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Inhibition of hydrogen sulfide synthesis reverses acquired resistance to 5-FU through miR-215-5p-EREG/TYMS axis in colon cancer cells

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ABSTRACT

Acquired resistance to 5-fluorouracil (5-FU) is a major barrier to benefit from chemotherapy in colon cancer patients. Hydrogen sulfide (H_2S), mainly produced by cystathionine-β-synthase (CBS), has been reported to promote the proliferation and migration of colon cancer cells. In this study, the effect of inhibiting H_2S synthesis on the sensitivity of colon cancer cell lines to 5-FU was investigated. Increased expression of CBS was validated in online database and tissue microarrays. Inhibiting H_2S synthesis significantly sensitized colon cancer cell lines to 5-FU both *in vitro* and *in vivo*. Decreasing H_2S synthesis utilizing shRNA lentiviruses significantly reversed the acquired resistance to 5-FU. MicroRNA sequencing was performed and miR-215-5p was revealed as one of the miRNAs with most significantly altered expression levels after CBS knock down. Epiregulin (EREG) and thymidylate synthetase (TYMS) were predicted to be potential targets of miR-215-5p. Decreasing H_2S synthesis significantly decreased the expression of EREG and TYMS. These results demonstrate that inhibiting H_2S synthesis can reverse the acquired resistance to 5-FU in colon cancer cells.

1. Introduction

As the third most common cancer worldwide, colorectal cancer (CRC) remains the fourth leading cause of death from cancer [1]. The mortality rate of CRC has declined significantly in the past decade, however, more than half of the patients will eventually die from this disease [2]. Despite the significant benefit at the initial stage of the standard 5-FU based chemotherapies, the almost inevitable resistance to 5-FU acquired by tumor cells in the repeated treatment cycles will lead to recurrence and distant metastasis [3,4]. Hence, reversing the acquired resistance to 5-FU remains a promising approach for improving the overall prognosis of CRC patients.

Endogenous hydrogen sulfide (H_2S) is mainly produced enzymatically by cystathionine-β-synthase (CBS) and cystathionine γ-lyase (CSE) in intestinal epithelial cells, utilizing homocysteine and cystathionine as substrates [5]. Increased expression of CBS and consequently increased production of H_2S has been validated in multiple types of tumors, including CRC, ovarian cancer and breast cancer [6,7]. H_2S promoted the proliferation and metastatic potential of tumor cells mainly through improving the mitochondrial energy supply, inhibiting the apoptosis induced by oxidative stress and promoting the peritumor angiogenesis [8–10]. Endeavors have been made to targeting the endogenous production of H_2S in tumor cells [11]. However, the involvement of CBS-H_2S axis in the development of acquired resistance to 5-FU remains to be illustrated.

In this study, we determined whether H_2S and its main producer, CBS, is involved in the development of acquired resistance to 5-FU by performing both *in vitro* and *in vivo* experiments. MicroRNA sequencing was also performed to reveal the mechanisms underlying the effect of targeting CBS.

2. Materials and methods

2.1. Tissue microarrays and immunohistochemistry analysis

Tissue microarrays (CO1801) containing 90 colon cancer tissues and paired adjacent tissues were purchased from US Biomax and immunohistochemical analysis of CBS were performed as described previously utilizing rabbit anti-CBS antibodies (Proteintech, USA) [12].

