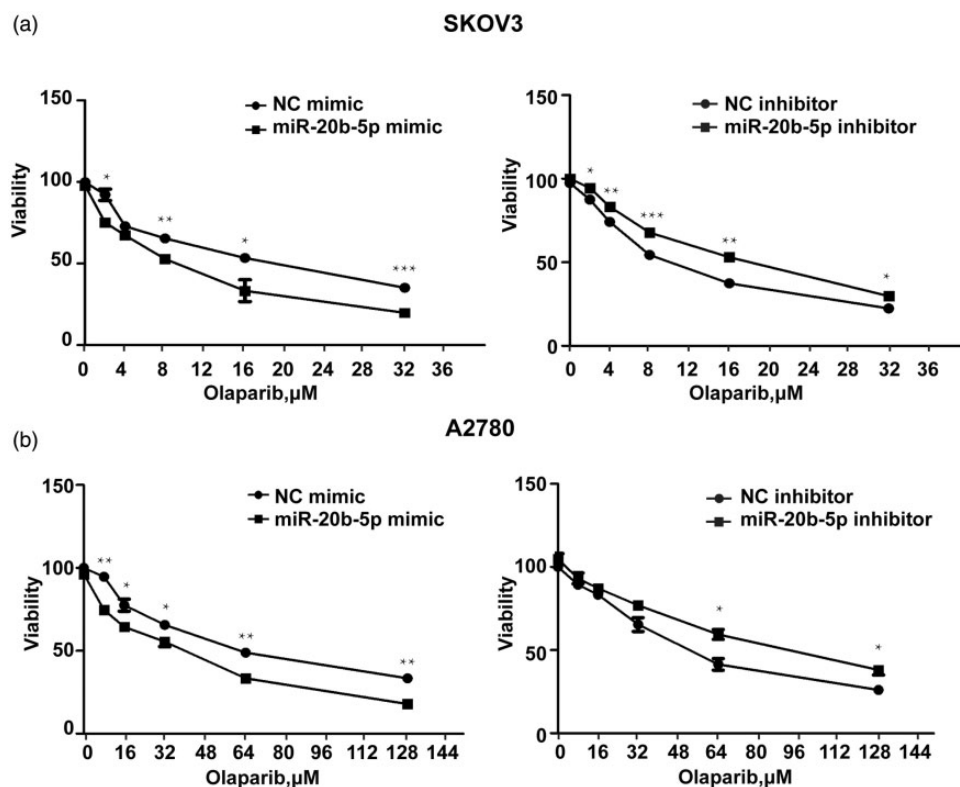


Figure 4. MiR-20b mimic or inhibitor treatment changed olaparib's response *in vitro*. Ovarian cancer cells treated with control mimic, miR-20b mimic, miR-20b inhibitor, or the control inhibitor were put in the 37°C incubator for one night, then incubated with olaparib for five days at different concentrations. MTT assay analyzed the influence of miR-20b on cell viability of olaparib treated A2780 and SKOV3 cells. Results here were mean \pm SD (repeated three times). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

The treatment groups were as follows: (1) control; (2) control mimic; (3) miR-20b mimic; (4) control mimic + olaparib; (5) miR-20b mimic + olaparib. In comparison to mimic control-treated mice (tumor weight = 0.56 ± 0.069 g), the miR-20b mimic-treated mice (tumor weight = 0.33 ± 0.061 g) carried significantly less tumor burden ($P < 0.001$). Although Olaparib + control mimic mice showed reduced tumor burden (tumor weight = 0.32 ± 0.029 g) compared with mimic control-treated mice, the combination of miR-20b mimic + olaparib led to significantly less tumor burden (tumor weight = 0.17 ± 0.024 g) compared with olaparib + control mimic mice ($P < 0.01$). The difference in tumor load between control mimic-treated mice and untreated mice (tumor weight = 0.56 ± 0.125 g) was not statistically significant (Figure 5(a) and (b)). These results showed that miR-20b may be a sensitizer of olaparib in ovarian cancer *in vivo*.

