

Circular RNA circ-CD44 regulates chemotherapy resistance by targeting the miR-330-5p/ABCC1 axis in colorectal cancer cells

Shuai Zhao*, Fei Xu*, Yiding Ji, Yuanyuan Wang, Ming Wei and Like Zhang

Department of General Surgery, Hebei Key Laboratory of Colorectal Cancer Precision Diagnosis and Treatment, The First Hospital of Hebei Medical University, Shijiazhuang City, Hebei Province, China.

*These authors contributed equally to this paper

Summary. Background. Colorectal cancer (CRC) is a common malignant tumor worldwide, ranking fourth for incidence. Recently, circular RNAs (circRNAs) have been demonstrated to play a key role in chemotherapy resistance to CRC treatment. Therefore, the role of circ-CD44 is investigated in CRC.

Methods. The expression levels of circ-CD44, miR-330-5p, and ATP binding cassette subfamily C member 1 (ABCC1) were quantified by real-time quantitative polymerase chain reaction (RT-qPCR) assay. The sensitivity of CRC cells to oxaliplatin (OXA) was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazol-3-ium bromide (MTT) assay. Colony-forming experiment was performed to measure the colony-forming ability of CRC cells. The apoptosis, migration, and invasion of CRC cells were determined by flow cytometry and transwell assays. A xenograft experiment was established to clarify the functional role of circ-CD44 silencing *in vivo*. The interactional relationship among circ-CD44, miR-330-5p, and ABCC1 was confirmed by dual-luciferase reporter and RNA immunoprecipitation assays. The protein expression of ABCC1 was quantified by western blot assay.

Results. Circ-CD44 was obviously upregulated in OXA-resistant colorectal cancer tissues and cells. Loss-of-function experiments revealed that inhibition of circ-CD44 suppressed proliferation, migration, and invasion

while it increased OXA sensitivity and apoptosis in OXA-resistant colorectal cancer cells, which was overturned by suppression of miR-330-5p; besides, silencing of circ-CD44 also slowed the tumor growth *in vivo*. Additionally, overexpression of miR-330-5p inhibited chemotherapy resistance, proliferation, migration, and invasion while it induced apoptosis by targeting ABCC1.

Conclusion. Mechanistically, circ-CD44 functioned as a miRNA sponge for miR-330-5p to upregulate the expression of ABCC1 and regulate chemotherapy resistance in CRC cells.

Key words: circ-CD44, miR-330-5p, ABCC1, CRC, OXA

Introduction

Colorectal cancer (CRC) is one of the most frequent neoplasms and an important reason for cancer-related death, which occurs in the colon and rectum (Arnold et al., 2017). Approximately 1.8 million CRC-related cases were reported in 2018 according to the relevant statistics, causing a major health burden in the world (Bray et al., 2018). Although clinical outcomes of CRC patients have improved due to chemotherapy strategies significantly improving, personalized treatment is necessary for better efficacy and toxicity (Kim, 2015). Hence, further understanding of the resistance mechanism of CRC progression is essential.

Over the last decade, the development of multidrug resistance continues to be one of the primary causes of treatment failure, promoting the characteristics of tumor metastasis (Vasan et al., 2019). Oxaliplatin (OXA) is a chemical medicine and is widely used after surgical resection, including advanced CRC (Petrelli et al. 2015; Goldstein et al., 2016). Functionally, OXA might segregate transcription factors or induce cell cycle arrest

Corresponding Author: Ming Wei, Department of General Surgery, Hebei Key Laboratory of Colorectal Cancer Precision Diagnosis and Treatment, The First Hospital of Hebei Medical University, No.89 Donggang Road, Shijiazhuang 050031, Hebei Province, China. e-mail: pngdwq@163.com or Like Zhang, Department of General Surgery, Hebei Key Laboratory of Colorectal Cancer Precision Diagnosis and Treatment, The First Hospital of Hebei Medical University, No.89 Donggang Road, Shijiazhuang 050031, Hebei Province, China. e-mail: sn7eny@126.com
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by the formation of interstrand and intrastrand cross-linking of DNA molecules (Arango et al., 2004). However, OXA resistance is involved in endogenous RNA networks (Meng et al., 2020), thus the underlying association between competing endogenous RNA networks and OXA treatment was investigated in this study.

Circular RNAs (circRNAs) are defined as RNA transcripts, lacking 5' caps and 3' tails with circular structures (Chen and Yang, 2015). More and more research has revealed that circRNAs participate in the cancer process and serve as tumor biomarkers, predicting poor prognosis (Li et al., 2019). For instance, a previous report suggested that circ_0067934 was highly related to the survival time of thyroid carcinoma patients (Wang et al., 2019). Circ-CD44 (hsa_circ_0000291) is derived from the CD44 gene and is located on chr11 (35163017-35163328). However, the functional effects of circ-CD44 have not been fully elucidated in CRC yet.

MicroRNAs (miRNAs) are RNA transcripts of approximately 20 nucleotides without protein-coding ability (Liu et al., 2014). Functionally, miRNAs can target the 3' untranslated region (3'UTR) of their target mRNAs, causing degradation or translation repression (Zealy et al., 2017). In addition, aberrant expression of miRNAs can act as oncogenes or cancer inhibitors in cancers by targeting special mRNAs to inhibit post-transcription translation of mRNAs (Vishnoi and Rani, 2017). MiR-330-5p might be a potential tumor inhibitor, since there is evidence that miR-330-5p was obviously decreased in cancer tissues and overexpression of miR-330-5p inhibited proliferation and invasiveness of tumor cells in glioma and cervical cancer (Chen et al., 2019; Zhao et al., 2019).

Notably, ATP binding cassette subfamily C member 1 (ABCC1), belongs to the ABCC subfamily and is also named resistance-associated protein 1, facilitating the drug-resistance of cancer cells, including hepatocellular carcinoma (Huang et al., 2018), breast cancer (Liang et al., 2010), and colon cancer (Lin et al., 2018). Importantly, Kunicka et al. displayed that targeting ABCC1 was a reliable candidate for the prediction of chemotherapy resistance in human cancers (Kunicka and Soucek, 2014).

Currently, some experimental evidence shows that the competing endogenous RNA hypothesis provided a new explanation for cancer initiation and progression (Qi et al., 2015). This hypothesis suggested that endogenous RNA transcripts with a host of miRNA-binding sites might function as miRNA sponges to alter gene expression (Sanchez-Mejias and Tay, 2015). Of interest, this mechanism has been considered the major regulation mechanism for circRNAs (Hansen et al., 2013; Panda, 2018). In this research, our data exhibited that there are some binding sites between miR-330-5p and circ-CD44 or ABCC1. Therefore, we hypothesized that circ-CD44 was implicated in OXA resistance in CRC by regulation of miR-330-5p and ABCC1.

Materials and methods

Tissue collection

We collected 38 samples of CRC tissues and paired adjacent tissues from CRC patients who received treatment at The First Hospital of Hebei Medical University. The patients did not receive preoperative treatments, including chemotherapy or local radiotherapy. This study obtained written informed consent from all patients, and it was permitted by the Ethics Committee of The First Hospital of Hebei Medical University. The entire investigation conformed to the principles outlined in the Declaration of Helsinki. After 2 months of OXA treatment (85 mg/m²; 3 times), CRC patients were divided into the chemotherapy-sensitive group (n=17; the increase of tumor growth <20%) and the resistant group (n=21; the appearance of new lesions or the increase of tumor growth >30%) in compliance with response evaluation guidelines. The clinicopathological characteristics of CRC patients are presented in Table 1.

Cell lines and cell culture

Human CRC cell lines (SW1116, Lovo, SW620, and HCT-116) and human normal intestinal epithelial cells (NCM460) were obtained from the American Type Culture Collection (Rockville, MD, USA). OXA-resistant CRC cells (SW620/OXA and HCT116/OXA cells) were purchased from Nanjing Key Gen Biotech (Nanjing, China). These cells were cultured within the RPMI 1640 medium (Wisent, Shanghai, China) that was supplemented with 10% (v/v) fetal bovine serum (FBS; Wisent) in 5% CO₂ and 90% humidity at 7°C. In addition, 3 μM OXA (Sigma, Louis, MO, USA) was used to incubate cells for 48h.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA and then dissolved in RNase-

Table 1. The clinicopathological characteristics of colorectal cancer patients.

Clinicopathological features	n Total=38	
Ages	≥50	27
	≤50	11
Tumor stage	I-II	25
	III-IV	13
Lymph node metastasis	Negative	23
	Positive	15
Tumor size	≤3 cm	21
	>3 cm	17

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free water. After that, 5 μ g of total RNA was converted by High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) using random primers in a 20 μ L reaction mixture. In addition, the cDNA primer for miR-330-5p was used: 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGCCTAAGA-3'. RT-qPCR assay was performed under Thermal Cycler CFX6 System (Bio-Rad, Hercules, CA, USA), including SYBR Green fluorescent dye, primers, qPCR mix, and cDNA. Relative transcript level was determined by the $2^{-\Delta\Delta C_t}$ method and standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or endogenous U6.

