Restoration of microRNA-218 increases cellular chemosensitivity to cervical cancer by inhibiting cell-cycle progression

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Abstract. We previously reported frequent loss of microRNA-218 (miR-218) in human cervical cancer, which was associated with tumor progression and poor prognosis. In this study, we investigated whether restoration of the miR-218 level is a valid strategy for the treatment of cervical cancer. The expression of miR-218 in cervical cancer samples and cell lines was quantified by reverse transcription TaqMan quantitative (RT-q)PCR. Overexpression of miR-218 was achieved by both transient and stable transfection, using a miR-218 mimic and a miR-218-expressing plasmid, respectively. Alterations in cellular proliferation and cell-cycle progression were measured by the MTT assay and flow cytometry analysis. Nude mice bearing SiHa xenografts were used to investigate the functions of miR-218 and carboplatin on tumor growth and weight. The expression of cycle-related proteins was detected by western blotting and immunohistochemical staining. *In vitro*, miR-218 significantly inhibited cellular growth in all four cell lines tested (P=0.021 for CaSki, P=0.009 for HeLa, P=0.016 for SiHa, and P=0.029 for C33A). Overexpression of miR-218 induced G1 phase arrest and reduced expression of cyclin D1 and CDK4. *In vivo*, restoration of miR-218 notably inhibited tumor growth and decreased tumor weight. In primary cultured samples, tumors with high levels of miR-218 were more sensitive to carboplatin (R^2=0.3319, P=0.0026); consistently, miR-218 overexpression suppressed tumor growth, induced cell-cycle arrest, and reduced the cyclin D1 level. Based on these and previous results, we conclude that restoration of the miR-218 level inhibits the growth of cervical cancer cells both *in vitro* and *in vivo*; furthermore, overexpression of miR-218 sensitizes cervical cancer cells to carboplatin. Our findings suggest a novel therapy for cervical cancer based on miR-218, especially in patients with reduced levels of miR-218.

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Introduction

Cervical cancer remains the second most common cancer in women worldwide. In 2008, there were >529,800 new cases and ~275,000 deaths caused by this malignancy (1,2). Unfortunately, up to date, the molecular mechanisms involved in the initiation and progression of cervical cancer have not been elucidated. Understanding of the epigenetic modifications involved in cervical cancer, such as those mediated by microRNAs, might shed light on this issue (3,4).

microRNAs are a family of small, single-stranded RNAs, which can degrade or block the transcription of mRNA via directly binding to the 3'-untranslated region of their target genes (5,6). microRNAs have been demonstrated to regulate cell proliferation, division, differentiation, invasion, and migration (7,8). A number of microRNAs, such as microRNA-218 (miR-218) have been shown to be down-regulated and play a role as tumor suppressors in human malignancies (9-13). miR-218 has been shown to suppress cellular proliferation and invasion and to induce apoptosis both *in vitro* and *in vivo* (13-15). In a previous study, we demonstrated that the circulating miR-218 level is significantly reduced in cervical cancer patients, and associates with the late tumor stage, lymphatic node metastasis and poor prognosis (16). Here, using a mouse model and primary cultured tumor tissues, we aimed to explore the effects of miR-218 restoration in human cervical cancer.

Materials and methods

Clinical specimens and primary cultures. A total of 25 fresh cervical cancer tissues were collected from patients who underwent surgery at the Affiliated Hospital of Jiangnan University (the Fourth People's Hospital of Wuxi, Jiangsu Province, China). For RNA extraction, the tissues were instantly stored in RNAlater solution (Qiagen, Valencia, CA, USA) at -80˚C.

For primary cultures, fresh tumor tissues were washed with Hank's buffered salt solution (HBSS) and gently minced with a sterilized scalpel. Then, the tissues were digested with 0.1% collagenase IA (product no., C5894; Sigma-Aldrich, Minneapolis, MN, USA) for 2 h at 37˚C. Following a wash with phosphate buffer saline, the mixture was resuspended to a density of ~1x10^5 cells/ml. Next, 2x10^4 cells/well were seeded into 96-well plates and incubated for 24 h prior to carboplatin treatment. Permissions for this study were obtained from the Ethical Committee of the Fourth People's Hospital of Wuxi, and informed written consent was obtained from all the patients. Patient information was collected; this information is summarized in Table I.