We next performed real-time qPCR, Western blot and immunohistochemistry studies to examine cyclin D1 expression in tumors. Consistent with the *in vitro* results, tumors in mice treated with miR-20b mimic showed significantly lower cyclin D1 mRNA and protein expression compared with control mimic-treated mice ($P < 0.01$). Cyclin D1 expression was significantly reduced by the combined treatment of miR-20b mimic + olaparib, compared with control mimic + olaparib ($P < 0.05$) (Figure 5(c)). γ -H2AX is a marker for DNA double-strand breaks, and RAD51 plays an important role in HR-mediated DNA repair.^{18–20} MiR-20b mimic-treated tumors showed reduced RAD51

expression (IHC score = 0.719 ± 0.035) and increased expression of γ -H2AX (IHC score = 1.96 ± 0.167) compared with tumors in the control mimic group (RAD51 IHC score = 1.279 ± 0.246 , $P < 0.01$; γ -H2AX IHC score = 0.920 ± 0.047 , $P < 0.05$). RAD51 expression was significantly reduced in miR-20b mimic and olaparib co-treated tumors (IHC score = 0.307 ± 0.110) compared with tumors in the olaparib + control mimic group (IHC score = 0.837 ± 0.107) ($P < 0.01$). We further found that γ -H2AX expression was substantially increased in tumors from the miR-20b mimic + olaparib group (IHC score = 6.216 ± 1.201) compared with tumors in the olaparib + control mimic group (IHC score = 2.512 ± 0.286) ($P < 0.001$) (Figure 6(a) and (b)). These data suggested that miR-20b overexpression down-regulates *CCND1* in ovarian cancer *in vivo*, thereby affecting DNA repair, which ultimately causes increased sensitivity to olaparib.

miR-20b expression and progression-free survival in ovarian cancer

We next investigated the relationship between miR-20b expression and survival of ovarian cancer patients by analyzing the TCGA dataset, which contains 367 high-grade serous ovarian cancer samples.²¹ Increased miR-20b expression was significantly associated with longer progression-free survival in ovarian cancer ($P = 0.027$) (Figure 7).