The sequences of primers:

circ-CD44 (sense 5'-CAGTAGCAATAACTGCCCCG-3'; antisense 5'-GTCAGGAGTCAGAATGCCCAA-3'); miR-330-5p (sense 5'-GCCGAGTCTCTGGGCC TCTG-3'; Reverse, 5'-CAGTGCAGGGTCCGAGGTA T-3'); ABCC1 (sense 5'-CTTACGGAGTCGCTTAG ATGAGA-3'; antisense 5'-ATGCCCAGAAGTGCAT CGAAA-3'); GAPDH (sense 5'-GTGTTCCCTACCCC CAATGTG-3'; antisense 5'-CATCGAAGGTGGAAGA GTGG-3'); U6 (sense 5'-ATCCTTACGCACCCAGT CCA-3'; antisense 5'-GAACGCTTCACGAATTTGC-3').

Transfection assay

To silence circ-CD44 expression, small interfering RNA (siRNA) targeting circ-CD44 (si-circ-CD44) and si-NC were designed by RiboBio (Shanghai, China). Circ-CD44-overexpression vector (circ-CD44) and empty vector (vector), ABCC1-overexpression vector (ABCC1) and negative control (pcDNA), mimic miR-330-5p and control (miR-330-5p and miR-NC), and inhibitor of miR-330-5p and negative control (anti-miR-330-5p and anti-miR-NC) were bought from Biossci Company (Wuhan, China). The oligonucleotides and vectors were transfected into CRC by Lipofectamine 2000 (Promega, Madison, WI, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazol-3-ium bromide (MTT) and colony-forming assays

CRC cells were plated into 96-well plates (3000 cells/well) and incubated with different doses of OXA. After 48-h incubation, cell viability was measured by MTT assay as previously described (Tang et al., 2018). The concentration (IC_{50}) of OXA inducing 50% inhibition of growth of CRC cells was estimated by the relative survival curve. As for the colony-forming assay, CRC cells were inoculated into 6-well plates and then incubated for 14 d. The clone was fixed with methanol, stained, and then imaged under the microscope (Olympus, Tokyo, Japan).

Cell apoptosis assay

Apoptotic CRC cells were measured by Annexin V-

FITC Apoptosis Detection Kit (Invitrogen). In brief, CRC cells were collected and then washed in cold phosphate buffer saline (PBS). After that, single-cell suspension at a density of 1×10^6 /mL was re-suspended in Annexin V labeled with fluorescein isothiocyanate (FITC; 5 μ L) and propidium (PI; 5 μ L) and then incubated for 30 min. Apoptotic cells were monitored under the FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Transwell assay

The migration of CRC cells was measured by 24-well transwell chambers (8- μ m pore size; Corning, Franklin Lakes, NJ, USA), while an invasion assay was performed in presence of Matrigel (BD Biosciences). CRC cells were re-suspended in 200 μ L of FBS-free medium (2×10^6 cells/mL) and then added into the apical chambers, while 600 μ L of complete medium was added to the bottom chambers. After 24h of incubation, the cells on the lower chambers were fixed with paraformaldehyde and stained with 0.4% crystal violet (Invitrogen). Fields were randomly selected and then photographed under a microscope (Olympus; 100 \times).

In vivo experiment

The animal assay was approved by the Institutional Animal Care and Use Committee of The First Hospital of Hebei Medical University, following the instruction of the American Veterinary Medical Association guidelines. 1×10^7 SW620/OXA stably transfected with short hairpin RNA targeting circ-CD44 (sh-circ-CD44; RiboBio) and sh-NC were subcutaneously vaccinated into the left-back of mice of BALB/c nude mice from Vital River Laboratory (Beijing, China), with 6 mice per group. Notably, the OXA group was intraperitoneally injected with OXA (3.0 mg/kg) for one cycle of 3 d after 8 d, with PBS as solvent control. Tumor growth was monitored based on volume = $1/2$ (length \times width 2). After 23 d, flowing CO₂ was used for euthanasia of animals.

Dual-luciferase reporter assay

The possible targets of circ-CD44 and miR-330-5p were predicted by circinteractome (<https://circinteractome.irp.nia.nih.gov/>) and starBase v2.0 (<http://starbase.sysu.edu.cn/starbase2/>), respectively. CRC cells were seeded into 24-well plates (4×10^5 cells per well) and incubated for 24h. Then cells were co-transfected with miRNA mimic or miR-NC and luciferase report vectors (Ambion, Foster City, CA, USA) containing wild-type sequences of circ-CD44 (WT-circ-CD44) or 3'UTR of ABCC1 (ABCC1 3'UTR-WT), with MUT-circ-CD44 and ABCC1 3'UTR-MUT as controls. After 48h, firefly luciferase activity was measured by a dual-luciferase assay kit (Thermo Fisher Scientific), with Renilla luciferase as the internal control.

RNA immunoprecipitation (RIP) assay

RIP was implemented using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Briefly, 48h after transfection, CRC cells were lysed by RIP lysis buffer, and the lysates (200 μ L) interacted with magnetic beads embracing Ago2 or IgG (Millipore) at 4°C for 4h. After subjecting to Proteinase K digestion, immunoprecipitated RNA was extracted for RT-qPCR assay.

Western blot assay

The protein samples from tissues or cells were isolated by lysis buffer (Invitrogen). After that, total protein was transferred to nitrocellulose membranes (Millipore) after separating by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were shaken in 5% skim milk solution, followed by incubation with antibodies anti-ABCC1 (#72202S; 1:2000 dilution; Signaling Technology, Danvers, MA, USA) overnight at 4°C, with GAPDH (#2118S; 1:2000 dilution; Cell Signaling Technology) as control. The secondary antibody Anti-rabbit IgG (#7074S; 1:3000 dilution; Cell Signaling Technology) was diluted and

incubated with membranes for 1.5h. Eventually, the blots were captured under ChemiDoc MP imaging system (Bio-Rad) and analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Quantitative data are shown as mean \pm standard deviation from independent triplicate. All analyses were carried out using Student's *t*-test (two-tailed) or one-way analysis of variance with multiple comparisons with the post hoc Bonferroni test (SPSS 21.0; IBM, Somers, NY, USA). Significance was chosen as *P*-value less than 0.05.

Results

Circ-CD44 was overexpressed in OXA-resistant CRC tissues and cells

Analysis of GSE126094 differential circRNA expression in GEO data revealed a series of differentially expressed circRNAs in CRC tissues, including circ-CD44 (has-circRNA-000792) (Fig. 1A,B). These results were confirmed in our results; CRC