Table I. Clinical features of cervical cancer patients.

Variable	Value
Total number	25
Median age (range)	53.5 (39-68)
Low miR-218 level	49.2±7.8
High miR-218 level	55.1±9.1
Stage[a]	
I	8
II	7
III	6
IV	4
Lymph node metastasis	
Negative	20
Positive	5
Tumor size (cm)	
≤4.0	13
>4.0	12

[a]Based on the the FIGO 2009 criteria. ±, standard deviation.

Cervical cancer cell lines, culture and reagents. Four human cervical cancer cell lines were used in this study, HeLa, SiHa, C33A and CaSki, obtained from the American Type Culture Collection (ATCC; Manassas, VI, USA). The cells were cultured in the following media: RPMI-1640 supplemented with 10% fetal bovine serum (FBS) for CaSki, Dulbecco's Modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) with 10% FBS for HeLa, SiHa and C33A. All cell lines were cultured at 37˚C, with 5% CO_2. Carboplatin was purchased from Sigma-Aldrich (product no., C2538) and was freshly prepared before each experiment at a 5 mM stock concentration. The miR-218 mimic and its negative control were purchased form GenePharma Co., Ltd. (Shanghai, China).

Transient and stable transfection with miR218. The pGenesil-1-miR-218 expression plasmid was obtained as previously described (15). Transient transfection was performed with the miR-218 mimic and negative control in all four cell lines. A total of 1x10^5 cells/well were plated into the 6-well plates and incubated overnight. Transfection with miR-218 mimic (or the negative control) was performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA, USA), and G418 (0.4 mg/ml, Sigma-Aldrich) was used to select the stably transfected colonies.

MTT assay. Cells (2x10^3 or 2x10^4 cells/well for primary cultured cells) were seeded into 96-well plates and incubated overnight, then treated with different doses of carboplatin for 72 h. Next, 5 μl MTT (5 mg/ml; Sigma-Aldrich) and 100 μl dimethyl sulfoxide were sequentially added into each well, and the absorbance was measured at 570 nm. All procedures were performed in triplicate.

Cell-cycle analysis. Cells (3x10^6) were plated into a 60-mm dish and incubated overnight before treatment with the miR-218 mimic or carboplatin. Twenty-four hours later, the cells were harvested and suspended in 1 ml of staining solution, consisting of 50 μg/ml propidium iodide and 20 μg/ml RNase A (Thermo Fisher Scientific). Then, the cells were analyzed on a FACScalibur flow cytometer (BD Biosciences, San Diego, CA, USA). Flow cytometry data were analyzed using CellQuest™ software (BD Biosciences). The procedures were repeated in triplicate.

microRNA enrichment and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using the Invitrogen TRIzol reagent (Thermo Fisher Scientific). Then, the total RNA sample was enriched in microRNAs using the Applied Biosystems® mirVana miRNA Isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The expression of miR-218 was measured by the Applied Biosystems Taqman miRNA assay kit that is specific to miRNA-218, on an Applied Biosystems StepOnePlus™ Real-Time PCR system (all from Thermo Fisher Scientific). U6 RNA was used as the endogenous control. The relative level of miR-218 was calculated with the $2^{-\Delta\Delta Ct}$ method (17). All the procedures were repeated in triplicate.

Western blotting. Total protein was extracted using RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the protein concentration was measured using the BCA Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories). The membranes were incubated overnight at 4˚C with the primary antibodies rabbit monoclonal anti-cyclin D1 and rabbit monoclonal anti-CDK4 (1:1,000 for anti-cyclin D1 and 1:500 for anti-CDK4; Cell Signaling Technology, Beverly, MA, USA), and then incubated for 2 h at room temperature with the secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG (monoclonal; Cell Signaling Technology). The specific protein bands were detected using an Enhanced Chemiluminescence system (Beyotime Institute of Biotechnology).