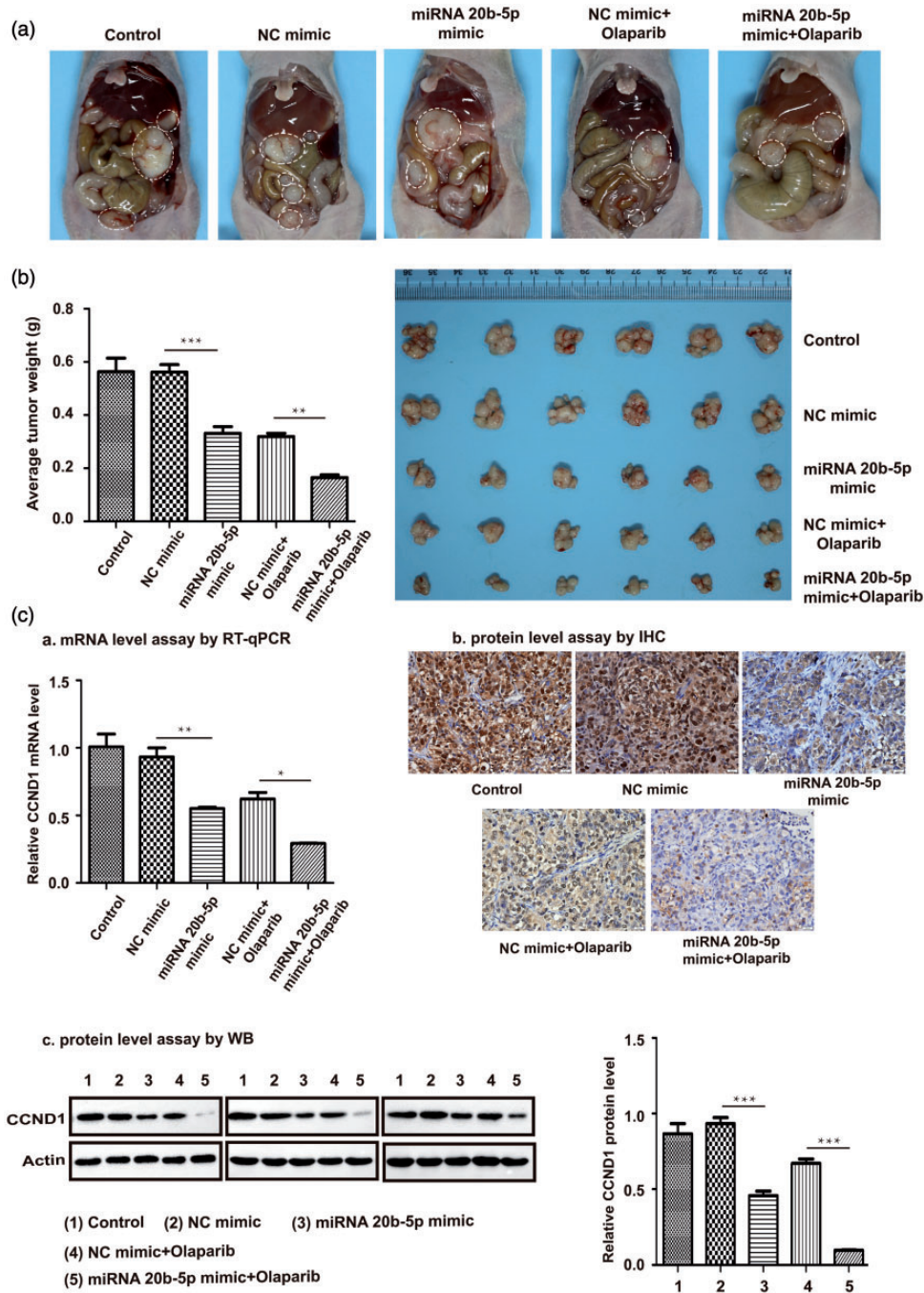


Figure 5. Combined treatment of miR-20b and olaparib against ovarian cancer *in vivo*. (a) and (b) Images of tumors inside the abdomen and tumor weight from five groups: 5% glucose solution (control), control mimic, miR-20b mimic, control mimic + olaparib and miR-20b mimic + olaparib group, respectively. (c) RT-qPCR analyzed CCND1 mRNA level in tumor tissue. Cyclin D1 protein level was shown by Western blot. Immunohistochemistry was done to verify CCND1 expression in tumor tissue, scale bar = 20 μ m. Results here were mean \pm SD ($n=3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (A color version of this figure is available in the online journal.)

Discussion

Ovarian cancer carrying methylated or mutated BRCA1 or mutated BRCA2, account for 30% of all ovarian cancers, exhibit abnormal HR repair, are sensitive to PARP inhibitors, and show increased sensitivity to DNA-damaging anticancer agents.²² Cyclin D1 is overexpressed in many cancers,²³ including ovarian cancer. Previous studies showed that cyclin D1 is a vital prognostic indicator for ovarian cancer.²⁴ Cyclin D1 plays a vital function in

regulating the cell cycle transition from G1 phase to S phase. Another study showed that cyclin D1 plays a non-kinase-dependent role in DNA repair and affects HR repair through interacting with RAD51.²⁵ Our previous work demonstrated that downregulation of cyclin D1 expression augmented the response of BRCA1 wild-type ovarian cancer to olaparib by impairing DNA double-strand break repair.¹¹ However, the mechanisms underlying cyclin D1 overexpression in ovarian cancer have not been