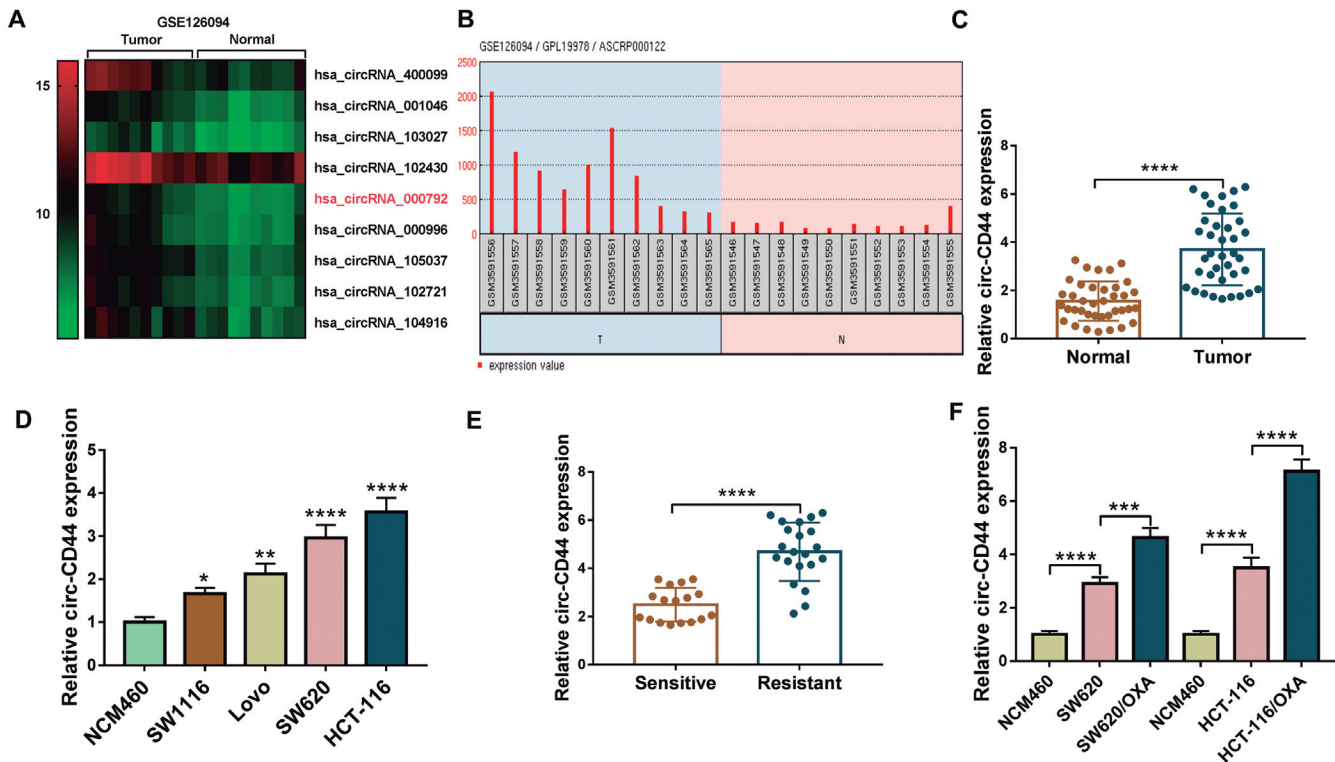


Fig. 1. The expression level of circ-CD44 in colorectal cancer tissues and cells. **A-B.** Heatmap of the differentially expressed circRNAs was shown according to the GEO accession (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126094>). **C-D.** The expression level of circ-CD44 was determined by RT-qPCR assay in colorectal cancer tissues and cells along with matched control groups. **E-F.** RT-qPCR assay was used to measure the expression of circ-CD44 in OXA-resistant colorectal cancer tissues and cells. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

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tissues and cells showed a high expression of circ-CD44 compared with negative controls (Fig. 1C,D). Interestingly, a significant increase was found in OXA-resistant CRC tissues and cells compared to controls (Fig. 1E,F). We hypothesized that circ-CD44 was implicated in the development of CRC.

Knockdown of circ-CD44 inhibited OXA resistance, proliferation, migration, and invasion while it induced apoptosis in OXA-resistant CRC cells

As upregulation of circ-CD44 was associated with OXA resistance in CRC cells, we knocked down the expression of circ-CD44 in OXA-resistant CRC cells. Firstly, MTT assay suggested that SW620/OXA and HCT-116/OXA cells exhibited a low sensitivity to OXA (Fig. 2A). Moreover, circ-CD44 was downregulated in SW620/OXA and HCT-116/OXA cells transfected with si-circ-CD44 compared with the control group (Fig. 2B). Importantly, the silencing of circ-CD44 increased the sensitivity of SW620/OXA and HCT-116/OXA cells to OXA (Fig. 2C). Colony-forming experiment assay

showed that circ-CD44 depletion obviously decreased the colony number of SW620/OXA and HCT-116/OXA cells (Fig. 2D). The downregulation of circ-CD44 also increased the apoptotic cell rate significantly (Fig. 2E). We also observed that the migration and invasion of OXA-resistant CRC cells were inhibited by knockdown of circ-CD44 (Fig. 3A,B). In summary, circ-CD44 knockdown might suppress the CRC process.

Inhibition of circ-CD44 increased the OXA sensitivity in vivo

A xenograft experiment was established to investigate the effect of circ-CD44 silencing on tumor growth. We noticed that SW620/OXA stably transfected with sh-circ-CD44 obviously repressed the expression of circ-CD44 (Fig. 4A). The tumor xenografts in the sh-circ-CD44+OXA group grew most slowly and their average weight was noticeably lower compared to those in other groups (Fig. 4B,C). In addition, the expression level of circ-CD44 was decreased in dissected tumor tissues from sh-circ-CD44+PBS and sh-circ-

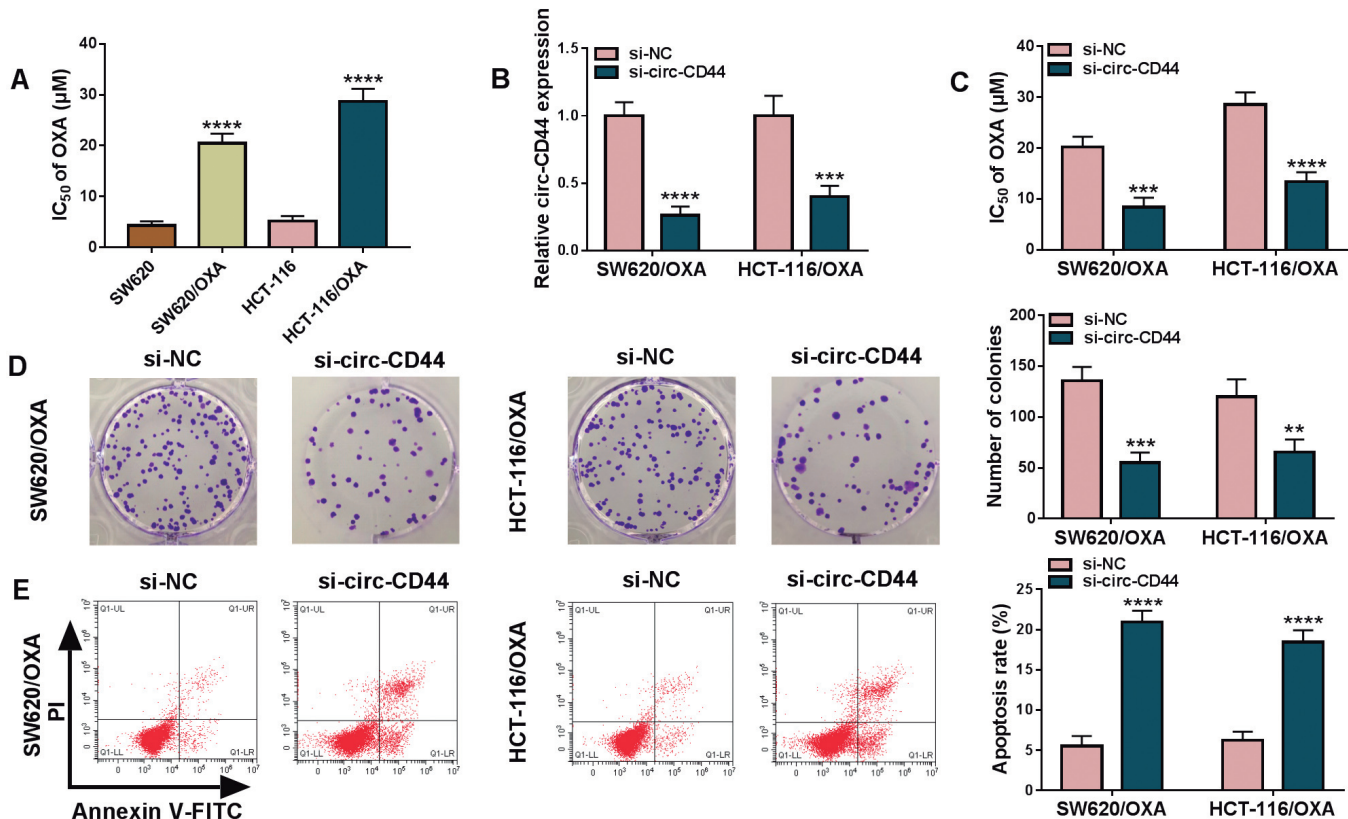


Fig. 2. Effects of circ-CD44 silencing on OXA sensitivity, proliferation, and apoptosis in OXA-resistant colorectal cancer cells. **A.** The sensitivity of colorectal cancer cells and OXA-resistant cells to OXA was assessed by MTT assay. **B-E.** SW620/OXA and HCT-116/OXA cells were transfected with si-NC or si-circ-CD44. **B.** Transfection efficiency of si-circ-CD44 was examined by RT-qPCR assay. **C.** The IC₅₀ of OXA was measured by MTT assay in transfected SW620/OXA and HCT-116/OXA cells. **D.** Colony-forming experiment was performed in SW620/OXA and HCT-116/OXA cells. **E.** The apoptosis rate of SW620/OXA and HCT-116/OXA cells was measured by flow cytometry assay. ***P*<0.01, ****P*<0.001, *****P*<0.0001.