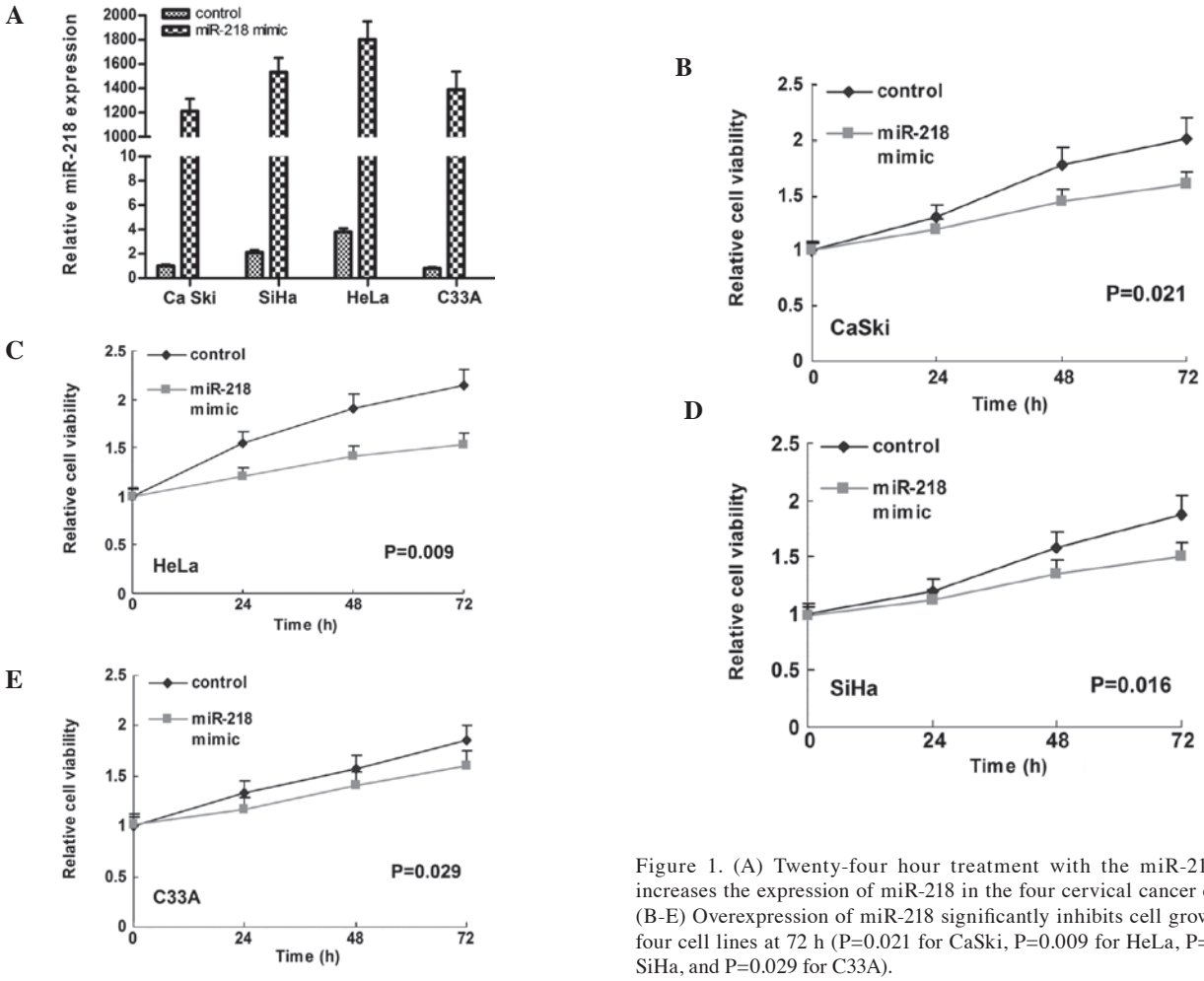

Figure 1. (A) Twenty-four hour treatment with the miR-218 mimic increases the expression of miR-218 in the four cervical cancer cell lines. (B-E) Overexpression of miR-218 significantly inhibits cell growth in the four cell lines at 72 h (P=0.021 for CaSki, P=0.009 for HeLa, P=0.016 for SiHa, and P=0.029 for C33A).