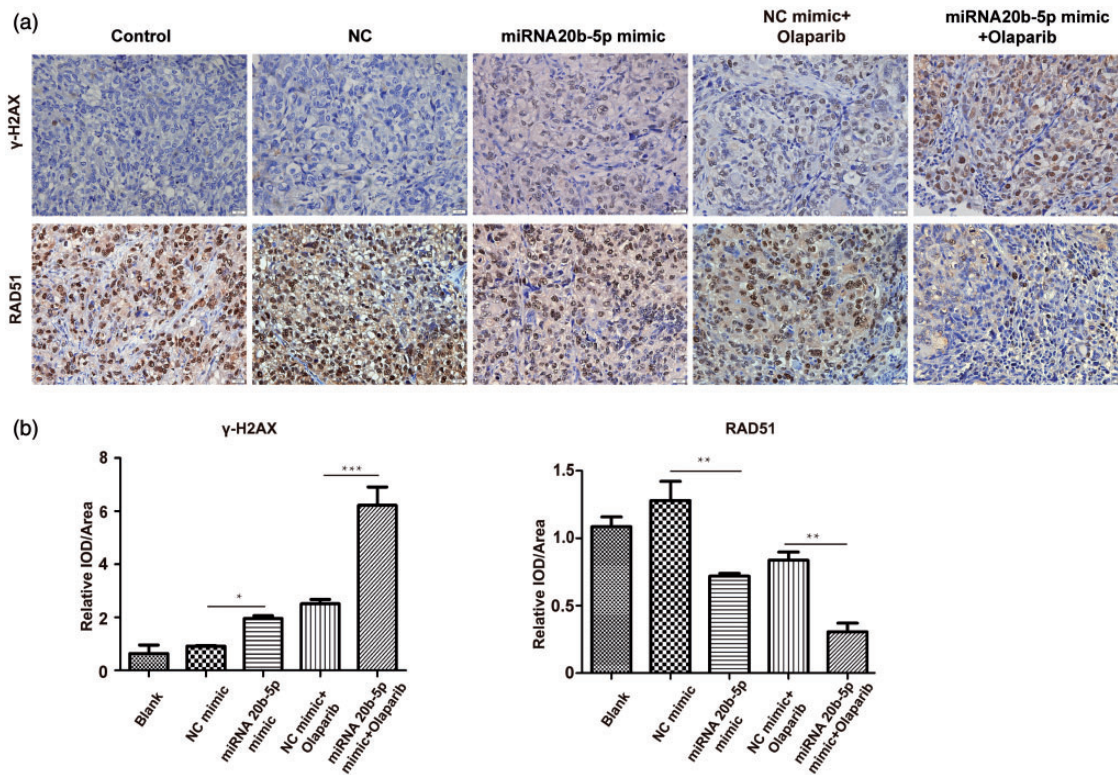


Figure 6. RAD51 and γ -H2AX level inside tumor tissue. (a) The typical sections from tumor tissue in various groups. (b) RAD51 and γ -H2AX expression were demonstrated by immunohistochemical scores (0–9). For instance, the scores were equivalent to the chroma of the signal (negative=0; light yellow=1; light brown=2; dark brown=3) \times percentage of positive cells (no signal=0; weak signal=0~25%; intermediate signal=25~50%; strong signal=above 50%). Scale bar: 20 μ m. Results here were mean \pm SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. (A color version of this figure is available in the online journal.)

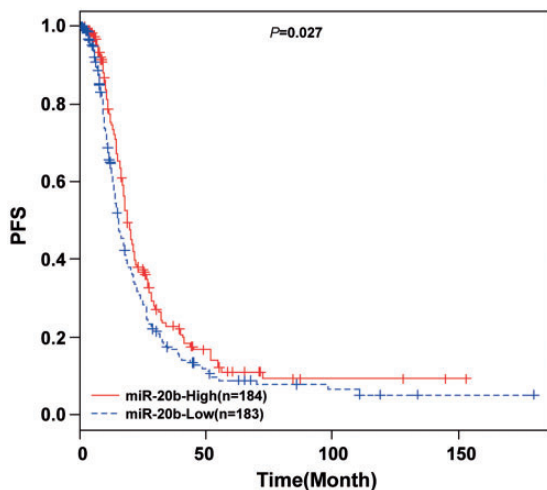


Figure 7. MiR-20b expression and ovarian cancer progression-free survival. Progression-free survival of 367 ovarian cancer patients in TCGA dataset with high miR-20b expression (red line) and low miR-20b expression (blue line) was analyzed by Kaplan-Meier survival curve. (A color version of this figure is available in the online journal.)