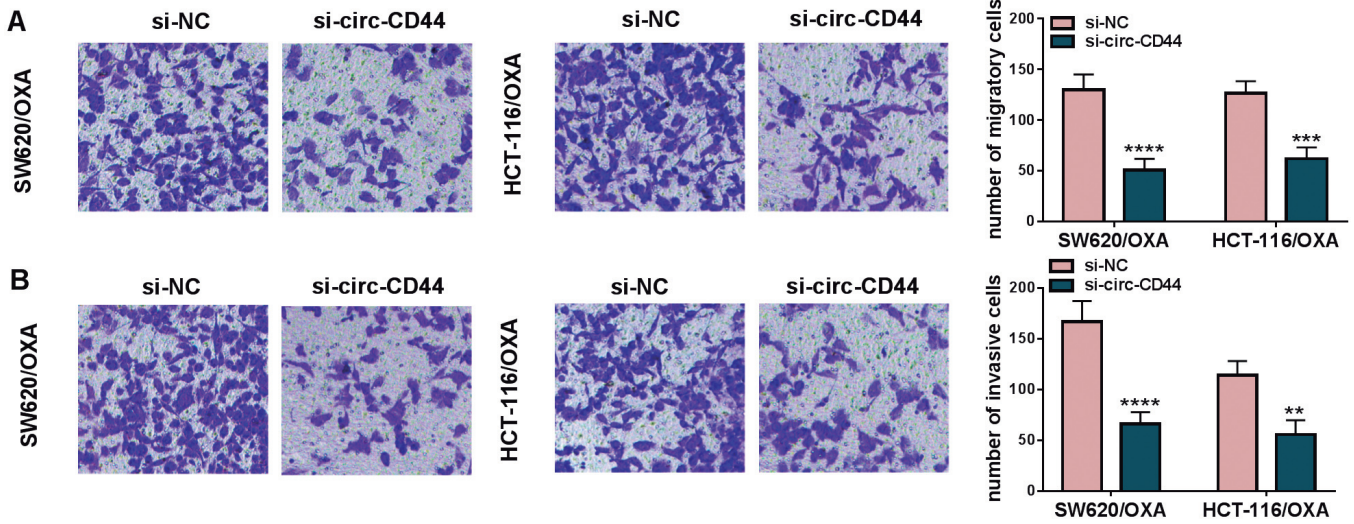


Fig. 3. Effects of circ-CD44 silencing on OXA migration and invasion in OXA-resistant colorectal cancer cells. **A-B.** Transwell assay was employed to measure migration and invasion abilities in SW620/OXA and HCT-116/OXA cells transfected with si-NC or si-circ-CD44. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

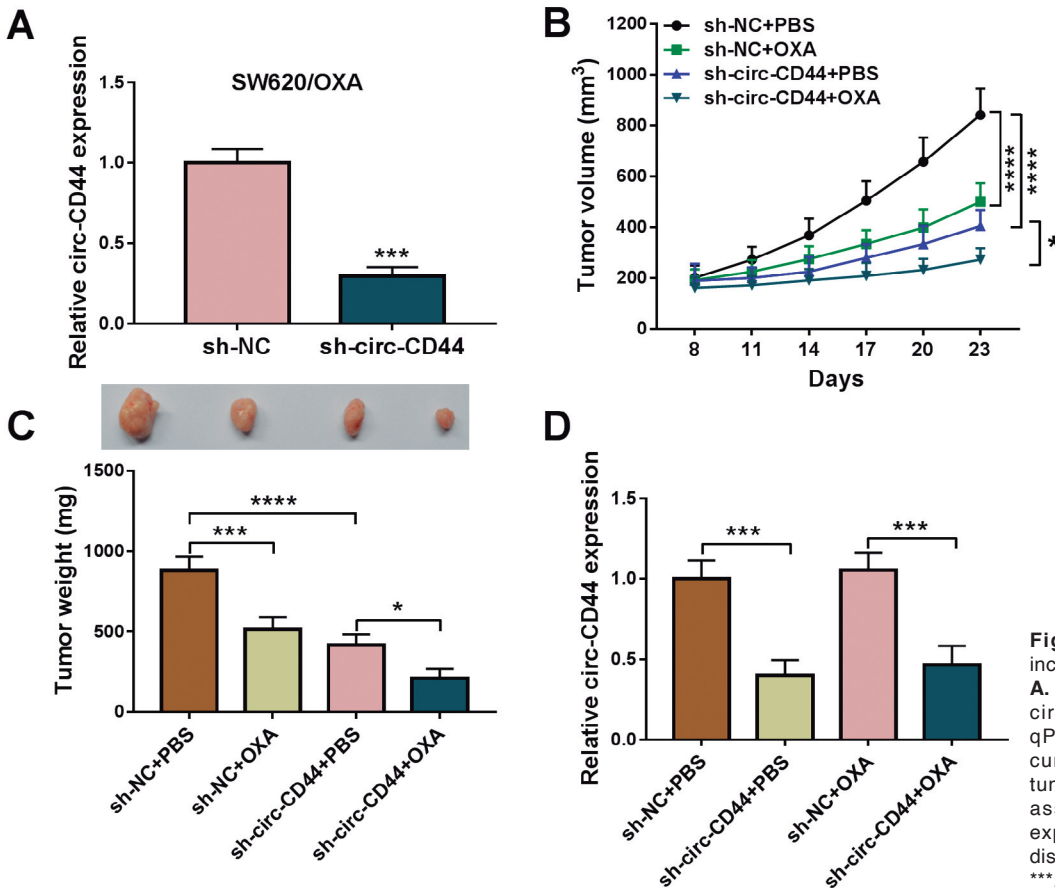


Fig. 4. Silencing of circ-CD44 increased OXA sensitivity in vivo. **A.** The knockdown efficiency of sh-circ-CD44 is examined by RT-qPCR assay. **B-C.** The growth curves and weight of xenograft tumors are shown. **D.** RT-qPCR assay was used to assess the expression level of circ-CD44 in dissected tumor tissues. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

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CD44+OXA group compared with matched controls (Fig. 4D). Thus, the suppression of circ-CD44 decreased the OXA resistance *in vivo*.

Circ-CD44 targeted miR-330-5p in OXA-resistant CRC cells

Bioinformatics analysis was conducted to predict the putative target miRNA of circ-CD44. As shown in Fig. 5A, miR-330-5p was predicted to be a target of circ-CD44. In addition, miR-330-5p increased in miR-330-5p mimic-transfecting cells while it decreased in anti-miR-330-5p-transfecting cells, suggesting the transfection experiment was successful (Fig. 5B). After transfection with plasmid into OXA-resistant CRC cells, luciferase activity decreased in CRC cells co-transfected with miR-

330-5p mimic and the WT-circ-CD44, while there was no difference in luciferase activity in MUT-circ-CD44 compared with control (Fig. 5C,D). RIP also revealed the prominent enrichment of miR-330-5p and circ-CD44 in the Ago2-immunoprecipitated complex when compared with the control group (Fig. 5E,F). Importantly, miR-330-5p was downregulated by OXA-resistant CRC tissues and cells, and it negatively correlated with the expression of circ-CD44 (Fig. 5G-I). In addition, circ-CD44 was overexpressed in SW620/OXA and HCT-116/OXA cells transfected with circ-CD44 when compared with the vector group (Fig. 5J). Interestingly, silencing of circ-CD44 increased the expression of miR-330-5p, while overexpression of circ-CD44 suppressed miR-330-5p in OXA-resistant CRC cells (Fig. 5K). Therefore, the above data indicated that

