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RAPID COMMUNICATION

miR-126-5p affects the chemosensitivity of colorectal cancer cells by regulating *SPRED1*, ERK1/2 pathway and apoptosis

Chemoresistance is still one of the main obstacles to treat colorectal cancer (CRC). Substantial studies on biomarkers related to chemoresistance have emerged in recent years. Many microRNAs (miRNA) have been reported to involved in drug resistance and CRC.[1] MiR-126-5p participates in malignant behaviors of numerous cancers.[2,3] However, it is not known if miR-126-5p participates in multidrug resistance in CRC. Especially, the impact of miR-126-5p on drug resistance of CRC and its drug resistance mechanism have not been reported.

After microRNAomics assay and screening, we obtained 33 candidate miRNAs including 24 up-regulated miRNAs and 9 down-regulated miRNAs (Fig. 1A). The 24 up-regulated miRNAs including miR-126-5p were represented by heat map and the value of transcripts per million (TPM) of miR-126-5p in HCT8/T cells was about four times that in HCT8 cells (Fig. 1B; Fig. S1A). Quantitative reverse transcription-PCR (qRT-PCR) analysis verified that miR-126-5p was indeed substantially up-regulated in HCT8/T cells by comparison with HCT8 cells (Fig. 1C). HCT8/T cells were derived from their parental HCT8 with long-term Taxol (Tax) induction. If there is no TAX treatment to maintain cell resistance for three months, HCT8/T cell resistance will slowly decrease, but not to the level of HCT8 cells. In the process of decreasing after the first three months, both the IC_{50} value of TAX and miR-126-5p expression in HCT8/T cells were detected every two weeks and the results showed that they were positively correlated (Fig. S1B). MiR-126-5p expression in both HCT8 and HCT8/T cells was increased after TAX treatment in a concentration- and time-dependent manner (Fig. 1D, E). TAX was removed after 18 days of TAX treatment, and miR-126-5p expression in both cells maintained at a high level for a short time after withdrawal, and then decreased sharply (Fig. 1D, E). Moreover, in other CRC cells (DLD-1, HT29 and SW1463), miR-126-5p expression was concentration-dependently elevated after TAX treatment and was increased in varying degrees with long-term treatment of 1 nM TAX over time (Fig. S1C). We also observed that both the miR-126-5p expression level and IC_{50} value of TAX were higher in DLD-1/T than in DLD-1 (Fig. S1D, E). The results indicated that TAX could induce miR-126-5p expression in CRC cells in a concentration- and time-dependent fashion.

We investigated the possible role of miR-126-5p in regulating chemoresistance by overexpressing miR-126-5p in parental HCT8 cells and knockdown miR-126-5p in HCT8/T cells (Fig. S2A, B). *In vitro*, overexpressed miR-126-5p reduced the sensitivity of HCT8 cells to multi drugs (Fig. 1F; Fig. S2C). On the contrary, miR-126-5p knockdown promoted the sensitivity of HCT8/T cells (Fig. 1G; Fig. S2D). *In vivo*, we used miR-126-5p stably knockdown (shmiR) HCT8/T cells and control (shNC) cells to establish nude mice xenograft models. Paclitaxel (PTX) injection significantly inhibited the growing of tumors from inoculation of HCT8/T shmiR cells, compared with that of HCT8/T shNC cells (Fig. 1H, I; Fig. S3A). Chemotherapy drugs significantly reduced tumor volume with the help of miR-126-5p knockdown without affecting body weight (Fig. S3B, C). Moreover, the inducing effect of drug on miR-126-5p also exists *in vivo* (Fig. S3D). Above data demonstrated that the increase of miR-126-5p level reduced the sensitivity of HCT8 cells to drugs, and decreasing miR-126-5p can recover drug sensitivity and enhance the response of HCT8/T cells to drugs.