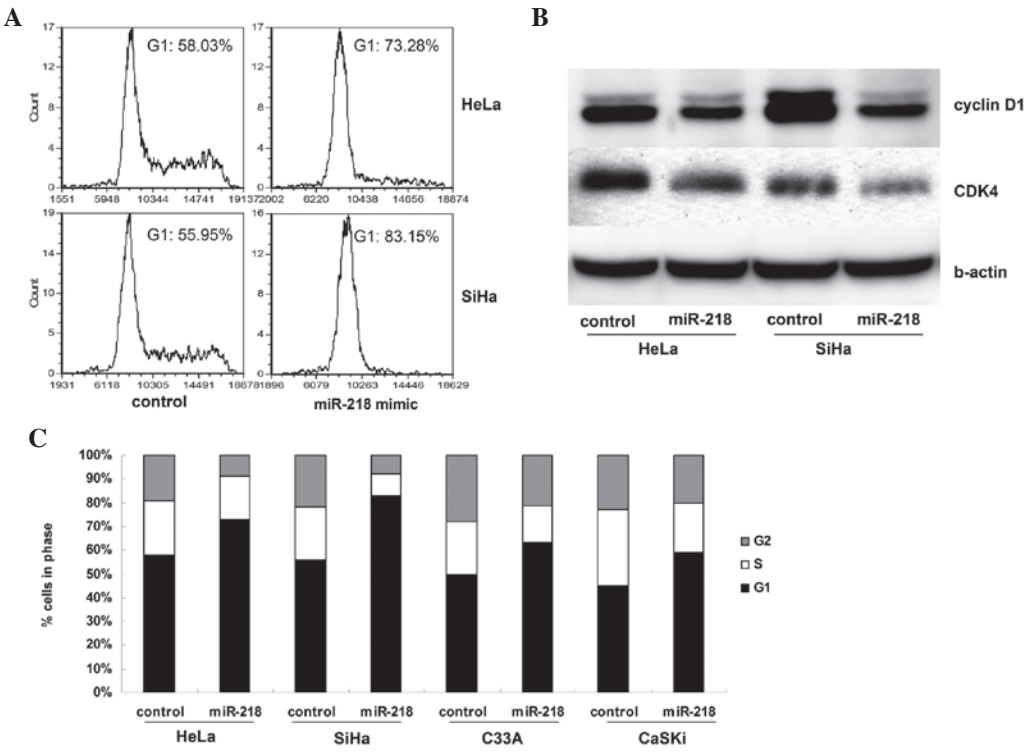

Figure 2. (A) In HeLa and SiHa cells, overexpression of miR-218 arrests the majority of the cells at the G1 phase. (B) Western blot analysis shows that overexpression of miR-218 decreases the levels of cyclin D1 and CDK4 in HeLa and SiHa cells. (C) The effects of miR-218 are more prominent in the HeLa and SiHa cell lines compared to C33A and CaSKi.

