Original Article
miR-659-3p is involved in the regulation of the chemotherapy response of colorectal cancer via modulating the expression of SPHK1

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Abstract: Colorectal cancer (CRC) is one of most prevalent malignant diseases worldwide. Metastasis and chemo-resistance are the two prominent death-related factors of CRCs. Thus, it is urgent to understand the mechanism responsible for the chemo-resistant properties of CRC and develop new therapeutic methods. Here, we found that the expression of miR-659-3p was significantly reduced in cisplatin (CDDP)-resistant HT29 and LOVO colorectal cancer cells and in CDDP-resistant clinical colorectal cancer samples compared with respective CDDP-sensitive counterparts. Sphingosine kinase 1 (SPHK1) is a direct target of miR-659-3p in colorectal cancer cells, and it is negatively regulated by miR-659-3p. We found that anti-miR-659-3p could increase the IC50 of CDDP in parental HT29 and LOVO colorectal cancer cells; additionally, miR-659-3p mimics decreased the IC50 of CDDP in HT29/CDDP and LOVO/CDDP colorectal cancer cells. Furthermore, we showed that the miR-659-3p/SPHK1 pathway was involved in the regulation of chemotherapy responses of colorectal cancer cells in vivo. In all, our findings suggest a new mechanism involved in the regulation of the chemotherapy response of CRC and might provide new targets for CRC prevention and treatment.

Keywords: miR-659-3p, SPHK1, colorectal cancer, CRC, CDDP, cisplatin

Introduction

Colorectal cancer (CRC) is one of the most prevalent malignant diseases worldwide, and it was the fifth most common cause of cancer-related deaths in China in 2015 [1, 2]. Although great advances have been made in the field of cancer prevention and diagnosis, the effects of treatment for CRC have remained unsatisfactory in the past decades. Surgical resection is the most effective therapeutic method for primary colorectal cancer [3-5], and for metastatic colorectal cancer, chemotherapy is the preferred adjuvant therapy [6]. Due to the heterogeneity of CRC, metastatic and recurrent CRCs resist conventional chemotherapy [3-5]. Thus, it is critical to elucidate the mechanisms responsible for the chemo-resistance of CRC.

MicroRNAs (miRNAs) are small non-coding RNAs, approximately 22 nt in length, that post-transcriptionally regulate the expression of target genes [7, 8]. miRNAs participate in the process of tumor oncogenesis, including in CRCs [9-11]. miR-659-3p is one of the miRNAs expressed in colorectal cells [12], and it participates in the metastasis of neuroblastoma [13] and the chemotherapy response of melanoma [14]. However, the role of miR-659-3p in the progression and chemotherapy response of colorectal cancer remains obscure.

Sphingosine-1-phosphate (S1P) is a critical enzyme of sphingolipid metabolism, and it is involved in many types of human cancers [15, 16]. Increasing evidence has demonstrated that the expression level and/or enzyme activity of SPHK1 was significantly increased in CRC [16-18]. The expression of SPHK1 was regulated at various levels [11, 19], and miRNAs have emerged as potent regulators of SPHK1 [11, 20-23]. The expression of SPHK1 is regulated by miR-101 in colorectal cancer cells [11]. The role of other miRNAs in regulating the expres-

sion of SPHK1 in colorectal cancer cells remains unclear.

Here, we demonstrated that the expression of miR-659-3p was significantly reduced in CDDP-resistant colorectal cancer cells, and SPHK1 was a direct target of miR-659-3p in colorectal cancer cells. We found that decreases in miR-659-3p and increases in SPHK1 were correlated with the chemo-resistant properties of colorectal cancer cells both in vitro and in vivo. Our findings could shed new light on the mechanism of chemo-resistance in CRC and could provide potential therapeutic targets for chemo-resistant CRC.

Materials and methods

Clinical colorectal cancer samples

CDDP-sensitive and -tolerant colorectal cancer samples were obtained from the Tianjin Union Medical Center with informed consent from the patients. The use of tissue samples was approved by the ethical committee of the Tianjin Union Medical Center.

Cell culture

LOVO and HT29 colorectal cancer cells were maintained in F-12k medium and McCoy's 5a medium supplemented with 10% FBS in a water-jacketed CO_2 incubator at 37°C and 5% CO_2. Transfection was performed with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. For screening CDDP-tolerant LOVO and HT29 cells (LOVO/CDDP and HT29/CDDP), 80% confluent LOVO and HT29 cells were treated with culture medium containing 10 µM CDDP for one month.

RNA extraction and real-time PCR

Total RNA was extracted from the cells with Trizol, following the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription. Real-time PCR was performed using a Bio-Rad iQ5 system, and the fold change in expression of each target RNA relative to U6 snRNA or beta-actin mRNA was calculated based on the threshold cycle (Ct) as $2^{-(Ct)}$, where $\Delta Ct = Ct_{target} - Ct_{U6/actin}$, and $\Delta (\Delta Ct) = \Delta Ct$ sample$-\Delta Ct$ control.