Four bioinformatics databases were used to predict the potential targets of miR-126-5p, and *SPRED1* was obtained (Fig. S4A). qRT-PCR and western blotting analysis showed that the mRNA level and protein expression of *SPRED1* was suppressed by up-regulated miR-126-5p, and promoted by down-regulated miR-126-5p (Fig. S4B,

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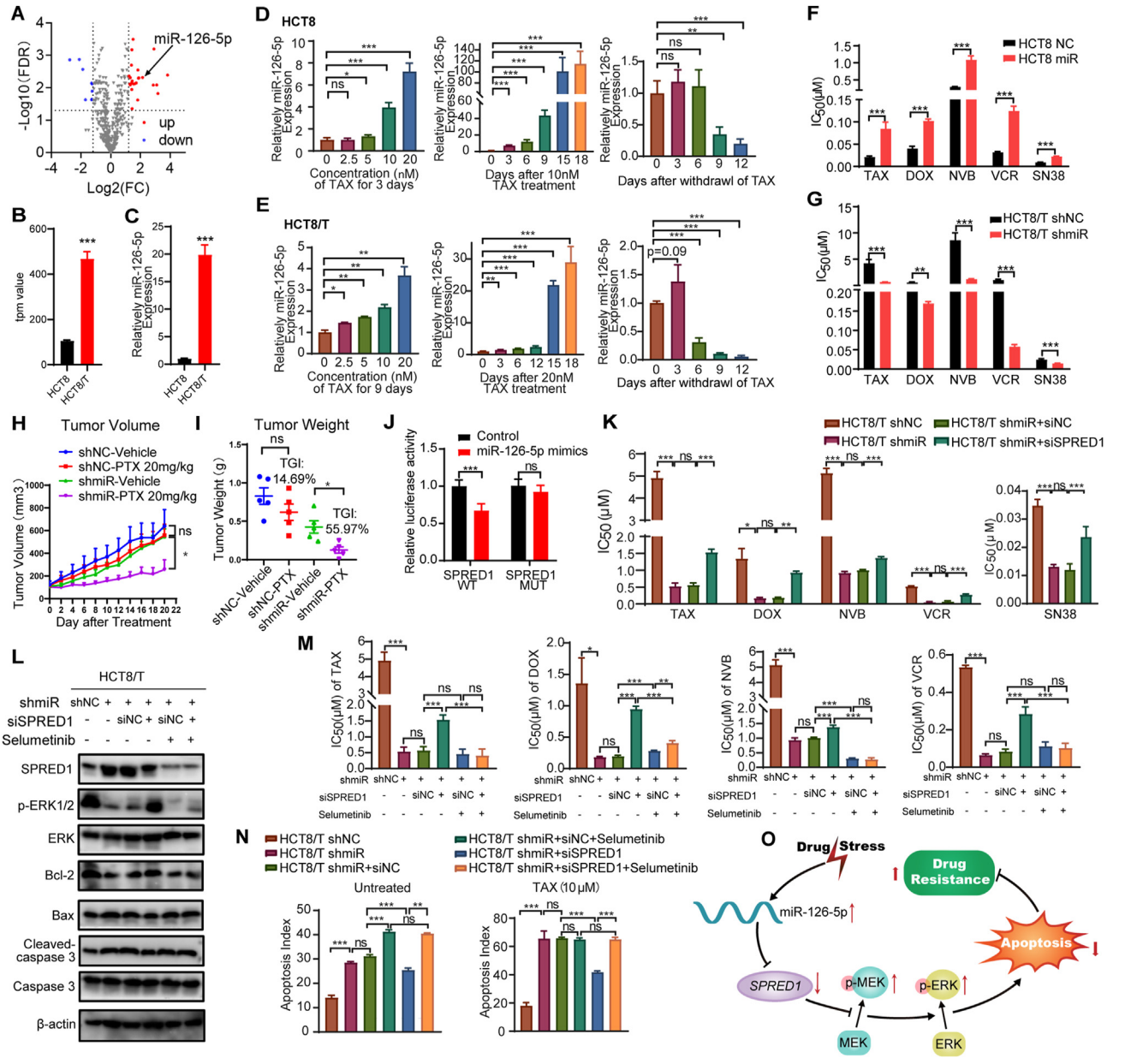