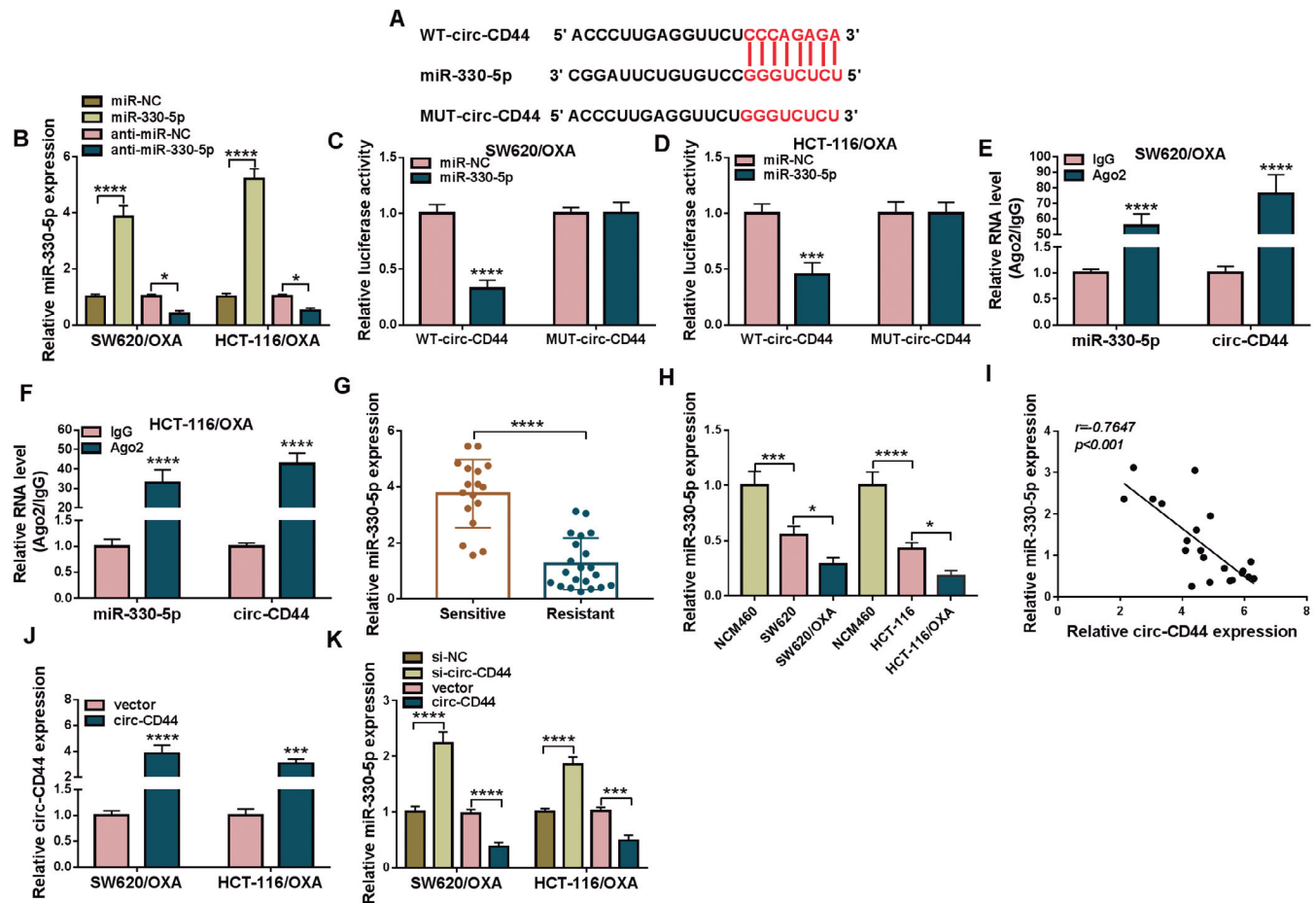


Fig. 5. MiR-330-5p was a direct target of circ-CD44. **A.** The binding region between miR-330-5p and circ-CD44 is shown. **B.** The expression level of miR-330-5p was determined by RT-qPCR assay in colorectal cancer tissues in SW620/OXA and HCT-116/OXA cells transfected with miR-NC, miR-330-5p, anti-miR-NC, or anti-miR-330-5p. **C-F.** The association between circ-CD44 and miR-330-5p was confirmed by dual-luciferase report and RIP assays. **G-H.** The expression level of miR-330-5p was estimated by RT-qPCR assay in OXA-resistant colorectal cancer tissues and cells. **I.** Pearson's correlation analysis was used to analyze the correlation between miR-330-5p and circ-CD44. **J.** The expression of circ-CD44 was determined by RT-qPCR assay in SW620/OXA and HCT-116/OXA cells transfected with vector and circ-CD44. **K.** The expression of miR-330-5p was assessed by RT-qPCR assay in SW620/OXA and HCT-116/OXA cells transfected with si-NC, si-circ-CD44, vector, or circ-CD44. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

miR-330-5p was a direct target of circ-CD44.

Knockdown of circ-CD44-mediated effects on OXA-resistant CRC cells was abolished by silencing miR-330-5p

We knocked down the expression of miR-330-5p by miR-330-5p inhibitor in circ-CD44-silencing SW620/OXA and HCT-116/OXA cells to investigate the role of miR-330-5p. The upregulation of miR-330-5p in circ-CD44-silencing cells was abolished by transfection with miR-330-5p inhibitor (Fig. 6A). The MTT results revealed that silencing of circ-CD44 inhibited OXA resistance, which was overturned by silencing of miR-330-5p in SW620/OXA and HCT-116/OXA cells (Fig. 6B). Moreover, the colony-forming ability was inhibited following circ-CD44 silencing and abrogated by miR-330-5p inhibition (Fig. 6C). The flow cytometry assay showed that circ-CD44 silencing together with miR-330-5p inhibition could inhibit cell apoptosis compared with circ-CD44 silencing alone (Fig. 6D). Inhibition of miR-330-5p reversed the suppressive effects on migration and invasion of SW620/OXA and HCT-116/OXA cells induced by circ-CD44 silencing (Fig. 6E,F). Collectively, silencing of miR-330-5p reversed circ-CD44-mediated effects on OXA-resistant CRC cells.

ABCC1 was a target gene of miR-330-5p

As presented in Fig. 7A, miR-330-5p had the complementary sequences on ABCC1. The dual-luciferase report showed that the luciferase activity in the ABCC1 3'UTR-WT transfected cells was notably decreased compared with that in the mutant and negative control cells (Fig. 7B,C). RIP assay suggested that miR-330-5p could directly bind to ABCC1 in an Ago2-dependent manner (Fig. 7D,E). In addition, we also found that ABCC1 was upregulated in OXA-resistant CRC tissues and cells compared with controls (Fig. 7F,G). A negative correlation between miR-330-5p and ABCC1 was found in OXA-resistant CRC tissues (Fig. 7H). The expression of ABCC1 was inhibited by overexpression of miR-330-5p, while ABCC1 was upregulated in OXA-resistant CRC cells transfected with miR-330-5p inhibitor (Fig. 7I). Conclusively, ABCC1 was a functional target of miR-330-5p.

MiR-330-5p regulated OXA sensitivity, proliferation, apoptosis, migration, and invasion in OXA-resistant CRC cells by targeting ABCC1

To elucidate whether ABCC1 was a functional target of miR-330-5p, ABCC1 was overexpressed in miR-330-

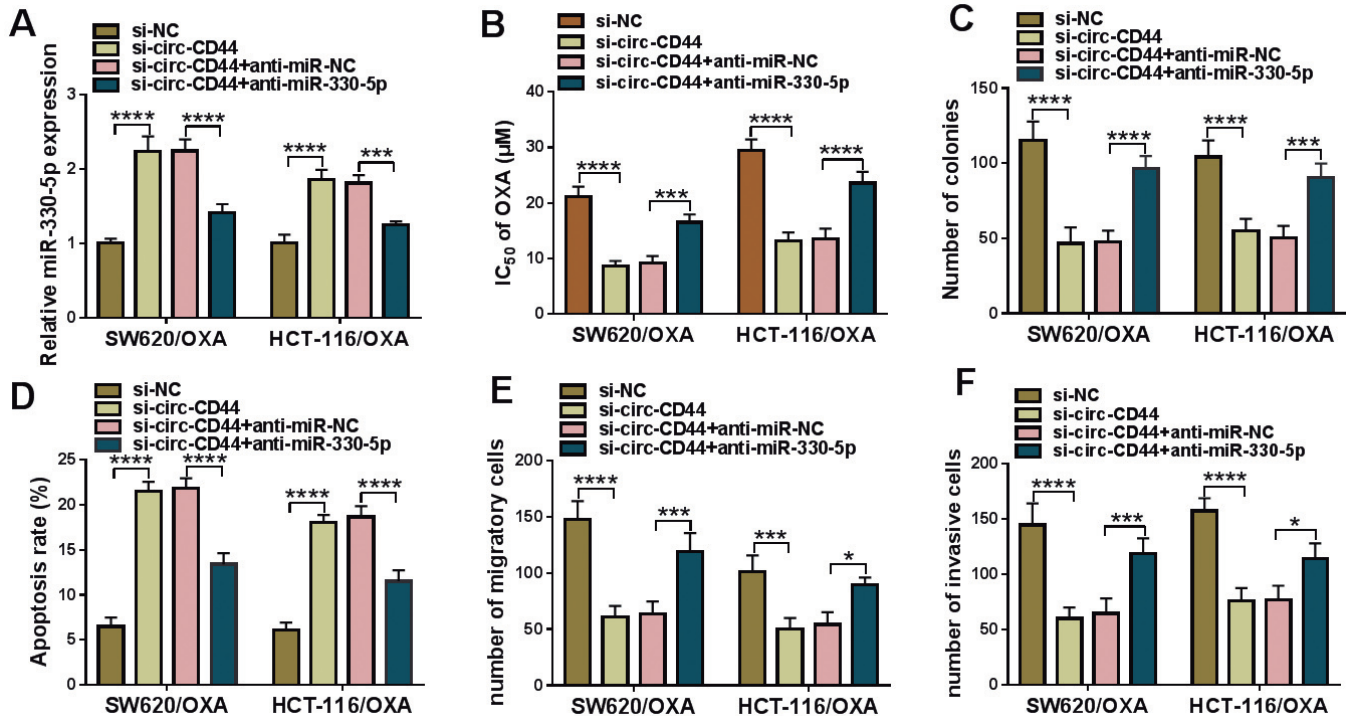


Fig. 6. Circ-CD44 regulated OXA sensitivity, proliferation, apoptosis, migration, and invasion in OXA-resistant colorectal cancer cells by targeting miR-330-5p. **A-F.** SW620/OXA and HCT-116/OXA cells were transfected with si-NC, si-circ-CD44, si-circ-CD44+anti-miR-NC, or si-circ-CD44+anti-miR-330-5p. **A.** The expression level of miR-330-5p was examined by RT-qPCR assay. **B.** The IC₅₀ of OXA was assessed by MTT assay. **C.** Colony-forming ability of SW620/OXA and HCT-116/OXA cells was measured by colony-forming assay. **D.** Flow cytometry assay was performed in SW620/OXA and HCT-116/OXA cells. **E-F.** Transwell assay was used to measure migration and invasion of SW620/OXA and HCT-116/OXA cells. **P*<0.05, ****P*<0.001, *****P*<0.0001.