Western blot

Equal amounts of total protein were separated by SDS-PAGE gel, and then the proteins were transferred to a PVDF membrane. After blocking, anti-SPHK1 and anti-GAPDH antibodies were added and incubated at 4°C overnight. An HRP-conjugated secondary antibody was used, and the bands were visualized by chemiluminescence.

Reporter assay

Wild-type SPHK1-3'UTR containing a miR-659-3p binding site or mutant-form 3'UTR was inserted downstream of the CDS of the firefly luciferase gene. The reporter gene was transfected into HT29 or LOVO cells with different miR-659-3p levels, simultaneously with Renilla luciferase. The activity of firefly luciferase or Renilla luciferase was measured 24 hours post-transfection.

IC50 detection

HT29 or LOVO colorectal cancer cells transfected with miR-659-3p-specific antisense oligonucleotides, alone or simultaneously with si-SPHK1, were seeded in a 96-well plate and then were treated with increasing concentrations of CDDP for 24 hours, and the survival of cells was estimated by MTT. The data of the non-treated group were set to 100%, and the concentration of CDDP in the group with 50% the survival rate of the non-treated group was regarded as the IC50 value. A similar experiment was performed with HT29/CDDP or LOVO/CDDP cells transfected with miR-659-3p mimics, alone or simultaneously with an SPHK1 expression plasmid.