Figure 1 MiR-126-5p directly targets and inhibits *SPRED1*, acting on ERK1/2 signaling pathway and affecting cell apoptosis and cell sensitivity to chemotherapy drugs. **(A)** Volcano plot for the comparison between HCT8 and HCT8/T cells. The cutoff values log2FC < -1.2 or >1.2 and FDR <0.05 were set to screen differentially expressing miRNAs. Red indicates up-regulated miRNAs, grey indicates unchanged miRNAs and green indicates down-regulated miRNAs. **(B)** The transcripts per million (TPM) values of miR-126-5p in HCT8 and HCT8/T from histological data. **(C)** qRT-PCR analysis of relative miR-126-5p expression levels in HCT8 and HCT8/T cells. **(D)** qRT-PCR analysis of relative miR-126-5p expression levels in HCT8 cells treated with increasing concentrations of Taxol (TAX) for 3 days, with 10 nM TAX for incremental days or TAX removal after 18 days of TAX incubation. **(E)** qRT-PCR analysis of relative miR-126-5p expression levels in HCT8/T cells treated with increasing concentrations of TAX for 9 days, with 20 nM TAX for incremental days or TAX removal after 18 days of TAX incubation. **(F)** Overexpression of miR-126-5p desensitized HCT8 cells to chemotherapy drugs. The cells were treated with TAX, doxorubicin (DOX), vinorelbine (NVB), vincristine (VCR) or 7-Ethyl-10-hydroxycamptothecin (SN38) at incremental concentrations for 72 h. IC_{50} values were detected by CCK-8 assay. **(G)** Knockdown of miR-126-5p resensitized HCT8/T cells to chemotherapy drugs. The cells were treated with drugs at incremental concentrations for 72 h. IC_{50} values were detected by CCK-8 assay. **(H, I)** Nude mice xenografts models with HCT8/T shNC and HCT8/T shmiR were injected with paclitaxel (PTX, 20 mg/kg) intraperitoneally every two days for 28 or 20 days. Saline was used for control group. Tumor volume was measured every two days. **(H)** The change of tumor volume. **(I)** The tumor weight after treatments. **(J)** Verification of miR-126-5p directly targeting *SPRED1* by dual luciferase reporter assay. Luciferase activities after co-transfecting with empty vector or miR-126-5p and wild-type or mutant 3′ UTR

C). Knockdown of miR-126-5p also led to relatively high mRNA level of *SPRED1 in vivo* (Fig. S3E). Dual luciferase assay results verified that miR-126-5p targeted *SPRED1* (Fig. 1J; Fig. S4D). Furthermore, we transfected siRNA targeting *SPRED1* (si*SPRED1*) into HCT8 and HCT8/T shmiR cells to silence *SPRED1* (Fig. S4E, F). The CCK-8 assay showed that *SPRED1* loss depressed the sensitivity of HCT8 cells to TAX and Vincristine (VCR), and partial weakened the sensitizing influence of miR-126-5p knockdown on HCT8/T cells (Fig. 1K; Fig. S4G). Then, we detected the apoptosis of HCT8 and HCT8/T shmiR cells after silencing *SPRED1* expression in the chemotherapy setting. HCT8 cell apoptosis was weakened by silencing of *SPRED1,* and *SPRED1 loss* partial weakened the promoting influence of miR-126-5p knockdown on cell apoptosis of HCT8/T cell in the chemotherapy setting (Fig. S1N, 5BN). The increased protein ratio of Bcl-2/Bax and decreased protein ratio of Cleaved-Caspase3/Caspase3 were observed in HCT8 cells with *SPRED1* silencing (Fig. S5A), and the change of protein expression ratio caused by miR-126-5p knockdown in HCT8/T cells were also reversed to varying degree by *SPRED1* silencing (Fig. 1L, Fig. S5C). Western blotting analysis of *in vivo* experiment also showed that knockdown miR-126-5p caused the down-regulated expression of Bcl-2 and the up-regulated protein ratio of Cleaved-Caspase 3/Caspase3 (Fig. S3E). These results revealed that miR-126-5p regulated cell chemosensitivity and apoptosis in HCT8 and HCT8/T cells through targeting *SPRED1*.