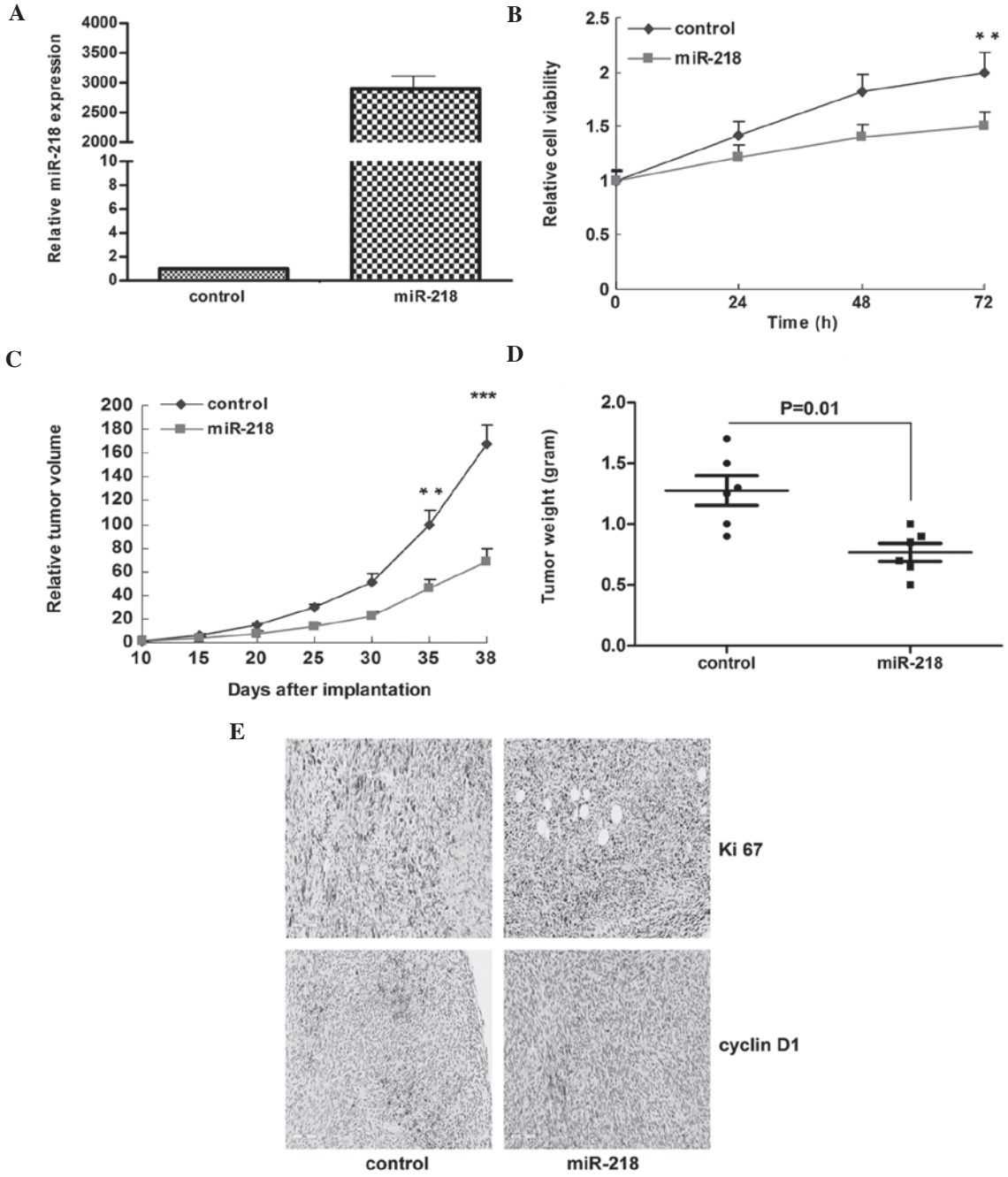

Figure 3. (A) Stable transfection with the miR-218 expression plasmid increases the level of miR-218 as much as 3,000-fold in SiHa cells. (B) Overexpression of miR-218 inhibits the growth of SiHa cells (**P<0.01 compared to the corresponding parental cells). (C) Compared to the control, miR-218 overexpression significantly inhibits tumor growth *in vivo* (n=6 for each group, **P<0.01, ***P<0.001). (D) Tumor weight is markedly reduced in the miR-218-overexpressing group compared to the control (P=0.01). (E) In the tissues overexpressing miR-218, the levels of Ki 67 and cyclin D1 appear reduced.