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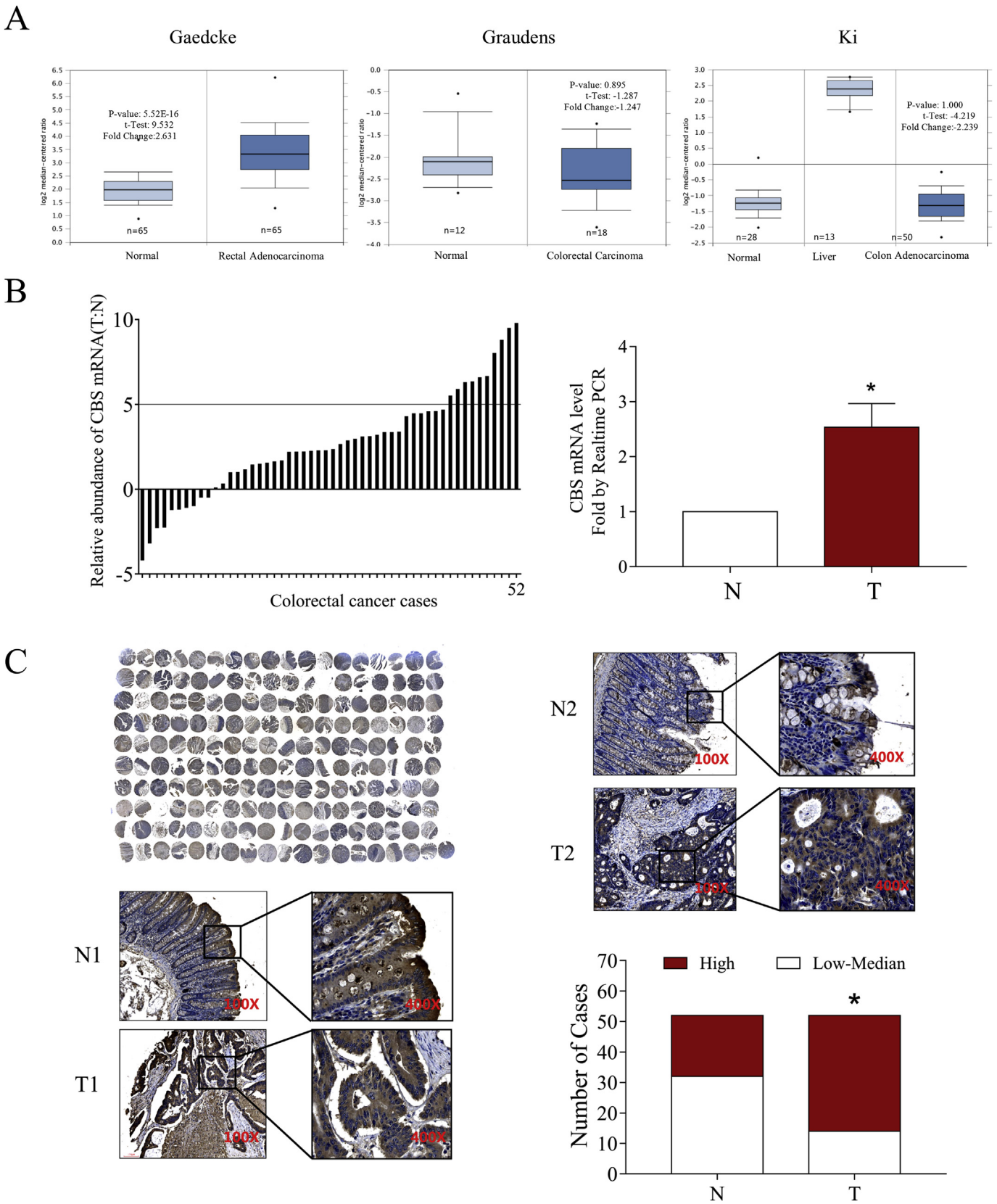

Fig. 1. Increased expression of CBS in colon cancer tissues. A, Analysis of TCGA online database with *Oncomine* revealed increased expression of CBS mRNA in colon cancer tissues samples compared with paired normal tissues. 2.6 fold increase of CBS mRNA was revealed in the study performed by Gaedcke et al. B, Increased expression of CBS mRNA in 52 colon cancer tissues and paired adjacent normal colon tissues. C, Tissue microarray (n = 90) analysis indicated increased expression of CBS in colon cancer tissues compared with paired adjacent normal tissues. (*$P < 0.05$).