Xenograft tumor formation assay

Approximately 5×10^6 HT29 cells transfected with miR-659-3p mimics or control mimics, with or without cisplatin treatment, were subcutaneously injected into the flanks of nude mice. A mock group was also used. The volume of xenograft tumors was measured once per week until the mice were sacrificed, and during the course of the experiment, the cisplatin-treated group was injected with 10 µM cisplatin twice per week. The tumor weight was measured at the time that the mice were sacrificed.
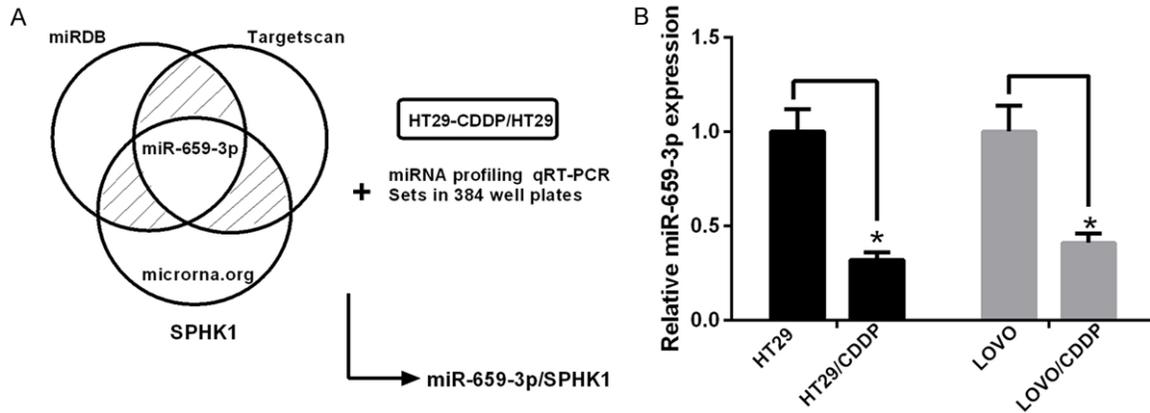
Figure 1. miR-659-3p is downregulated in CDDP-resistant colorectal cancer cells. A. A schematic indicating the strategy used in screening the candidate microRNAs that can regulate the expression of SPHK1 is shown. B. The expression of miR-659-3p in CDDP-resistant HT29 and LOVO colorectal cancer cells, as well as respective parental cells, was determined by qRT-PCR. The expression level of miR-659-3p in parental cells was normalized to one. *P<0.05.
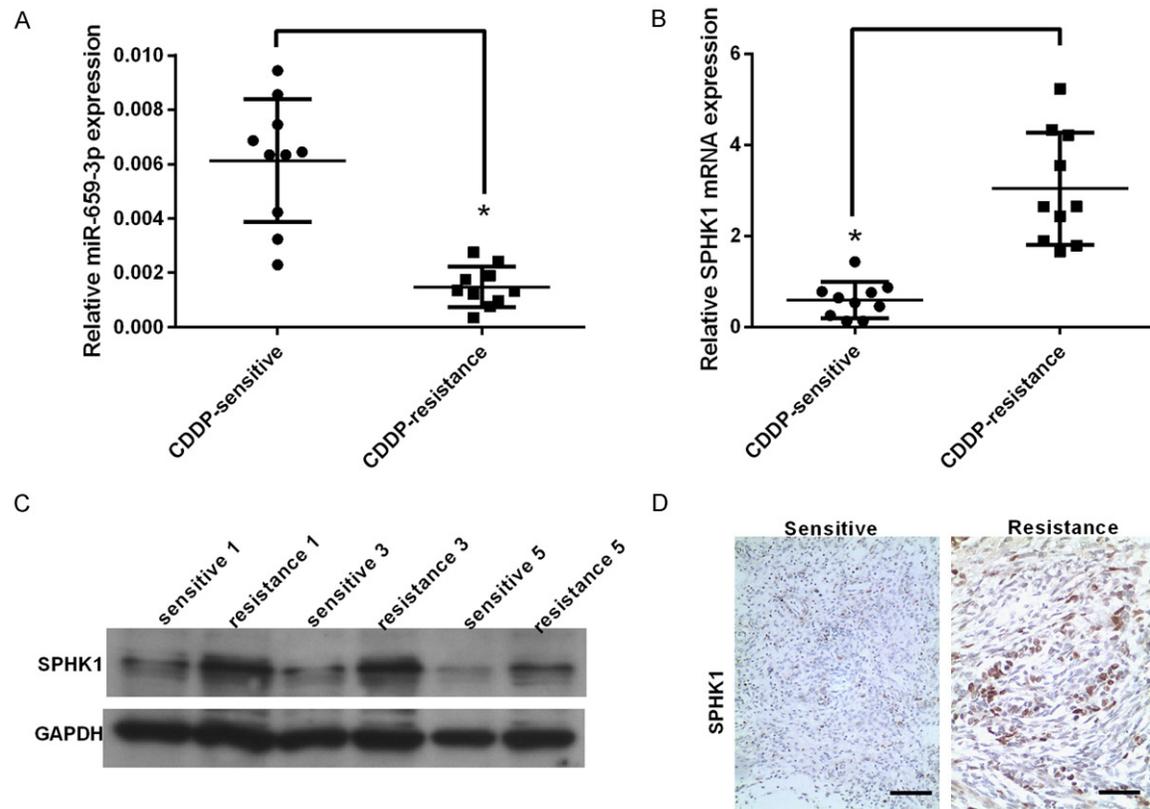
Figure 2. The expression of miR-659-3p and SPHK1 is negatively correlated with clinical colorectal cancer tissues. A. The expression of miR-659-3p in CDDP-sensitive and -resistant clinical colorectal cancer samples was detected by qRT-PCR. The expression of miR-659-3p was normalized to U6 snRNA. *P<0.05. B. The expression of SPHK1 in CDDP-sensitive and -resistant clinical colorectal cancer samples was detected by qRT-PCR. The expression of SPHK1 was normalized to beta-actin *P<0.05. C. The expression of SPHK1 in CDDP-sensitive and -resistant clinical colorectal cancer samples was detected by western blot. GAPDH served as a loading control. D. The expression of SPHK1 in CDDP-sensitive and -resistant clinical colorectal cancer samples was detected by immunohistochemical methods.
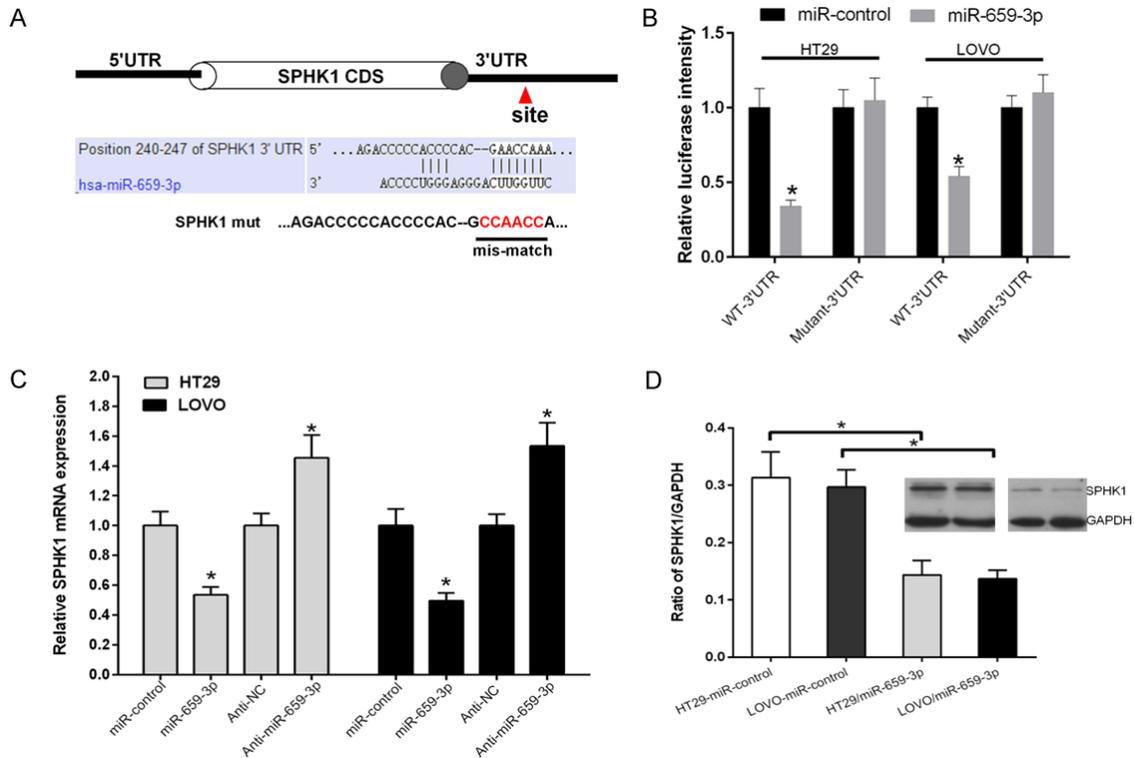
Figure 3. miR-659-3p negatively regulates the expression of SPHK1 in colorectal cancer cells. A. A schematic indicating the target site of miR-659-3p in the 3'UTR of SPHK1 mRNA and a mutant form of the SPHK1 3'UTR is shown. B. A luciferase reporter containing wild-type or a mutant form of SPHK1 3'UTR was transfected into colorectal cancer cells simultaneously with miR-659-3p mimics or control mimics, and the luciferase activity was detected 24 hours post-transfection. The luciferase activity in control mimic-transfected cells was normalized to one. *P<0.05. C. The expression of SPHK1 mRNA in HT29 and LOVO colorectal cancer cells transfected with miR-659-3p mimics or control mimics was determined by qRT-PCR. The expression level of SPHK1 mRNA in control mimic-transfected cells was set to one. *P<0.05. D. The expression of SPHK1 protein in HT29 and LOVO colorectal cancer cells transfected with miR-659-3p mimics or control mimics was determined by western blot. For quantification, the band intensity of SPHK1 protein was normalized to that of GAPDH. *P<0.05.