Immunohistochemical staining (IHC). IHC was performed as previously described (18). The primary antibodies used were: anti-Ki 67 (1:200; Boster Biological Technology, Ltd., Wuhan, China) and anti-cyclin D1 (1:300; Cell Signaling Technology). Staining was performed with the Histostain-Plus IHC kit (Mingrui Biotech Co., Ltd., Shanghai, China). The stained tissues were visualized on an optical microscope (Leica Microsystems GmbH, Wetzlar, Germany) and were separately evaluated by two pathologists.

The tumor xenograft growth assay. The experimental procedures using animals in this study were approved by the Animal Ethics Committee of the Jiangnan University. Six- to eight-week-old female BALB/c nude mice were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). SiHa cells ($1x10^6$) stably expressing miR-218 were subcutaneously injected into the left posterior flanks of the nude mice. As a negative control, an equal amount of SiHa cells transfected with the empty vector was injected into the right flanks. To test the effects of miR-218 on cellular sensitivity to carboplatin, another group of nude mice bearing SiHa cells was established following the same procedure, and carboplatin (intraperitoneal injection, 30 mg/kg) was administrated at days 10, 17, 24 and 31. From 10 days after implantation, the tumor size was measured

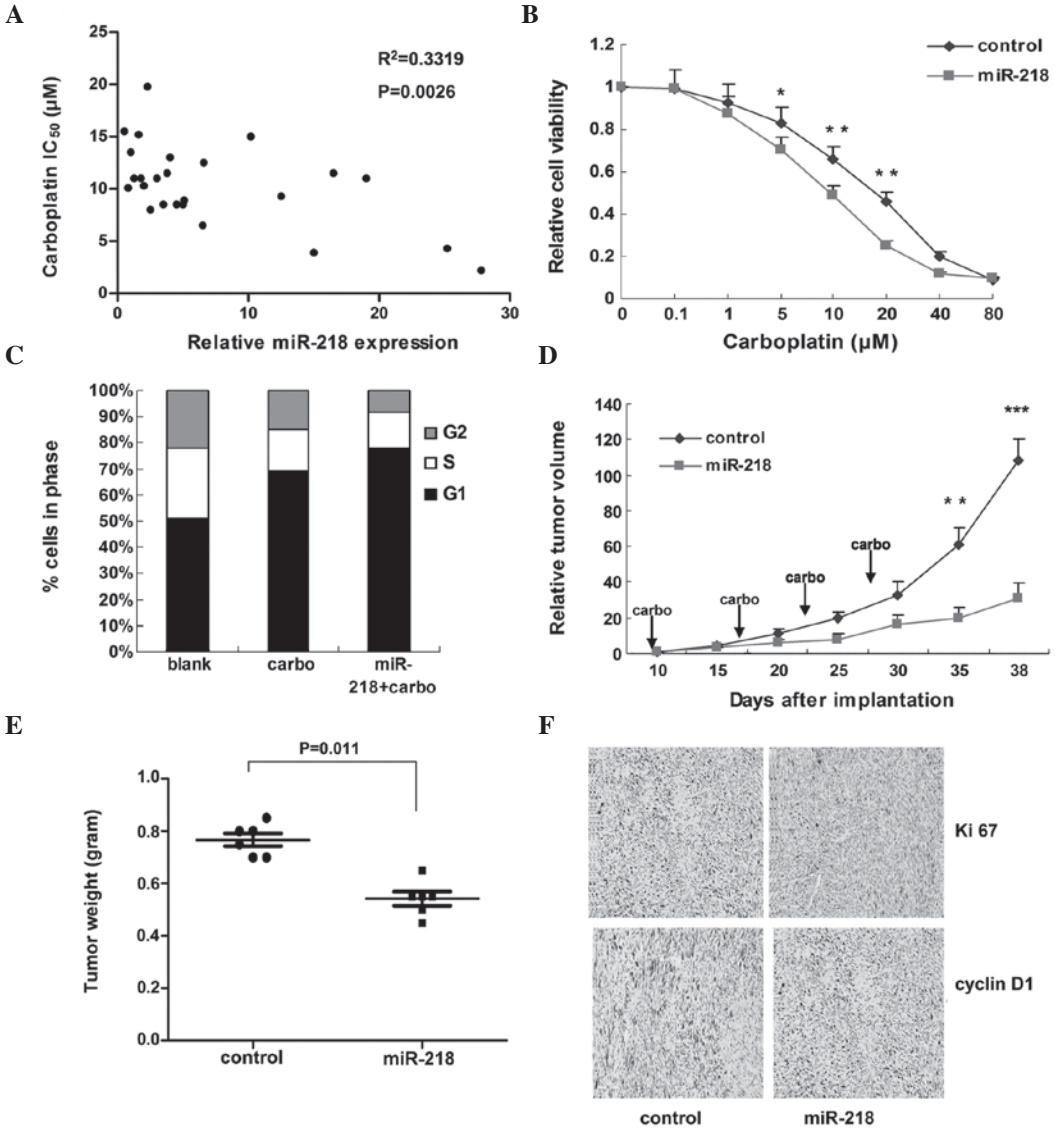

Figure 4. (A) High expression of miR-218 correlates to cellular sensitivity to carboplatin (carbo) in primary cultured cervical cancer samples (R^2=0.3319, P=0.0026). IC_{50}, half maximal inhibitory concentration. (B) miR-218 overexpression increases cellular sensitivity to carboplatin (*P<0.05, **P<0.01). (C) miR-218 overexpression induces G1-phase cycle arrest in SiHa cells. Blank, cells cultured in normal media. (D) Restoration of miR-218 sensitizes SiHa xenografts to carboplatin in the mouse model (n=6 for each group, **P<0.01, ***P<0.001). (E) Weekly treatment with carboplatin significantly decreases tumor weight in the miR-218 group compared to the control group (P=0.011). (F) Combined with overexpression of miR-218, carboplatin further reduces the detected levels of Ki 67 and cyclin D1.