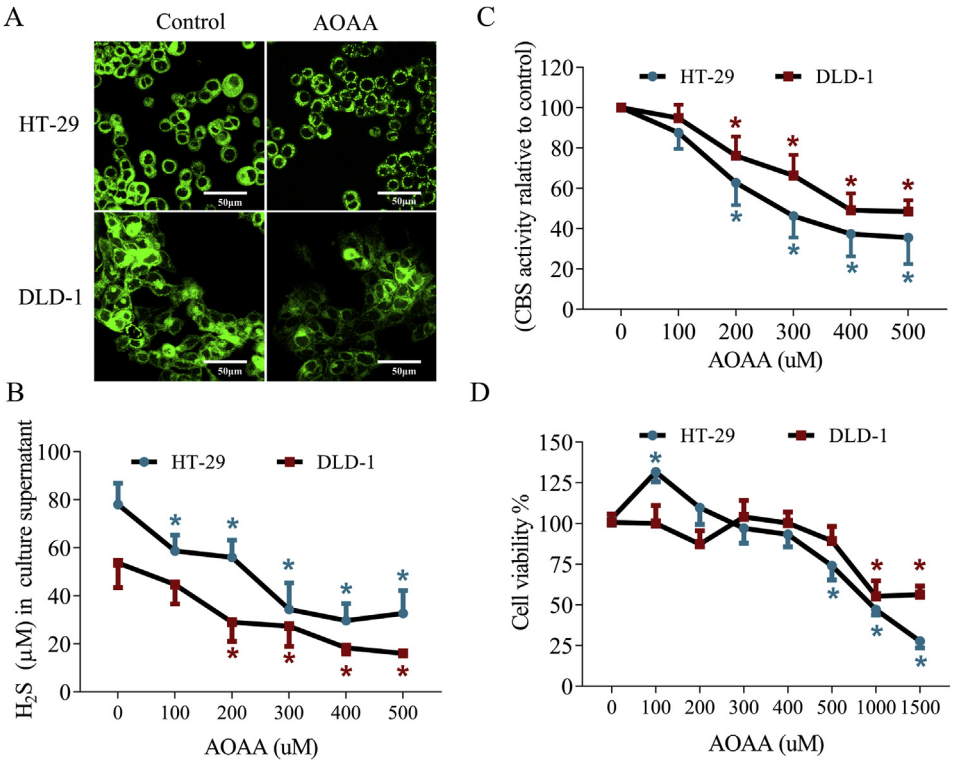

Fig. 2. The inhibitory effect of AOAA on the production of H_2S and proliferation in colon cancer cells. A, Immunofluorescence analysis of endogenous H_2S with AzMC probe depicted the inhibitory effect of AOAA on the endogenous levels of H_2S. B, AOAA significantly decreased the H_2S concentrations in culture supernatant in a dose-dependent manner in both cell lines. C, AOAA significantly inhibited the enzymatic activity of CBS in a dose-dependent manner. D, AOAA, added at the concentrations above 500 μM, inhibited the cell viability of both cell lines. (*$P < 0.05$).

2.2. mRNA and miRNA detection

For mRNA, total RNA isolation and cDNA synthesis were performed with Trizol (Thermofisher, USA) and RevertAid First Strand cDNA Synthesis Kit (Thermofisher, USA). MicroRNA was extracted using the miRNeasy Mini Kit (Qiagen, German) and reverse transcribed into cDNA using the kit (QuantiMir™ RT Kit, USA) according to the manufacturer's recommendations. Quantitative real-time PCR analysis was performed with 7500 real-time PCR System (Applied Biosystems). RNA relative expression was calculated as fold change using the comparative threshold cycle (CT) method ($2^{-\triangle\triangle CT}$) with GAPDH serving as the internal control for mRNA and U6 serving as the internal control gene for miRNA. The primers and are listed in the supplementary materials and methods.

2.3. Fluorescent detection of intracellular H_2S

Cells were washed twice with HBSS and incubated with 7-Azido-4-Methylcoumarin (AzMC) at the dose of 0.01 mM for 30 min s and the fluorescence was visualized under CarlZeiss LSM710 confocal microscope (CarlZeiss, German).

2.4. Measurement of supernatant H_2S levels

A sulfur ion specific electrode (51344800, METTLER, Switzerland) and Seven2Go S7 conductivity meter (METTLER, Switzerland) were used to detect the H_2S levels in the culturing supernatant with standard curves generated with serial dilution of sodium sulfide solution (1 mM).