Results

miR-659-3p is a potential regulator of SPHK1 and is downregulated in CDDP-tolerant colorectal cancer cells

SPHK1 is a critical oncogene that participates in many types of human cancers, including CRC. SPHK1 influences not only the proliferation and migration of CRC, but it also affects the chemotherapy response of CRC. However, regulation of SPHK1 expression, especially regulation by miRNA under chemo-resistant conditions, has not been well elucidated. Here, we used qRT-PCR-based miRNA profiling and bioinformatics methods to identify potential miRNAs that might regulate the expression of SPHK1 under chemo-resistant conditions. We found that miR-659-3p was the only miRNA simultaneously predicted by miRDB, Targetscan and microrna.org (**Figure 1A**) as a potential regulator of SPHK. Moreover, we found that miR-659-3p was significantly reduced in CDDP-resistant HT29 (HT29/CDDP) colorectal cancer cells compared with parental HT29 cells (**Figure 1A**), as detected by qRT-PCR-based miRNA profiling methods. To validate the result of the miRNA profiling, we used qRT-PCR to detect the expression of miR-659-3p in HT29/CDDP and LOVO/CDDP colorectal cancer cells and their respective parental cells. We found that miR-659-3p was significantly reduced in both HT29/CDDP and LOVO/CDDP cells compared with the parental cells (**Figure 1B**), supporting the miRNA profiling result. In a subsequent study, we investigate the role of miR-659-3p in the regulation of SPHK1 and the chemotherapy response of HT29 and LOVO colorectal cancer cells to CDDP.
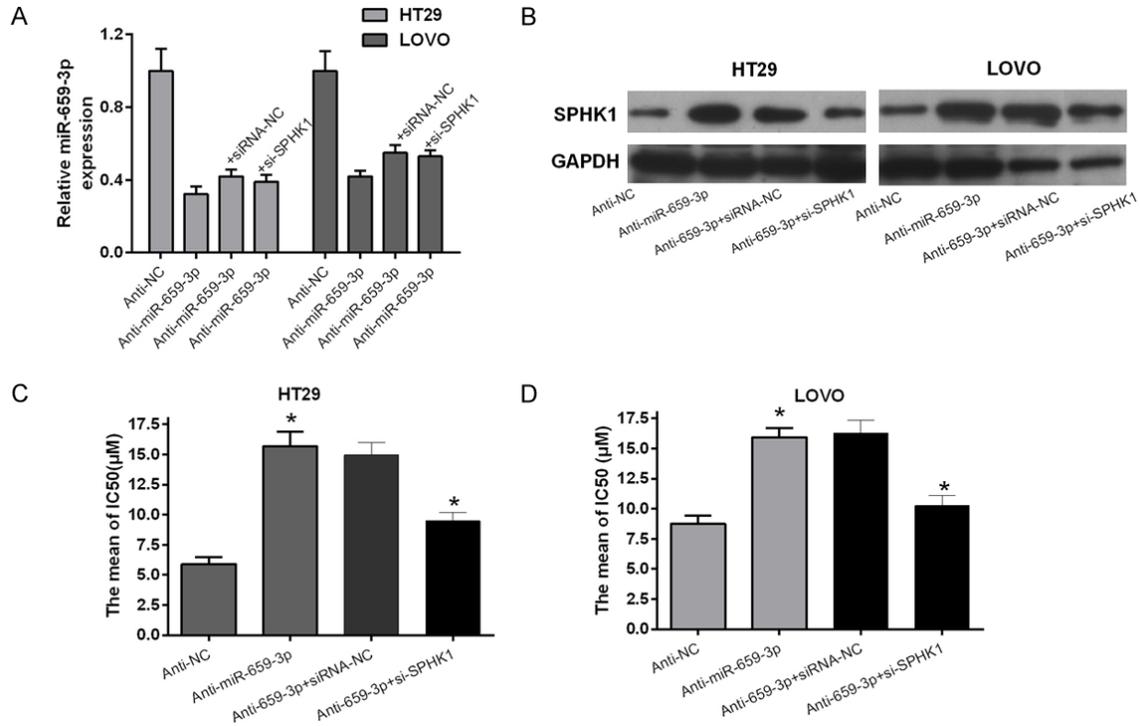
Figure 4. Knockdown of SPHK1 re-sensitizes HT29 and LOVO colorectal cancer cells transfected with anti-miR-659-3p to CDDP. A. The relative expression of miR-659-3p with the indicated treatments was measured by qRT-PCR. The expression of miR-659-3p was normalized to U6 snRNA. *P<0.05. B. The expression of SPHK1 protein in HT29 and LOVO colorectal cancer cells with the indicated treatment was determined by western blot. GAPDH served as a loading control. C. HT29 colorectal cancer cells were first transfected with the indicated oligonucleotides and then treated with increasing concentrations of CDDP. The mean IC50 of CDDP is shown. *P<0.05. D. LOVO colorectal cancer cells were first transfected with the indicated oligonucleotides and then treated with increasing concentrations of CDDP. The mean IC50 of CDDP is shown. *P<0.05.