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5p-transfected SW620/OXA and HCT-116/OXA cells. As shown in Fig. 8A, the downregulation of ABCC1 in miR-330-5p-transfected SW620/OXA and HCT-116/OXA cells was rescued by transfecting with ABCC1. IC₅₀ of SW620/OXA and HCT-116/OXA cells to OXA was decreased after overexpression of miR-330-

5p, which was overturned by overexpression of ABCC1 (Fig. 8B). Furthermore, overexpression of ABCC1 abolished miR-330-5p-induced inhibitory effects on colony-forming (Fig. 8C). The upregulation of miR-330-5p induced apoptosis and inhibited migration and invasion of SW620/OXA and HCT-116/OXA cells,

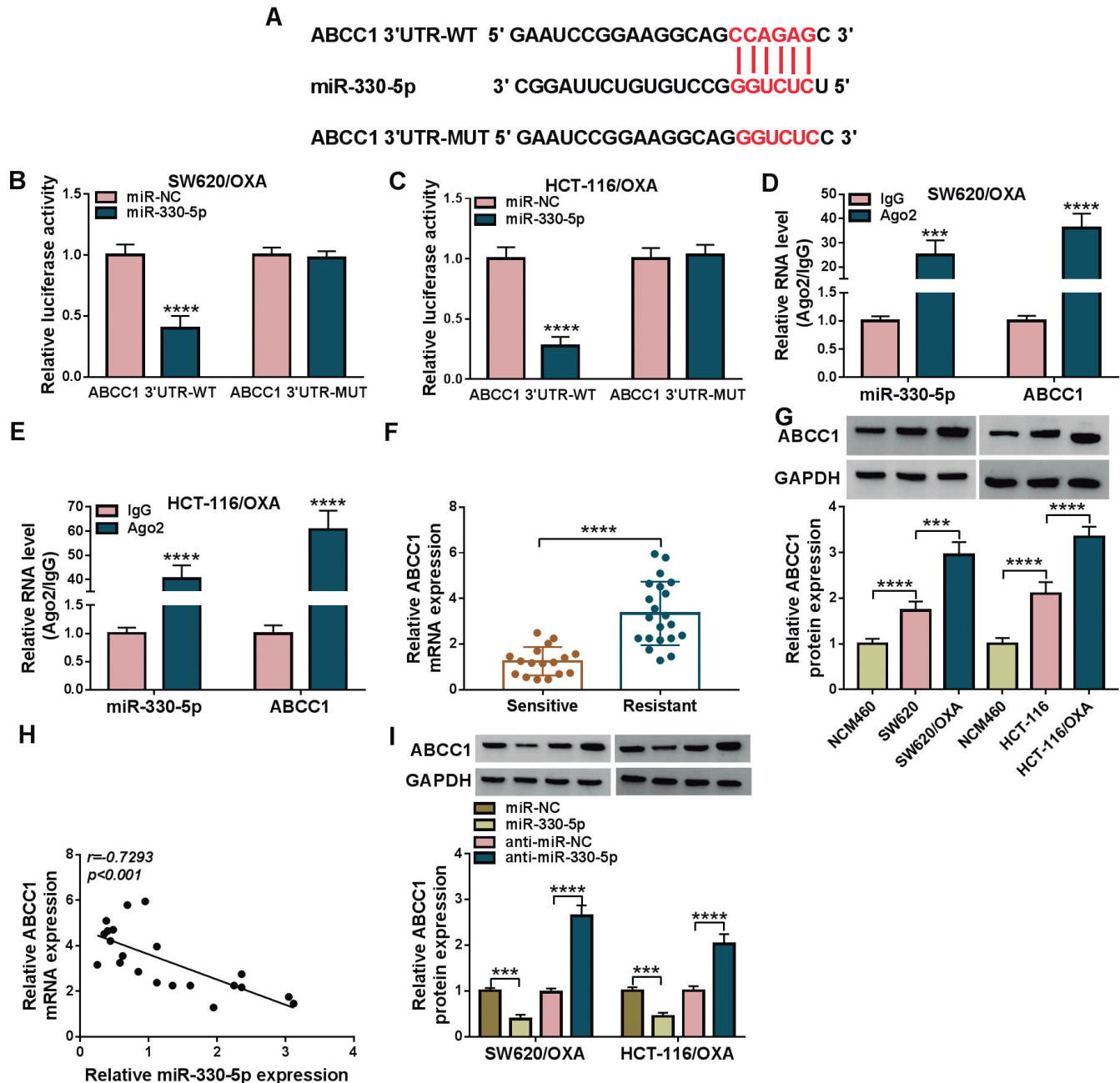


Fig. 7. miR-330-5p targeted ABCC1 in OXA-resistant colorectal cancer cells. **A.** The complementary sequences between miR-330-5p and ABCC1 are shown. **B-E.** The dual-luciferase report and RIP assays were conducted in SW620/OXA and HCT-116/OXA cells. **F-G.** mRNA and protein expression levels of ABCC1 were measured by RT-qPCR and western blot assays in colorectal cancer tissues and cells. **H.** Pearson's correlation analysis was used for analyzing the correlation between miR-330-5p and ABCC1. **I.** The protein expression of ABCC1 was detected by western blot assay in SW620/OXA and HCT-116/OXA cells transfected with miR-NC, miR-330-5p, anti-miR-NC, or anti-miR-330-5p. ** $P < 0.001$, **** $P < 0.0001$.

which was overturned by the re-expression of ABCC1 (Fig. 8D-F). Collectively, the regulator role of miR-330-5p was partially dependent on regulating ABCC1.

Circ-CD44/miR-330-5p/ABCC1 axis in OXA-resistant CRC cells

The association between circ-CD44, miR-330-5p, and ABCC1 was explored in CRC cells. The expression levels of ABCC1 were decreased in si-circ-CD44-transfected SW620/OXA and HCT-116/OXA cells, which were abolished by silencing of miR-330-5p (Fig. 9A,B). Therefore, circ-CD44 was involved in the CRC process by regulation of miR-330-5p and ABCC1.

Discussion

The current results suggested that circ-CD44 increased OXA resistance in CRC by targeting miR-330-5p, which in turn regulates ABCC1 expression. Taken together, circ-CD44 functioned as a tumor promoter in CRC by targeting the miR-330-5p/ABCC1 axis, which will be helpful to further understand the theoretical basis and molecular mechanisms of OXA-based chemo-

therapy in CRC.

It has been well established that circRNAs take part in gene expression by targeting the binding regions of miRNAs as miRNA sponges, resulting in negative regulation of miRNAs (Panda, 2018). Inhibition of circ-CD44 suppressed the malignant phenotype of gastric cancer cells by targeting miR-183 (Cao et al., 2019). Analogously, the oncogenic function of circ-CD44 was reported in breast cancer through sponging with miR-326 (Min et al., 2020). Here, miR-330-5p was identified as a target of circ-CD44.

Previous research has reported that miR-330-5p was a key miRNA that participated in various cell functions in different types of tumors. Consistently, recent studies also revealed that miR-330-5p was obviously decreased in CRC (Yoo et al., 2016; Lu et al., 2020; Shirjang et al., 2020). Nevertheless, the functional effects of miR-330-5p were controversial; the carcinogenic role of miR-330-5p was revealed in hepatocellular carcinoma (Zhang et al., 2018a), which might contribute to different environments of hepatocellular carcinoma and CRC. Interestingly, miR-330-5p was closely related to resistance to chemotherapeutic drugs. Previous report suggested that miR-330-5p might suppress the