2.5. In vitro CBS activity measurement

H_2S production was measured to compare CBS activity with AzMC as described preciously [13]. The volume of the working buffer was set at 180 μL and contained 200 mmol/L Tris–HCl pH8.0, 5 μmol/L pyridoxal 5′-phosphate (PLP), 10 mmol/L glutathione and 0.5 mg/mL BSA. AOAA was added to each well in 10 μL volume. Total protein (150 μg) collected from different groups of cells was added to all wells in a 10 μL volume. AzMc (10 μmol/L) and the CBS substrates L-cysteine and homocysteine (each at 2.5 mmol/L) were added in a 10 μL volume. The mixture was incubated at 37 ˚C for 1 h, and fluorescence of the AzMC was measured using Synergy H2 (Biotek, USA) microplate reader (excitation λ = 365, emission λ = 450 nm).

2.6. Cell viability assays

Cellular activity was tested by the CCK-8 assay (Sangon Biotech, China). Briefly, 5000 cells were added to each well of a 96-well plate (Corning, USA). Cells were treated with gradient concentrations of AOAA with or without 5-FU for 48 h and the IC50 value of 5-FU was measured. Combination index was calculated with Chou-Talalay method as described previously [14].

2.7. Colony formation assays

Cells were seed into 6-well plates at density of 1000 cells per well and incubated with or without 5-FU and AOAA for 21 days. After fixation with 4% formaldehyde, cells were stained with 0.05% crystal violet for 2 min at room temperature, followed by washing with PBS and drying. Colony numbers were measured with Image J software.

2.8. Flowcytometry analysis

Cell cycle was evaluated based on flow cytometric analysis of propidium-iodide stained nuclei utilizing a cell cycle detection kit (KeyGen Biotech, China). Flowcytometric analysis were performed using FACS Aria II (BD Biosciences, USA) and further analysis was completed with FlowJo software (Treestar, USA).

2.9. Western blotting

Total protein was extracted using RIPA buffer and the extracts containing equal quantities of protein (30 μg) were electrophoresed in 6 or 10% polyacrylamide gel, transferred with PVDF membranes and blocked for 1 h (5% bovine serum albumin (BSA) in TBS-Tween 20