completely investigated. miRNAs are crucial for the expressions of many mRNAs.²⁶ Abnormal expression of miRNAs is often found in cancer.²⁷ Several miRNAs play an important role in regulating DNA repair and the susceptibility of cells to DNA-damaging drugs.^{26,28–30} The relationship between miRNAs and ovarian cancer has recently been investigated,^{13,31,32} and these studies have suggested

that miRNAs may represent possible targets or strategies for prevention, early detection, and treatment of the disease. In this study, we performed a series of *in vitro* experiments and showed that miR-20b targets the *CCND1* gene by binding to the 3'-UTR of the *CCND1* mRNA in BRCA1 wild-type ovarian cancer cells. We found that overexpression of miR-20b increased G0/G1 cells and decreased S phase cells through the downregulation of cyclin D1. Conversely, cells in G0/G1 phase were significantly decreased after miR-20b inhibitor treatment, which was consistent with the results after overexpression of *CCND1*. miR-20b also inhibited the colony formation of ovarian cancer cells. We further demonstrated that miR-20b interfered with RAD51 accumulation, thereby affecting HR efficacy (as indicated by decreased γ -H2AX expression) and altered the sensitivity of ovarian cancer cells to olaparib. Finally, TCGA data demonstrated that high miR-20b expression was significantly associated with longer progression-free survival of ovarian cancer patients.

The miR-106a-363 cluster encodes miR-20b and belongs to a large family called the miR-17 family, which contains many highly similar miRNAs.³³ miR-20b is involved in the pathogenesis of many cancers and has been shown to inhibit or promote the proliferation, invasion, and metastasis of different types of tumors.^{34–40} These studies on miR-20b yielded controversial results, and the downstream target genes of miR-20b were not fully examined. Our current results suggest that miR-20b may function as a tumor suppressor miRNA in ovarian cancer. Other studies reported

that miR-20b blocks bladder cancer EJ cells in G1 phase by targeting cyclin D1,³⁸ enhances the sensitivity of non-small cell lung cancer or breast cancer cells to cisplatin or taxol,^{34,39} and works as a tumor suppressor in papillary thyroid carcinoma.⁴⁰ These results were consistent with our findings.

The correlation between miR-20b and ovarian cancer has never been investigated before, let alone research on miR-20b and drug sensitivity in ovarian cancer. This report confirmed the potential of miR-20b combined with olaparib for targeted therapy of cyclin D1 in ovarian cancer without BRCA1 gene mutation. The combination therapy caused reduced tumor load and cell viability, induced cell cycle arrest, and blocked DNA repair in ovarian cancer cells. Furthermore, increased expression of miR-20b was related to the favorable progression-free survival of patients with ovarian cancer. To the best of our knowledge, this is the first report showing that miR-20b directly regulates *CCND1* expression in ovarian cancer. Our data suggest the possibility for miRNAs as markers to predict drug sensitivity and the prognoses of patients in ovarian cancer. Our findings also indicate that PARPi application may be effective in treating BRCA wild-type tumors with the development of a miRNA-based drug delivery system.

Authors' contributions

All the authors participated in the design, implement of the experiment and data analysis. QZ conceptualized the study, analyzed results and drafted the article. YX conducted the experiments. CL did statistical analysis. YPQ did literature search. XZ edited the article. HSY engaged in the experiment design, instructed students.

Acknowledgements

We thank Dr. Zhongyi Hu from Center for Research on Reproduction & Women's Health, Perelman School of Medicine, University of Pennsylvania, who analyzed the TCGA dataset for us.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was financially supported by the Science and Technology Support Program of Sichuan Province of China (2020YJ0295).

ORCID iD

Qian Zhong  <https://orcid.org/0000-0001-8354-5839>

Supplemental material

Supplemental material for this article is available online.