miR-659-3p is reduced while SPHK1 is increased in CDDP-resistant clinical colorectal cancer samples, compared with CDDP-sensitive samples

To further investigate whether miR-659-3p participates in the process of the chemotherapy response of CRC to CDDP, we measured the expression of miR-659-3p in clinically CDDP-sensitive and CDDP-resistant colorectal cancer samples. Compared with CDDP-sensitive samples, the expression of miR-659-3p was significantly reduced in CDDP-resistant samples (**Figure 2A**), indicating a potential role of miR-659-3p in the CDDP response. Above, we demonstrated that miR-659-3p might be a regulator of SPHK1; thus, we detected the expression of SPHK1 in clinical colorectal cancer samples. As shown in **Figure 2B**, the expression of SPHK1 was significantly increased in CDDP-resistant samples, compared with CDDP-sensitive samples. We further performed western blot and immunochemistry to determine the expression of SPHK1 in clinical samples. Compared

with CDDP-sensitive samples, the protein level of SPHK1 was significantly increased in CDDP-resistant samples (**Figure 2C** and **2D**). Together, these results indicated that both miR-659-3p and SPHK1 were involved in the chemotherapy response of CRC to CDDP and that miR-659-3p might be involved in the regulation of SPHK1.

miR-659-3p negatively regulates the expression of SPHK1 in colorectal cancer cells