every five days. Tumor volume = (length x width^2)/2. Four weeks later, all mice were sacrificed, and the tumor tissues were collected for further analysis.

Statistical analysis. The software SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The χ^2 and the t-test were used for categorical and quantitative data comparisons, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-218 inhibits the growth of cervical cancer cells. Compared to the negative control, 24-h treatment with the miR-218 mimic increased the expression of miR-218 by >1,000-fold in all four cell lines (Fig. 1A), and significantly inhibited cellular growth (Fig. 1B-E, P=0.021 for CaSki, P=0.009 for HeLa, P=0.016 for SiHa, and P=0.029 for C33A, respectively).

miR-218 induces G1 phase cell-cycle arrest and inhibits cyclin D1 and CDK4. In HeLa and SiHa cells, overexpression of miR-218 arrested the majority of the cells at the G1 phase (Fig. 2A), but this arrest was not prominent in the C33A and CaSKi cell lines (Fig. 2C). In addition, the expression of cyclin D1 and CDK4 was reduced in miR-218-overexpressing HeLa and SiHa cells (Fig. 2B).

Stable overexpression of miR-218 inhibits tumor growth in vivo. By transfection of SiHa cells with the miR-218-expressing plasmid, we stably overexpressed miR-218, as much as 3,000-fold (Fig. 3A). The growth of the transfected SiHa cells was significantly reduced (Fig. 3B, P<0.01), similarly to the transient transfection experiments. In the mouse model

established by injection of the miR-218-transfected SiHa cells, growth of the xenograft was also impaired (Fig. 3C, P<0.001); moreover, the tumor weight in these mice was reduced compared to the control group (Fig. 3D, P=0.01). Overexpression of miR-218 also inhibited the expression of Ki 67 (a marker of proliferation) and cyclin D1 (Fig. 3E).

miR-218 expression correlates to tumor sensitivity to carboplatin in primary cultured cervical cancer samples. As shown in Fig. 4A, reduced expression of miR-218 was associated with cervical cancer cell resistance to carboplatin, and as the level of miR-218 increased, the half maximal inhibitory concentration (IC_{50}) of carboplatin (72-h treatment) gradually decreased. The correlation between miR-218 expression and IC_{50} was significant (R^2=0.3319, P=0.0026).