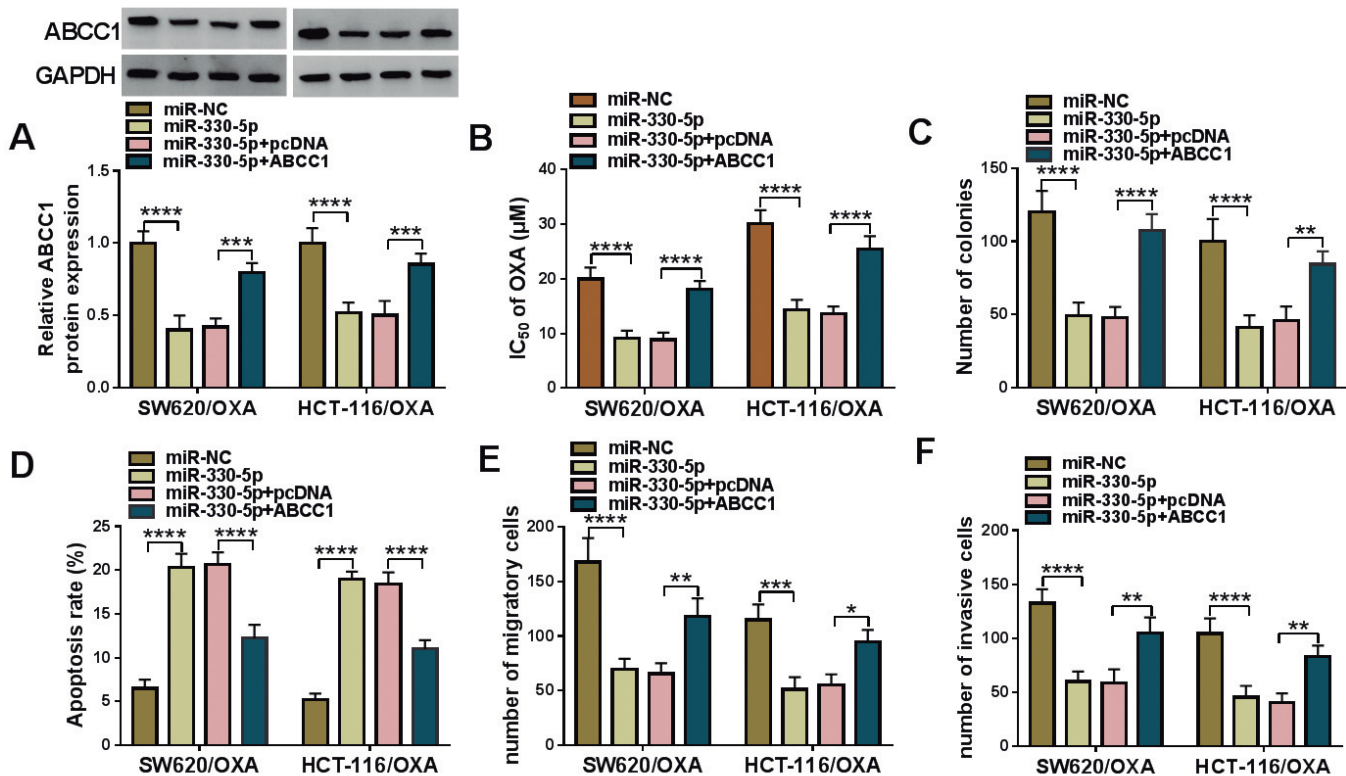


Fig. 8. Overexpression of ABCC1 reversed miR-330-5p-induced effects on OXA-resistant colorectal cancer cells. **A-F.** SW620/OXA and HCT-116/OXA cells were transfected with miR-NC, miR-330-5p, miR-330-5p+pcDNA, or miR-330-5p+ABCC1. **A.** The protein expression level of ABCC1 was examined by western blot assay. **B.** MTT assay was used to measure IC₅₀ of OXA. **C.** Colony number was measured by colony-forming assay. **D.** Apoptotic cells were monitored by flow cytometry assay. **E-F.** The migration and invasion of SW620/OXA and HCT-116/OXA cells were assessed by transwell assay. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

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gemcitabine resistance of pancreatic cancer by interacting with RASSF1 (Liu et al., 2020). Similarly, miR-330-5p might function as a possible regulator of chemo-radiotherapy resistance in esophageal adenocarcinoma (Staff, 2015). As we expected, miR-330-5p was confirmed to increase the sensitivity of CRC cells to OXA by targeting ABCC1.

Overexpression of ABCC1 frequently occurs in human cancers, and it is associated with the multidrug-resistance phenomenon (Yin and Zhang, 2011). It has been established that miR-1268a is capable of modulating temozolomide resistance by regulation of ABCC1 expression in glioblastoma (Li et al., 2018). Gain of miR-7 might inhibit the chemo-resistance of small cell lung cancer by decreasing ABCC1 expression (Liu et al., 2015). Certainly, ABCC1 also functions as a

carcinogenic gene and is involved in multidrug resistance in CRC; multiple miRNAs inhibit the drug resistance of CRC cells by directly targeting ABCC1 (Zhang et al., 2018b; Shan et al., 2019). In line with these studies, our data also suggested that miR-330-5p interacted with ABCC1 in OXA-resistant CRC cells, suggesting that ABCC1 was a functional target of miR-330-5p. Notably, ABCC1 can confer resistance to cytotoxic and targeted chemotherapy by drug transport functions (Cole, 2014). The functional effects of ABCC1 need to be investigated in multidrug resistance in chemotherapy of multiple cancers.

To conclude, overexpression of circ-CD44 was closely associated with the development of CRC by regulation of the miR-330-5p/ABCC1 pathway, which also increased resistance to OXA in CRC cells.

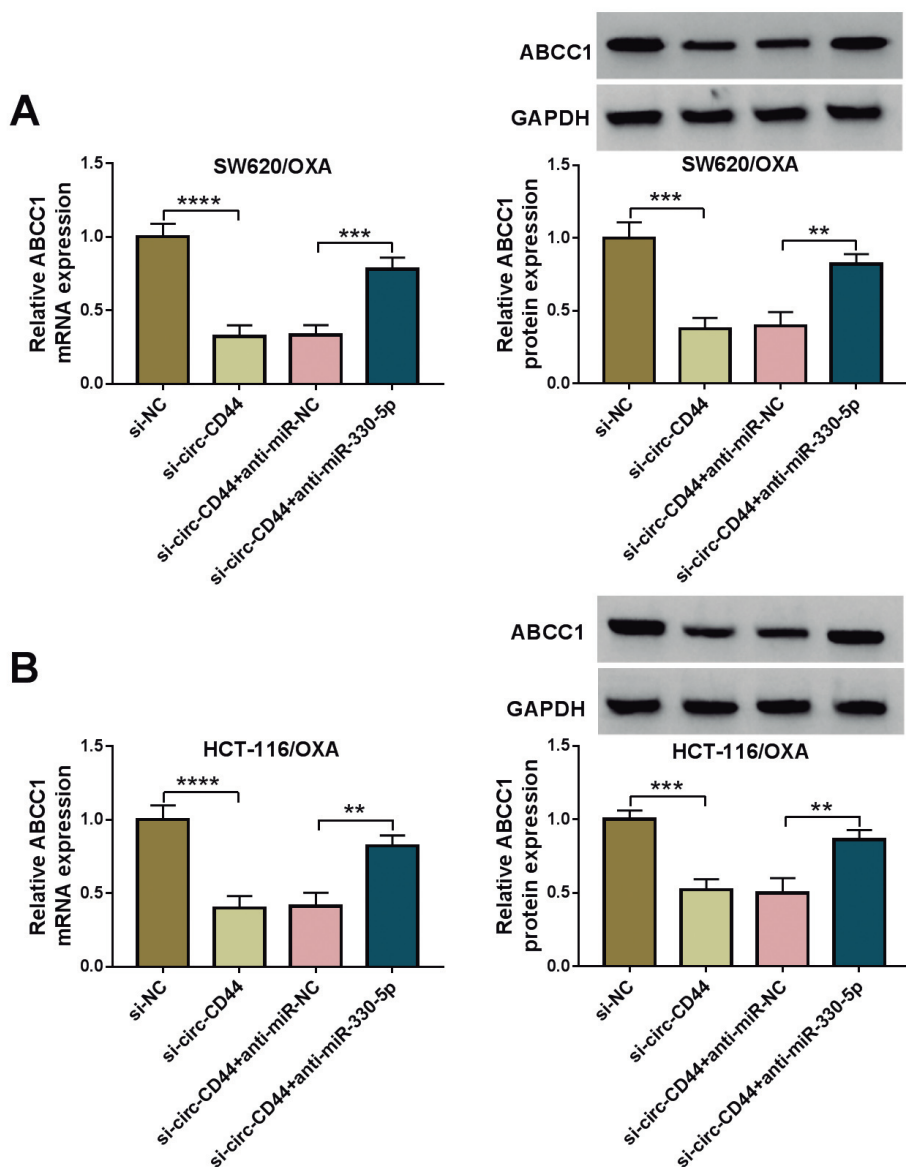


Fig. 9. Circ-CD44 targeted the miR-330-5p/ABCC1 axis in OXA-resistant colorectal cancer cells. **A-B.** RT-qPCR and western blot assays were carried out to examine ABCC1 level in SW620/OXA and HCT-116/OXA cells transfected with si-NC, si-circ-CD44, si-circ-CD44+anti-miR-NC, or si-circ-CD44+anti-miR-330-5p. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Conclusion

Overall, these data demonstrated that circ-CD44 was upregulated in OXA-resistant CRC tissues and cells and that it could sponge miR-330-5p to restore ABCC1 expression, suggesting that the circ-CD44/miR-330-5p/ABCC1 axis might have potential use in OXA-resistant CRC therapy, revealing an important mechanism of chemotherapy resistance.

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Data Availability Statement. Please contact the corresponding author for data request.