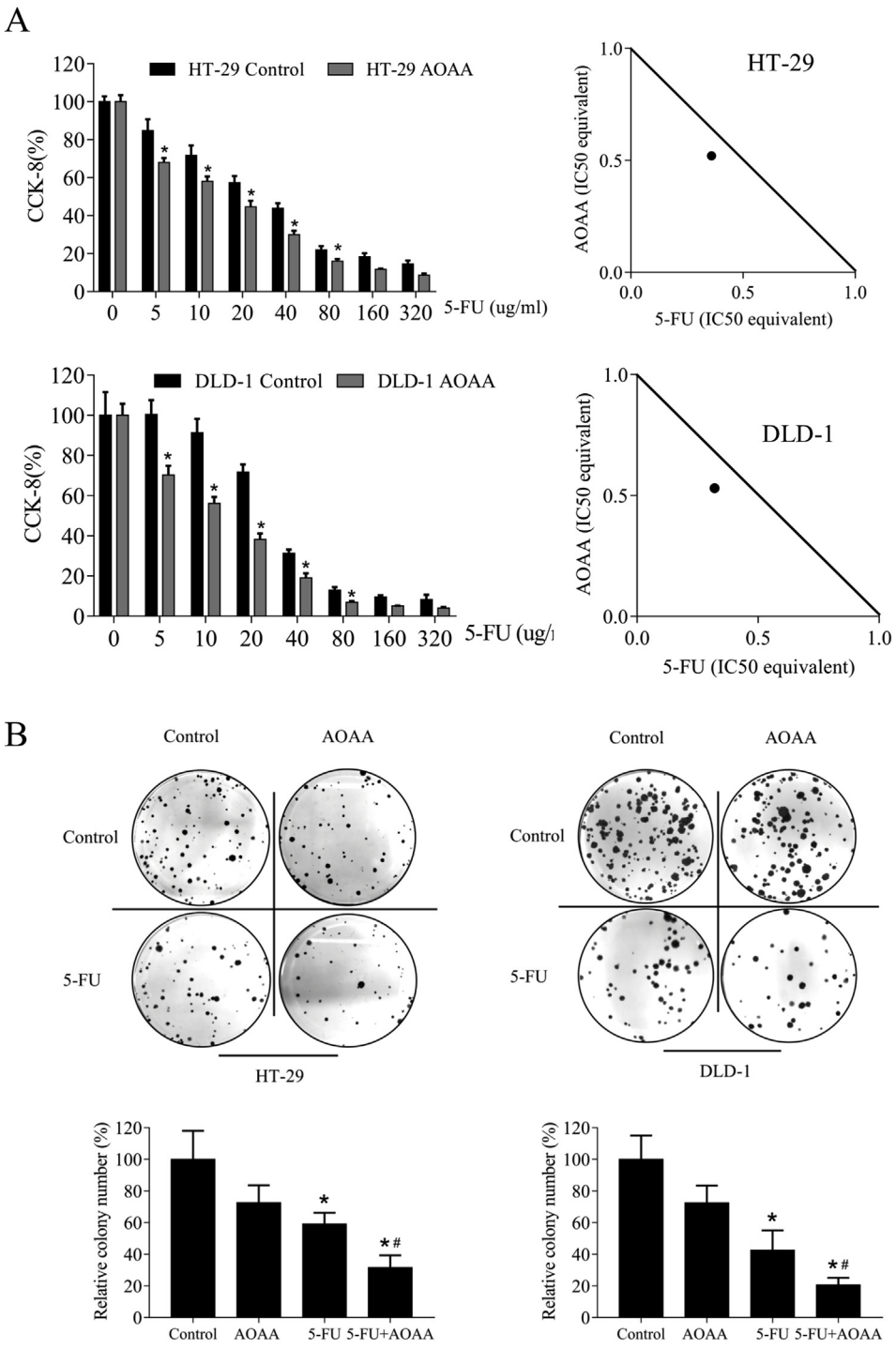

Fig. 3. Synergistic effect between AOAA and 5-FU in inhibiting the proliferation and clonogenicity of colon cancer cells. A, IC50 isobolograms for 5-FU and AOAA-treated HT-29 and DLD-1 cells (CI = 0.88 for HT-29 and 0.85 for DLD-1). B, AOAA (200 μM) significantly increased the inhibitory effect of 5-FU on the clonogenicity of both colon cancer cell lines. (*$P < 0.05$ vs Control, # $P < 0.05$ vs 5-FU).

buffer) at room temperature. Incubations with primary antibodies to detect Bcl-2, Bax, GAPDH, TYMS, EREG (CST, USA) and CBS (Proteintech, USA) were followed by incubations with the secondary antibodies conjugated with horseradish peroxidase (CST, USA). Blots were developed with ECL detection reagents (Millipore, USA). Images were collected utilizing Syngene GeneGenius gel imaging system (Syngene, UK) following the manufacturer's instructions.

2.10. Tumorigenesis in nude mice and in vivo imaging

This study has been approved by the institute review board at Peking University First Hospital (No. 201821). Male nude mice (3 weeks old) were purchased from Vital River Inc. (Beijing, China) and raised in the containment unit of the Laboratory Animal Center at the Peking University First Hospital. DLD-1 cells (10^6 cells/mouse, 0.2 ml PBS) with stably transfection with luciferase were subcutaneously injected into the flank fat pads of 4-week-old male nude mice. Five days later when the xenografts were palpable, mice were randomly assigned