The above data indicated that miR-659-3p might be an authentic regulator of SPHK1 in colorectal cancer cells. To investigate whether miR-659-3p was truly involved in the regulation of SPHK1, we first inserted the SPHK1 3'UTR containing a miR-659-3p target site or a mutant form of SPHK1 3'UTR downstream of firefly luciferase CDs (**Figure 3A**). Then, we transfected the reporter plasmid into HT29 or LOVO colorectal cancer cells simultaneously with miR-659-3p mimics or control mimics. We found that miR-659-3p significantly inhibited the expression of the wild-type luciferase
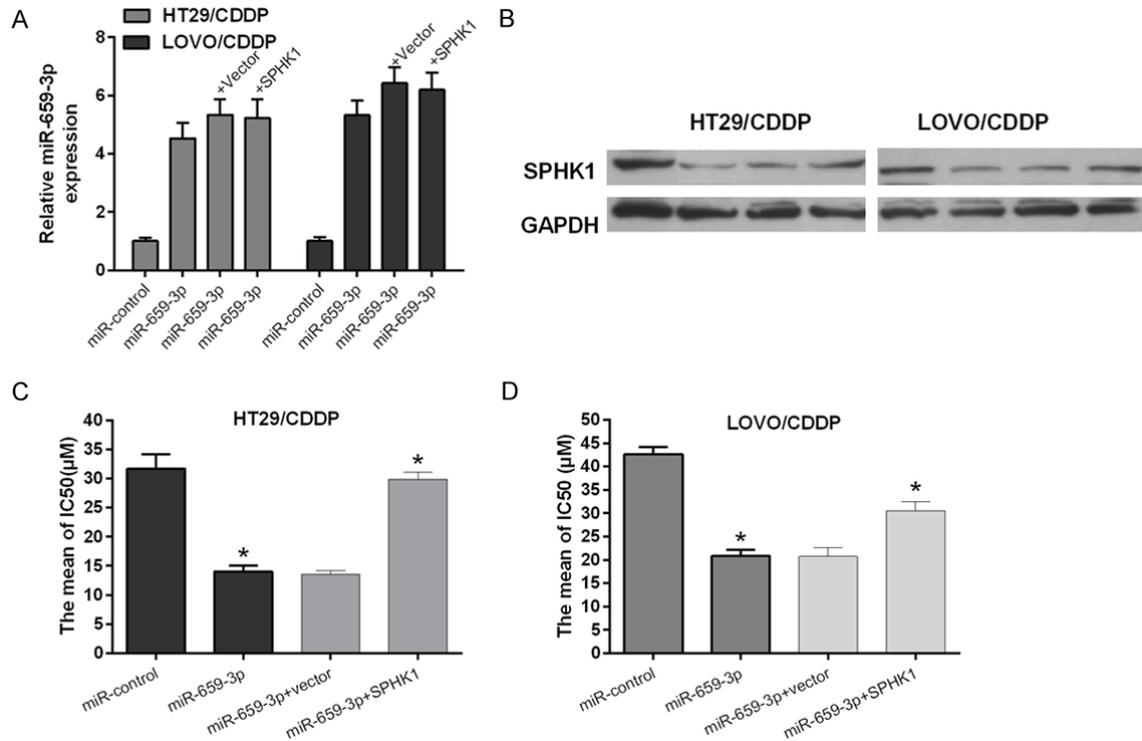
Figure 5. Exogenous SPHK1 re-introduces CDDP tolerance to HT29/CDDP and LOVO/CDDP colorectal cancer cells transfected with miR-659-3p mimics. A. The relative expression of miR-659-3p with the indicated treatments was measured by qRT-PCR. The expression of miR-659-3p was normalized to U6 snRNA. *P<0.05. B. The expression of SPHK1 protein in HT29/CDDP and LOVO/CDDP colorectal cancer cells with the indicated treatments was determined by western blot. GAPDH served as a loading control. C. HT29/CDDP colorectal cancer cells were first transfected with the indicated mimics and plasmid and then treated with increasing concentrations of CDDP. The mean IC50 of CDDP is shown. *P<0.05. D. LOVO/CDDP colorectal cancer cells were first transfected with the indicated mimics and plasmid and then treated with increasing concentrations of CDDP. The mean IC50 of CDDP is shown. *P<0.05.

reporter plasmid but had no effects on the mutant plasmid in both cell lines (**Figure 3B**), indicating direct regulation of the luciferase reporter plasmid by miR-659-3p. We further determined the impact of miR-659-3p on endogenous SPHK1. As shown in **Figure 3C**, miR-659-3p decreased while anti-miR-659-3p increased the mRNA levels of SPHK1 in both HT29 and LOVO cells (**Figure 3C**). Moreover, the results of the western blot analysis showed that miR-659-3p could reduce the protein levels of SPHK1 in both cell lines (**Figure 3D**). In all, these results demonstrated that miR-659-3p could inhibit the expression of SPHK1 in colorectal cancer cells.

Knockdown of SPHK1 re-sensitized HT29 and LOVO colorectal cancer cells transfected with anti-miR-659-3p to CDDP

To investigate the role of miR-659-3p in the chemotherapy response of colorectal cancer cells to CDDP and whether SPHK1 is the func-