References

- Arango D., Wilson A.J., Shi Q., Corner G.A., Aranes M.J., Nicholas C., Lesser M., Mariadason J.M. and Augenlicht L.H. (2004). Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. *Br. J. Cancer* 91, 1931-1946.
- Arnold M., Sierra M.S., Laversanne M., Soerjomataram I., Jemal A. and Bray F. (2017). Global patterns and trends in colorectal cancer incidence and mortality. *Gut* 66, 683-691.
- Bray F., Ferlay J., Soerjomataram I., Siegel R.L., Torre L.A. and Jemal A. (2018). Global cancer statistics 2018: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394-424.
- Cao C., Han S., Yuan Y., Wu Y., Lian W., Zhang X., Pan L. and Li M. (2019). Downregulated circular RNA hsa_circ_0000291 suppresses migration and proliferation of gastric cancer via targeting the miR-183/ITGB1 axis. *Cancer Manag. Res.* 11, 9675-9683.
- Chen J., Chen T., Zhu Y., Li Y., Zhang Y., Wang Y., Li X., Xie X., Wang J., Huang M., Sun X. and Ke Y. (2019). CircPTN sponges miR-145-5p/miR-330-5p to promote proliferation and stemness in glioma. *J. Exp. Clin. Cancer Res.* 38, 398.
- Chen L.L. and Yang L. (2015). Regulation of circRNA biogenesis. *RNA Biol.* 12, 381-388.
- Cole S.P. (2014). Targeting multidrug resistance protein 1 (MRP1, ABCC1): Past, present, and future. *Annu. Rev. Pharmacol. Toxicol.* 54, 95-117.
- Goldstein DA, Zeichner SB, Bartnik CM, Neustadter E and Flowers CR. (2016). Metastatic colorectal cancer: A systematic review of the value of current therapies. *Clin. Colorectal Cancer* 15, 1-6.
- Hansen T.B., Jensen T.I., Clausen B.H., Bramsen J.B., Finsen B., Damgaard C.K. and Kjems J. (2013). Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384-388.
- Huang H., Chen J., Ding C.M., Jin X., Jia Z.M. and Peng J. (2018). LncRNA NR2F1-AS1 regulates hepatocellular carcinoma oxaliplatin resistance by targeting ABCC1 via miR-363. *J. Cell. Mol. Med.* 22, 3238-3245.
- Kim J.H. (2015). Chemotherapy for colorectal cancer in the elderly. *World J. Gastroenterol.* 21, 5158-5166.
- Kunicka T and Soucek P. (2014). Importance of abcc1 for cancer therapy and prognosis. *Drug Metabol. Rev.* 46, 325-342.
- Li Y., Liu Y., Ren J., Deng S., Yi G., Guo M., Shu S., Zhao L., Peng Y. and Qi S. (2018). MiR-1268a regulates ABCC1 expression to mediate temozolomide resistance in glioblastoma. *J. Neurooncol.* 138, 499-508.
- Li C., He X., Zhang L., Li L. and Zhao W. (2019). A pair-wise meta-analysis highlights circular RNAs as potential biomarkers for colorectal cancer. *BMC Cancer* 19, 957.
- Liang Z., Wu H., Xia J., Li Y., Zhang Y., Huang K., Wagar N., Yoon Y., Cho H.T., Scala S. and Shim H. (2010). Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1. *Biochem. Pharmacol.* 79, 817-824.
- Lin H., Yang G., Yu J., Wang J., Li Q., Guo S. and Cao B. (2018). KDM5c inhibits multidrug resistance of colon cancer cell line by down-regulating ABCC1. *Biomed. Pharmacother.* 107, 1205-1209.
- Liu B., Li J. and Cairns M.J. (2014). Identifying miRNAs, targets and functions. *Brief. Bioinform.* 15, 1-19.
- Liu H., Wu X., Huang J., Peng J. and Guo L. (2015). MiR-7 modulates chemoresistance of small cell lung cancer by repressing MRP1/ABCC1. *Int. J. Exp. Pathol.* 96, 240-247.
- Liu Y., Xia L., Dong L., Wang J., Xiao Q., Yu X. and Zhu H. (2020). CircHIPK3 promotes gemcitabine (gem) resistance in pancreatic cancer cells by sponging miR-330-5p and targets RASSF1. *Cancer Manag. Res.* 12, 921-929.
- Lu C., Fu L., Qian X., Dou L. and Cang S. (2020). Knockdown of circular rna circ-FARSA restricts colorectal cancer cell growth through regulation of miR-330-5p/LASP1 axis. *Arch. Biochem. Biophys.* 689, 108434.
- Meng X., Sun W., Yu J., Zhou Y., Gu Y., Han J., Zhou L., Jiang X. and Wang C. (2020). LINC00460-miR-149-5p/miR-150-5p-Mutant p53 feedback loop promotes oxaliplatin resistance in colorectal cancer. *Mol. Ther. Nucleic Acids* 22, 1004-1015.
- Min J., Pan X. and Lv G. (2020). The circRNA circ_0000291 acts as a sponge of microRNA 326 to regulate E26 transformation-specific sequence-1 expression and promote breast cancer progression. *Pathol. Int.* 70, 953-964.
- Panda A.C. (2018). Circular RNAs act as miRNA sponges. *Adv. Exp. Med. Biol.* 1087, 67-79.
- Petrelli F., Coinu A., Ghilardi M., Cabiddu M., Zaniboni A. and Barni S. (2015). Efficacy of oxaliplatin-based chemotherapy + bevacizumab as first-line treatment for advanced colorectal cancer: A systematic review and pooled analysis of published trials. *Am. J. Clin. Oncol.* 38, 227-233.
- Qi X., Zhang D.H., Wu N., Xiao J.H., Wang X. and Ma W. (2015). CeRNA in cancer: Possible functions and clinical implications. *J. Med. Genet.* 52, 710-718.
- Sanchez-Mejias A. and Tay Y. (2015). Competing endogenous RNA networks: Tying the essential knots for cancer biology and therapeutics. *J. Hematol. Oncol.* 8, 30.
- Shan C., Zhang Y., Hao X., Gao J., Chen X. and Wang K. (2019). Biogenesis, functions and clinical significance of circRNAs in gastric cancer. *Mol. Cancer* 18, 136.
- Shirjang S., Mansoori B., Mohammadi A., Shajari N., Duijf P.H.G., Najafi S., Abedi Gaballu F., Nofouzi K. and Baradaran B. (2020). MiR-330 regulates colorectal cancer oncogenesis by targeting BACH1. *Adv. Pharm. Bull.* 10, 444-451.
- Staff P.O. (2015). Correction: MicroRNA-330-5p as a putative modulator

Circ-CD44/miR-330-5p/ABCC1 axis regulates chemotherapy resistance

- of neoadjuvant chemoradiotherapy sensitivity in oesophageal adenocarcinoma. *PLoS One* 10, e0137155.
- Tang Y.C., Zhang Y., Zhou J., Zhi Q., Wu M.Y., Gong F.R., Shen M., Liu L., Tao M., Shen B., Gu D.M., Yu J., Xu M.D., Gao Y. and Li W. (2018). Ginsenoside RG3 targets cancer stem cells and tumor angiogenesis to inhibit colorectal cancer progression *in vivo*. *Int. J. Oncol.* 52, 127-138.
- Vasan N., Baselga J. and Hyman D.M. (2019). A view on drug resistance in cancer. *Nature* 575, 299-309.
- Vishnoi A. and Rani S. (2017). MiRNA biogenesis and regulation of diseases: An overview. *Methods Mol. Biol.* 1509, 1-10.
- Wang H., Yan X., Zhang H. and Zhan X. (2019). CircRNA circ_0067934 overexpression correlates with poor prognosis and promotes thyroid carcinoma progression. *Med. Sci. Monit.* 25, 1342-1349.
- Yin J. and Zhang J. (2011). Multidrug resistance-associated protein 1 (MRP1/ABCC1) polymorphism: From discovery to clinical application. *Zhong Nan Da Xue Xue Bao. Yi Xue Ban* 36, 927-938.
- Yoo H.I., Kim B.K. and Yoon S.K. (2016). MicroRNA-330-5p negatively regulates ITGA5 expression in human colorectal cancer. *Oncol. Rep.* 36, 3023-3029.
- Zealy R.W., Wrenn S.P., Davila S., Min K.W. and Yoon J.H. (2017). MicroRNA-binding proteins: Specificity and function. *Wiley Interdiscip. Rev. RNA* 8, e1414.
- Zhang Z., Yang T. and Xiao J. (2018a). Circular RNAs: Promising biomarkers for human diseases. *EBioMedicine* 34, 267-274.
- Zhang Z., Feng L., Liu P. and Duan W. (2018b). ANRIL promotes chemoresistance via disturbing expression of ABCC1 by regulating the expression of Let-7a in colorectal cancer. *Biosci. Rep.* 38, BSR20180620.
- Zhao H., Hu G.M., Wang W.L., Wang Z.H., Fang Y. and Liu Y.L. (2019). LncRNA TDRG1 functions as an oncogene in cervical cancer through sponging mir-330-5p to modulate ELK1 expression. *Eur. Rev. Med. Pharmacol. Sci.* 23, 7295-7306.

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