SPRED1 was a negative regulator of Ras/ERK.[4] In the xenograft model, the loss of miR-126-5p caused the up-regulation of *SPRED1* and the inhibition of ERK1/2 phosphorylation (Fig. S3E). Selumetinib, a MEK inhibitor, was chosen to block ERK1/2 signaling pathway. We first incubated HCT8/T cells with selumetinib, and this inhibitor raised the response of HCT8/T cells to multi drugs (Fig. S6A, E). Next, we incubated HCT8 miR and HCT8/T shmiR + si*SPRED1* cells with selumetinib (Fig. 1L; Fig. S6B—D). The inhibitor almost entirely counteracted the influence of miR-126-5p overexpression and restored the suppressed chemosensitivity caused by miR-126-5p (Fig. S6F). The loss of *SPRED1* caused a partly reversion in the sensitizing impact of miR-126-5p knockdown on HCT8/T cells, but selumetinib attenuated the desensitization effect of silencing *SPRED1* on HCT8/T shmiR cells (Fig. 1M). Then, we detected the apoptosis of HCT8/T, HCT8 miR and HCT8/T shmiR + si*SPRED1* cells treated with selumetinib in the chemotherapy setting by flow cytometry. The limited ERK1/2 phosphorylation significantly promoted HCT8/T cell apoptosis, down-regulated the protein ratio of Bcl-2/BAX, and up-regulated the protein ratio of Cleaved-Caspase3/Caspase3 (Fig. S7A, E). The inhibitory effect on ERK1/2 phosphorylation relieved the effect on cell apoptosis caused by miR-126-5p overexpression to a certain extent and the relative protein expression of Bcl-2/BAX down-regulated and Cleaved-Caspase3/Caspase3 up-regulated even if miR-126-5p overexpressed (Fig. S7B, C, F). The inhibited ERK1/2 phosphorylation also reversed the effect of silencing *SPRED1* on HCT8/T shmiR cells apoptosis in the chemotherapy setting (Fig. 1N; Fig. S7G). Interestingly, SPRED1 protein expression was also significantly inhibited when ERK1/2 signaling pathway was blocked (Fig. 1; Fig. S5A). We suspected that this may be owing to the positive feedback of SPRED1. The loss of *SPRED1* mainly activates Ras/MAPK signaling and *SPRED1* expression is positively regulated by Ras/MAPK signaling.[5] MiR-126-5p/*SPRED1* axis affects the cell apoptosis and sensitivity to multiple chemotherapeutic drugs by interfering ERK1/2 signaling pathway in HCT8 and HCT8/T cells.

Our studies suggested that miR-126-5p might play a crucial role in chemoresistance of HCT8 cells. The illustration of the possible mechanisms of miR-126-5p in inducing chemoresistance of HCT8 cells was shown in Figure 1O (Fig. S8). MiR-126-5p directly targets and decreases the expression of target gene *SPRED1*, acting on ERK1/2 signaling pathway and thus affecting cell apoptosis, and ultimately enhances cell endurance to chemotherapy drugs and leads to multidrug resistance. MiR-126-5p could act as an effective multi-targeting therapeutic agent, and it might be helpful for clinical treatment in CRC according to our study.

Author contributions

M. Xu conducted experiments, processed the data, and completed the manuscript. G. Zhang, X. Gu, F. Zhao and Z. Yang participated in some experiments. X. Zhang supervised and designed this study and revised the manuscript. All authors reviewed and approved the final manuscript.

of *SPRED1* in 293T cells. **(K)** Silencing *SPRED1* desensitized HCT8 shmiR cells to multiple chemotherapy drugs. The cells were treated with drugs at incremental concentrations for 72 h. IC_{50} values were detected by CCK-8 assay. **(L)** Western blotting analysis of Bax, Bcl-2, Caspase3 and Cleaced-Caspase3 protein expression in HCT8/T cell groups. **(M)** Selumetinib resensitized cells to chemotherapy agents. After transfection and/or incubation, the cells were treated with drugs at incremental concentrations for 72 h. IC_{50} values were detected by CCK-8 assay. **(N)** Silencing of *SPRED1* reduced cell apoptosis and selumetinib promoted cell apoptosis in the chemotherapy setting. After transfection and/or incubation, HCT8/T cellswere exposed to 10 μM TAX for another 24 h. The flow cytometry was used to detect cell apoptosis. **(O)** MiR-126-5p affects the chemosensitivity of colorectal cancer cells by regulating *SPRED1*, ERK1/2 pathway and apoptosis. All data were expressed as mean ± SD, except that the data of **H, I** expressed as mean ± SEM. ns, $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Conflict of interests

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.05.024.

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