tional target of miR-659-3p during this process, we determined the IC50 of CDDP with doxorubicin in parental HT29 and LOVO colorectal cancer cells transfected with anti-miR-659-3p, alone or simultaneously with si-SPHK1 or respective controls. The qRT-PCR results showed that anti-miR-659-3p could reduce miR-659-3p levels in HT29 and LOVO cells and that simultaneous treatment with si-SPHK1 had no additional effects on miR-659-3p levels (**Figure 4A**). Anti-miR-659-3p significantly increased the protein levels of SPHK1 in both cell lines, and simultaneous treatment with si-SPHK1 reversed the impact of anti-miR-659-3p on SPHK1 protein (**Figure 4B**). Transfection with anti-miR-659-3p increased the CDDP-resistant abilities of parental HT29 and LOVO colorectal cancer cells, as indicated by the significant increases in the IC50 values of CDDP in both cell lines (**Figure 4C** and **4D**). Simultaneous knockdown of SPHK1 could reverse the influences of anti-miR-659-3p on CDDP responses in both cell lines
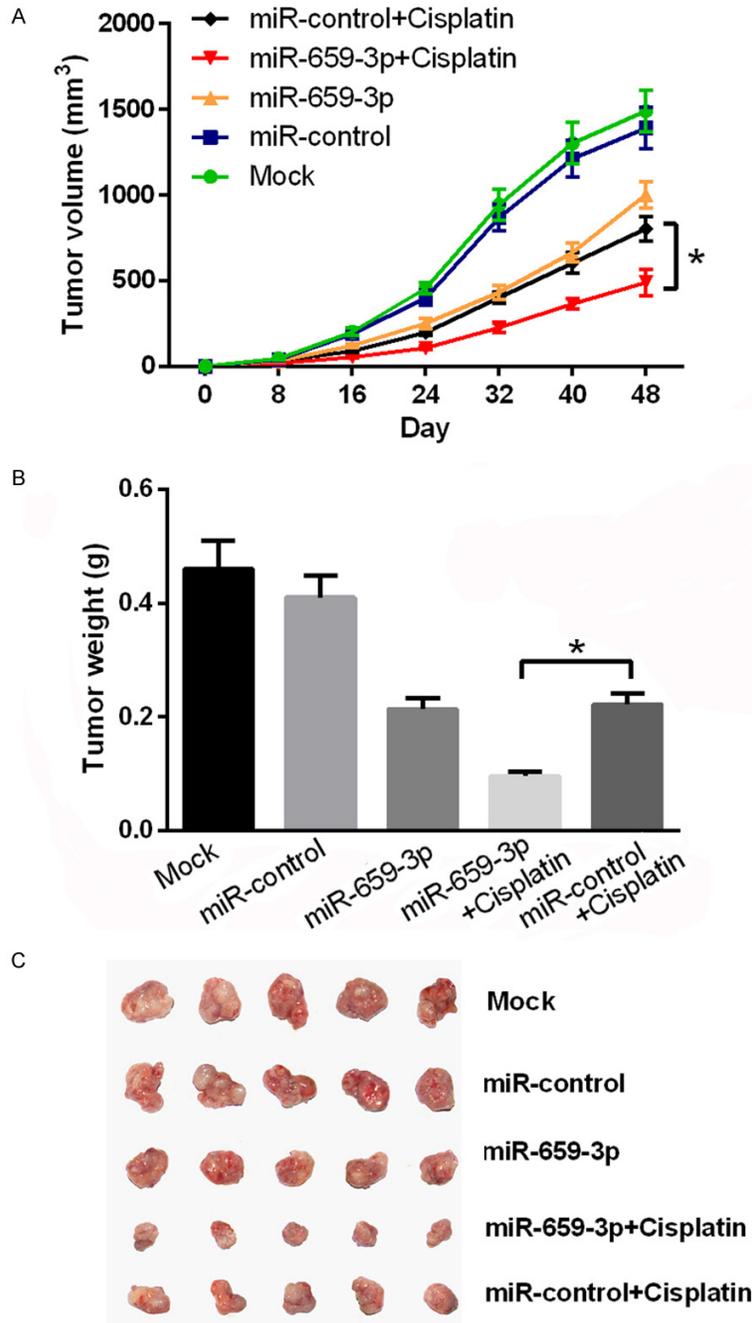
Figure 6. miR-659-3p sensitizes xenograft tumors to cisplatin. A. HT29 colorectal cancer cells with the indicated treatment were subcutaneously injected into the flanks of nude mice, and the growth curve of the tumors is shown. *P<0.05. B. The mean weight of the xenograft tumors at the time the mice were sacrificed is shown. *P<0.05. C. A photograph of the xenograft tumors at the time the mice were sacrificed is shown.

functional target of miR-659-3p during this process.

Exogenous SPHK1 restored CDDP tolerance to HT29/CDDP and LOVO/CDDP colorectal cancer cells transfected with miR-659-3p mimics

To further validate the role of miR-659-3p in the CDDP response of HT29 and LOVO colorectal cancer cells and the involvement of SPHK1 during this process, we treated HT-29/CDDP and LOVO/CDDP cells with miR-659-3p mimics, alone or simultaneously with a SPHK1 expression plasmid or respective controls. miR-659-3p mimics significantly increased miR-659-3p levels in both HT29/CDDP and LOVO/CDDP cells, and simultaneous transfection with an SPHK1 expression plasmid had no additional effect on miR-659-3p levels (**Figure 5A**). miR-659-3p reduced the protein levels of SPHK1 in HT29/CDDP and LOVO/CDDP cells, and the SPHK1 expression plasmid could rescue the expression of SPHK1 in both cells (**Figure 5B**). Transfection with miR-659-3p mimics obviously reduced the CDDP-resistant abilities of HT29/CDDP and LOVO/CDDP colorectal cancer cells, as indicated by the significant increases in the IC50 of CDDP in both cell lines (**Figure 5C** and **5D**). Re-expression of SPHK1 could reverse the influences of anti-miR-659-3p on the CDDP response of both cell lines (**Figure 5C** and **5D**). In all, these results