Restoration of miR-218 sensitizes SiHa cells to carboplatin. *In vitro*, miR-218 overexpression increased cellular sensitivity to carboplatin and induced cell-cycle arrest (Fig. 4B and C). *In vivo*, tumor growth in the group overexpressing miR-218 was markedly lower compared to the control group, where miR-218 expression was lower (Fig. 4D, P<0.001), while tumor weight was also reduced (Fig. 4E, P=0.011). Carboplatin treatment further reduced the expression of Ki 67 and cyclin D1 when combined with overexpression of miR-218 (Fig. 4F).

Discussion

Uncontrolled tumor growth and chemo-/radio- resistance remain the leading causes of cancer-related death, especially in cervical cancer, where patients often experience tumor recurrence and poor outcome (19). Thus, valid approaches to suppress tumor growth and improve cellular sensitivity to routinely used therapeutic agents are urgently needed. The group of small, single-stranded nucleotide chains, microRNAs, has been demonstrated to be a potent tool to target the genes involved in tumor growth and reactions to drugs (20). For instance, SPC3649, a miR-122 antagonist, is currently being evaluated in a phase II clinical trial (NCT01872936) and may constitute the first commercial microRNA-based drug (21). Another example is MRX34, a drug designed to mimic the activity of human miR-34, now under phase I clinical trials (NCT01829971) (22).

We previously reported that loss of miR-218 relates to late tumor stage and lymphatic node metastasis, indicating the roles of miR-218 in the progression of cervical cancer. We now investigated the relationship between the miR-218 level and tumor sensitivity to carboplatin, as well as the effects of miR-218 restoration in cervical cancer progression.

miR-218 has been shown to participate in numerous signaling pathways through which it can suppress tumor growth, migration and invasion, such as IKK-β, NF-κB, Slits-Robo, survivin and PI3K/AKT (9,23,24). Using two transfection protocols, we upregulated miR-218 in four human cervical cancer cell lines, and in both series of experiments, cellular growth and cell-cycle progression were significantly inhibited. *In vivo*, overexpression of miR-218 also significantly suppressed the growth of SiHa xenografts. Our findings are supported by previous studies on nasopharyngeal cancer (14), gastric carcinoma (15)and other malignancies (12,13).

For cervical cancer patients at the late stage, platinum-based chemotherapy is commonly combined with surgery or radiotherapy. Since severe dose-dependent toxicity is frequently observed, oncologists nowadays opt for weekly chemotherapy, which has been demonstrated to be more efficient and have limited side-effects (25,26). In this study, we found that the low miR-218 level correlates to resistance to carboplatin in the primary cultured human cervical cancer samples, which was consistent with the data observed in the mouse model. Tumors where miR-218 expression was restored were more sensitive to carboplatin treatment than those with relatively reduced expression of miR-218, which indicates that upregulating miR-218 may be a promising strategy for patients who both show low expression of miR-218 and resistance to carboplatin. Apart for its suppressive effects on tumor proliferation (as shown by the Ki 67 IHC detection), we also found the miR-218 overexpression or miR-218 overexpression combined with carboplatin treatment significantly inhibits cell cycle progression, in agreement with a previous study (27).

In other malignancies, microRNAs such as miR-193b and miR-21, were proved to predict tumor sensitivity to chemotherapy (28,29). Here, we demonstrated that miR-218 expression correlates to carboplatin sensitivity. Moreover, we showed that restoration of miR-218 allows to overcome carboplatin resistance when miR-218 expression is low. In the near future, we will focus on development of miR-218 as a predictive marker for resistance to chemotherapy in cervical cancer.

In summary, based on our previous and the present findings, we conclude that restoration of the reduced miR-218 level in the cells can significantly suppress the growth of cervical cancer, potentially via the induction of cell-cycle arrest; furthermore, we found that both *in vitro* and *in vivo,* the high level of miR-218 can sensitize cervical cancer cells to carboplatin. Our findings provide strong arguments for the development of a novel therapy for cervical cancer based on miR-218, especially for patients with a reduced miR-218 level.

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