REFERENCES

- Zhong Q, Wen YJ, Yang HS, Luo H, Fu AF, Yang F, Chen LJ, Chen X, Qi XR, Lin HG, Wan Y, Chen XC, Wei YQ, Zhao X. Efficient inhibition of cisplatin-resistant human ovarian cancer growth and prolonged survival by gene transferred vesicular stomatitis virus matrix protein in nude mice. *Ann Oncol* 2008;**19**:1584–91
- Kim G, Ison G, McKee AE, Zhang H, Tang S, Gwise T, Sridhara R, Lee E, Tzou A, Philip R, Chiu HJ, Ricks TK, Palmby T, Russell AM, Ladouceur G, Pfuma E, Li H, Zhao L, Liu Q, Venugopal R, Ibrahim A, Pazdur R. FDA approval summary: olaparib monotherapy in patients with deleterious germline BRCA-Mutated advanced ovarian cancer treated with three or more lines of chemotherapy. *Clin Cancer Res* 2015;**21**:4257–61
- Jaspers JE, Kersbergen A, Boon U, Sol W, van Deemter L, Zander SA, Drost R, Wientjens E, Ji J, Aly A, Doroshow JH, Cranston A, Martin NM, Lau A, O'Connor MJ, Ganesan S, Borst P, Jonkers J, Rottenberg S. Loss of 53BP1 causes PARP inhibitor resistance in Brca1-mutated mouse mammary tumors. *Cancer Discov* 2013;**3**:68–81
- Xu G, Chapman JR, Brandsma I, Yuan J, Mistrik M, Bouwman P, Bartkova J, Gogola E, Warmerdam D, Barazas M, Jaspers JE, Watanabe K, Pieterse M, Kersbergen A, Sol W, Celie PHN, Schouten PC, van den Broek B, Salman A, Nieuwland M, de Rink I, de Ronde J, Jalink K, Boulton SJ, Chen J, van Gent DC, Bartek J, Jonkers J, Borst P, Rottenberg S. REV7 counteracts DNA double-strand break resection and affects PARP inhibition. *Nature* 2015;**521**:541–4
- Ray Chaudhuri A, Callen E, Ding X, Gogola E, Duarte AA, Lee JE, Wong N, Lafarga V, Calvo JA, Panzarino NJ, John S, Day A, Crespo AV, Shen B, Starnes LM, de Rooter JR, Daniel JA, Konstantinopoulos PA, Cortez D, Cantor SB, Fernandez-Capetillo O, Ge K, Jonkers J, Rottenberg S, Sharan SK, Nussenzweig A. Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* 2016;**535**:382–7
- Liu J, Matulonis UA. New strategies in ovarian cancer: translating the molecular complexity of ovarian cancer into treatment advances. *Clin Cancer Res* 2014;**20**:5150–6
- Yang L, Zhang Y, Shan W, Hu Z, Yuan J, Pi J, Wang Y, Fan L, Tang Z, Li C, Hu X, Tanyi JL, Fan Y, Huang Q, Montone K, Dang CV, Zhang L. Repression of BET activity sensitizes homologous recombination-proficient cancers to PARP inhibition. *Sci Transl Med* 2017;**9**.
- Scott CL, Swisher EM, Kaufmann SH. Poly (ADP-ribose) polymerase inhibitors: recent advances and future development. *J Clin Oncol* 2015;**33**:1397–406
- Konstantinopoulos PA, Ceccaldi R, Shapiro GI, D'Andrea AD. Homologous recombination deficiency: exploiting the fundamental vulnerability of ovarian cancer. *Cancer Discov* 2015;**5**:1137–54
- Erratum for the Perspective: "Laying a trap to kill cancer cells: PARP inhibitors and their mechanisms of action" by Y. Pommier, M. J. O'Connor, J. de Bono. *Sci Transl Med* 2016;**8**:368er7
- Zhong Q, Hu Z, Li Q, Yi T, Li J, Yang H. Cyclin D1 silencing impairs DNA double strand break repair, sensitizes BRCA1 wildtype ovarian cancer cells to olaparib. *Gynecol Oncol* 2019;**152**:157–65
- Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 2005;**122**:553–63
- Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017;**16**:203–22
- Stordal B, Timms K, Farrelly A, Gallagher D, Busschots S, Renaud M, Thery J, Williams D, Potter J, Tran T, Korpany G, Cremona M, Carey M, Li J, Li Y, Aslan O, O'Leary JJ, Mills GB, Hennessy BT. BRCA1/2 mutation analysis in 41 ovarian cell lines reveals only one functionally deleterious BRCA1 mutation. *Mol Oncol* 2013;**7**:567–79
- Bai Y, Deng H, Yang Y, Zhao X, Wei Y, Xie G, Li Z, Chen X, Chen L, Wang Y, Su D, Qian Z, Zhong Q, Luo H, Yi T. VEGF-targeted short hairpin RNA inhibits intraperitoneal ovarian cancer growth in nude mice. *Oncology* 2009;**77**:385–94
- Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, Derksen PW, de Bruin M, Zevenhoven J, Lau A, Boulter R, Cranston A, O'Connor MJ, Martin NM, Borst P, Jonkers J. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor

- AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci USA* 2008;**105**:17079–84
17. Jelinic P, Levine DA. New insights into PARP inhibitors' effect on cell cycle and homology-directed DNA damage repair. *Mol Cancer Ther* 2014;**13**:1645–54
 18. Zhao W, Steinfeld JB, Liang F, Chen X, Maranon DG, Jian Ma C, Kwon Y, Rao T, Wang W, Sheng C, Song X, Deng Y, Jimenez-Sainz J, Lu L, Jensen RB, Xiong Y, Kupfer GM, Wiese C, Greene EC, Sung P. BRCA1-BARD1 promotes RAD51-mediated homologous DNA pairing. *Nature* 2017;**550**:360–5
 19. Yang Z, Waldman AS, Wyatt MD. Expression and regulation of RAD51 mediate cellular responses to chemotherapeutics. *Biochem Pharmacol* 2012;**83**:741–6
 20. Kuo LJ, Yang LX. Gamma-H2AX – a novel biomarker for DNA double-strand breaks. *In Vivo* 2008;**22**:305–9.
 21. Cancer Genome Atlas Research N. Integrated genomic analyses of ovarian carcinoma. *Nature* 2011;**474**:609–15
 22. Fong PC, Yap TA, Boss DS, Carden CP, Mergui-Roelvink M, Gourley C, De Greve J, Lubinski J, Shanley S, Messiou C, A'Hern R, Tutt A, Ashworth A, Stone J, Carmichael J, Schellens JH, de Bono JS, Kaye SB. Poly(ADP)-ribose polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. *J Clin Oncol* 2010;**28**:2512–9
 23. Dai J, Wei RJ, Li R, Feng JB, Yu YL, Liu PS. A study of CCND1 with epithelial ovarian cancer cell proliferation and apoptosis. *Eur Rev Med Pharmacol Sci* 2016;**20**:4230–5
 24. Bali A, O'Brien PM, Edwards LS, Sutherland RL, Hacker NF, Henshall SM. Cyclin D1, p53, and p21Waf1/Cip1 expression is predictive of poor clinical outcome in serous epithelial ovarian cancer. *Clin Cancer Res* 2004;**10**:5168–77
 25. Jirawatnotai S, Hu Y, Michowski W, Elias JE, Becks L, Bienvenu F, Zagodzón A, Goswami T, Wang YE, Clark AB, Kunkel TA, van Harn T, Xia B, Correll M, Quackenbush J, Livingston DM, Gygi SP, Sicinski P. A function for cyclin D1 in DNA repair uncovered by protein interactome analyses in human cancers. *Nature* 2011;**474**:230–4
 26. Crosby ME, Kulshreshtha R, Ivan M, Glazer PM. MicroRNA regulation of DNA repair gene expression in hypoxic stress. *Cancer Res* 2009;**69**:1221–9
 27. Wang Y, Huang JW, Calses P, Kemp CJ, Taniguchi T. MiR-96 down-regulates REV1 and RAD51 to promote cellular sensitivity to cisplatin and PARP inhibition. *Cancer Res* 2012;**72**:4037–46
 28. Lal A, Pan Y, Navarro F, Dykxhoorn DM, Moreau L, Meire E, Bentwich Z, Lieberman J, Chowdhury D. miR-24-mediated downregulation of H2AX suppresses DNA repair in terminally differentiated blood cells. *Nat Struct Mol Biol* 2009;**16**:492–8