(**Figure 4C** and **4D**). These results indicated that miR-659-3p was involved in the regulation of the CDDP responses of HT29 and LOVO colorectal cancer cells and that SPHK1 was a further supported a role of miR-659-3p in the CDDP response of HT29 and LOVO cells, and it also further confirmed that SPHK1 is a functional target of miR-659-3p.

miR-659-3p sensitizes xenograft tumors to cisplatin

The above results demonstrated that miR-659-3p played an important role in chemotherapy responses in vitro. Here, we attempted to determine whether miR-659-3p was involved in this process in vivo. Equal amounts of HT29 colorectal cancer cells with different treatments as indicated were subcutaneously injected into the flanks of nude mice. For cisplatin treatment, 10 μM of cisplatin was inter-tumorally injected eight days after the cells were implanted. miR-659-3p could significantly reduce the volume and the mean weight of xenograft tumors (**Figure 6A-C**). Cisplatin treatment inhibited the growth of xenograft tumors, and miR-659-3p further increased the influences of cisplatin on xenograft tumors (**Figure 6A-C**). Together, these results demonstrated that miR-659-3p participated in the regulation of the chemotherapy responses of HT29 and LOVO cells both in vitro and in vivo.

Discussion

Great advances have been made in the field of CRC in the past decades, but due to the chemo-resistance of CRC to conventional therapeutic agents, the death rate from CRC remains high [2]. Thus, it is urgent to determine the mechanisms responsible for the chemo-resistance of CRC. Here, we demonstrated that miR-659-3p participated in the regulation of the response of colorectal cancer cells to various chemotherapeutic agents both in vitro and in vivo by inhibiting the expression of SPHK1.

miR-659-3p, which is expressed in colorectal cells [12], has not been a well-studied miRNA. Until now, its role in colorectal cells has remained unclear [12]. Here, we found that the expression of miR-659-3p was significantly reduced in CDDP-resistant HT29 and LOVO colorectal cancer cells compared with the parental cells, indicating a role of miR-659-3p in the chemotherapy response of colorectal cancer cells. Further supporting our speculation, we found that miR-659-3p was also significantly reduced in clinically chemo-resistant colorectal cancer samples, compared with the chemo-sensitive samples. In fact, a previous study reported that the expression of miR-659-3p was correlated with the therapeutic response of melanoma to cisplatin and paclitaxel [14].

We found that re-expression of miR-659-3p in HT29/CDDP and LOVO/CDDP cells re-sensitized the cells to CDDP treatment and that blocking miR-659-3p endowed parental HT29 and LOVO colorectal cancer cells with CDDP-resistant properties. These findings further supported a critical role of miR-659-3p in the chemotherapy response of cancers.

SPHK1 is a critical enzyme involved in the transformation of pro-death ceramide/sphingosine into pro-survival S1P3 [15, 16, 24]. Increasing evidence has demonstrated that SPHK1 participates in many processes of carcinogenesis, including the proliferation of cancer cells [22, 25, 26] and metastasis [17, 26, 27], as well as the chemotherapy response of cancers [28, 29]. SPHK1 affects many aspects of colorectal cancers [17, 19, 30], and its expression is precisely regulated by tumor-suppressing genes, such as PRSS8 [19]. The expression of SPHK1 is regulated by various types of miRNA in many types of cancers, but until now, only miR-101 has been reported to regulate the expression of SPHK1 in colorectal cancer cells [11], which is involved in the proliferation of colorectal cancer cells. Here, we showed that miR-659-3p negatively regulated the expression of SPHK1 in colorectal cancer cells. We also demonstrated that the influences of miR-659-3p on the chemotherapy response were mediated by SPHK1. The finding that the expression of miR-659-3p was reduced while the expression of SPHK1 was increased in chemo-resistant clinical colorectal cancer samples compared with those of chemo-sensitive samples further supported the involvement of miR-659-3p-SPHK1 in the chemotherapy response of colorectal cancers. Moreover, in our study, we also found that miR-659-3p could modulate cisplatin efficiency in xenograft tumor models. All of these results indicated that miR-659-3p was involved in the chemotherapy response of CRC by negatively regulating the expression of SPHK1. Our findings might shed new light on the prevention and treatment of chemo-resistant CRC.

Acknowledgements

Disclosure of conflict of interest

None.

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