 29. Wang Y, Huang JW, Li M, Cavenee WK, Mitchell PS, Zhou X, Tewari M, Furnari FB, Taniguchi T. MicroRNA-138 modulates DNA damage response by repressing histone H2AX expression. *Mol Cancer Res* 2011;**9**:1100–11
 30. Hu H, Du L, Nagabayashi G, Seeger RC, Gatti RA. ATM is down-regulated by N-Myc-regulated microRNA-421. *Proc Natl Acad Sci U S A* 2010;**107**:1506–11
 31. Yang N, Kaur S, Volinia S, Greshock J, Lassus H, Hasegawa K, Liang S, Leminen A, Deng S, Smith L, Johnstone CN, Chen XM, Liu CG, Huang Q, Katsaros D, Calin GA, Weber BL, Butzow R, Croce CM, Coukos G, Zhang L. MicroRNA microarray identifies let-7i as a novel biomarker and therapeutic target in human epithelial ovarian cancer. *Cancer Res* 2008;**68**:10307–14
 32. Cai J, Yang C, Yang Q, Ding H, Jia J, Guo J, Wang J, Wang Z. Deregulation of let-7e in epithelial ovarian cancer promotes the development of resistance to cisplatin. *Oncogenesis* 2013;**2**:e75
 33. Tanzer A, Stadler PF. Molecular evolution of a microRNA cluster. *J Mol Biol* 2004;**339**:327–35
 34. Ao X, Nie P, Wu B, Xu W, Zhang T, Wang S, Chang H, Zou Z. Decreased expression of microRNA-17 and microRNA-20b promotes breast cancer resistance to taxol therapy by upregulation of NCOA3. *Cell Death Dis* 2016;**7**:e2463
 35. Zhang Y, Guan DH, Bi RX, Xie J, Yang CH, Jiang YH. Prognostic value of microRNAs in gastric cancer: a meta-analysis. *Oncotarget* 2017;**8**:55489–510
 36. Li C, Qin F, Hu F, Xu H, Sun G, Han G, Wang T, Guo M. Characterization and selective incorporation of small non-coding RNAs in non-small cell lung cancer extracellular vesicles. *Cell Biosci* 2018;**8**:2
 37. Guo J, Xiao Z, Yu X, Cao R. miR-20b promotes cellular proliferation and migration by directly regulating phosphatase and tensin homolog in prostate cancer. *Oncol Lett* 2017;**14**:6895–900
 38. Park SL, Cho TM, Won SY, Song JH, Noh DH, Kim WJ, Moon SK. MicroRNA-20b inhibits the proliferation, migration and invasion of bladder cancer EJ cells via the targeting of cell cycle regulation and Sp-1-mediated MMP-2 expression. *Oncol Rep* 2015;**34**:1605–12
 39. Jiang Z, Yin J, Fu W, Mo Y, Pan Y, Dai L, Huang H, Li S, Zhao J. MiRNA 17 family regulates cisplatin-resistant and metastasis by targeting TGFbetaR2 in NSCLC. *PLoS One* 2014;**9**:e94639
 40. Hong S, Yu S, Li J, Yin Y, Liu Y, Zhang Q, Guan H, Li Y, Xiao H. MiR-20b displays tumor-suppressor functions in papillary thyroid carcinoma by regulating the MAPK/ERK signaling pathway. *Thyroid* 2016;**26**:1733–43

(Received October 24, 2020, Accepted January 21, 2021)