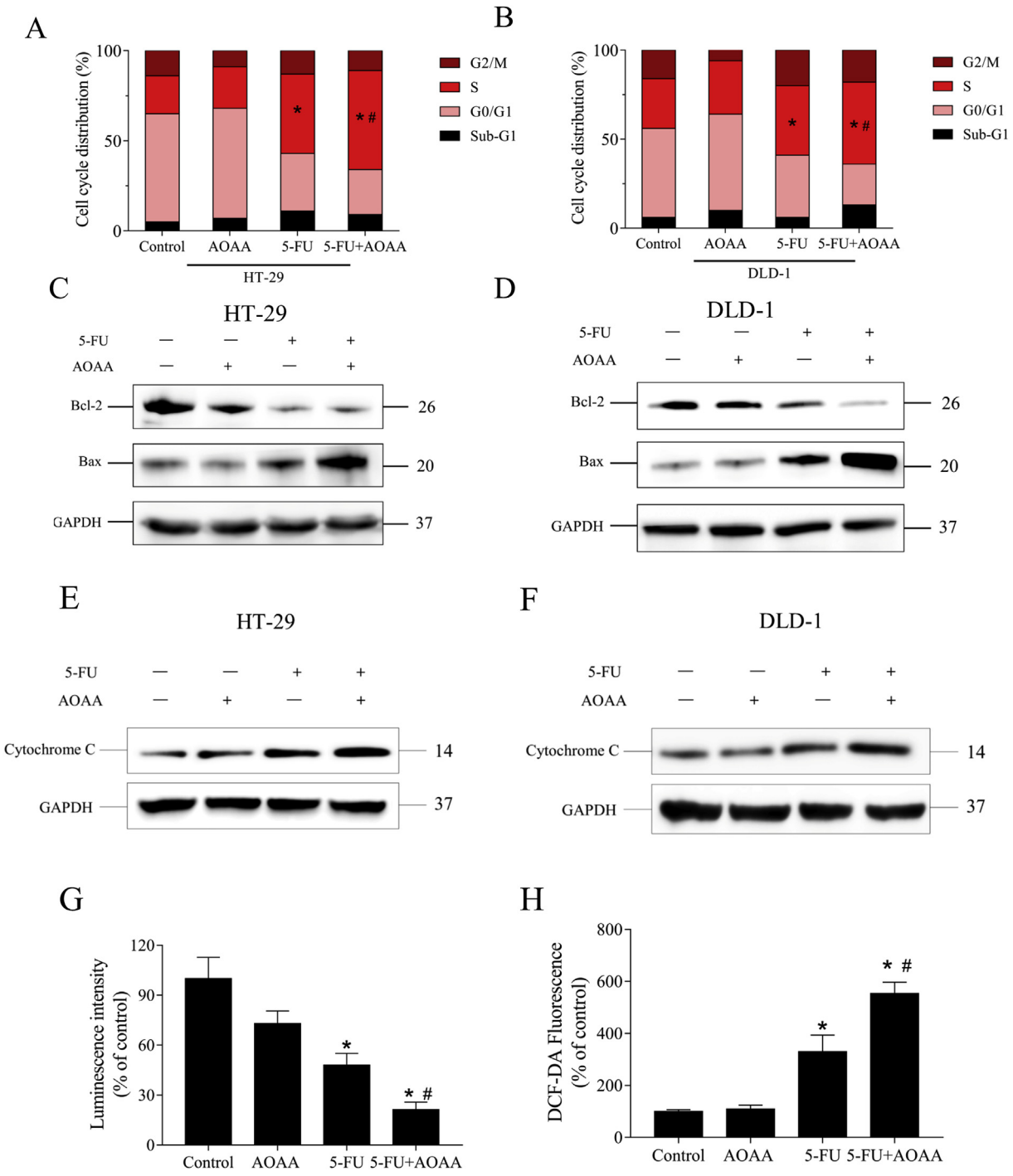

Fig. 4. Effect of AOAA and 5-FU on cell cycles, apoptosis, bioenergetic dysfunction and production of reactive oxygen species in colon cancer cells. A and B, Co-treatment with AOAA further increased the accumulation of cells in S phase compared with cells incubated with 5-FU alone. C and D, Co-treatment with AOAA exaggerated the effect of 5-FU on the expression of Bcl-2 and Bax. E and F, Co-treatment with AOAA exaggerated the effect of 5-FU on the release of cytochrome C. G, AOAA exaggerated the inhibitory effect of 5-FU on the endogenous levels of ATPs. Co-treatment with AOAA further decreased the levels of ATPs to 21.33% of control cells. H, AOAA sensitized colon cancer cells to the induction of reactive oxygen species by 5-FU in colon cancer cells. Co-treatment with AOAA further increased the levels of ROSs to 5.5-fold of control cells (*$P < 0.05$ vs Control, # $P < 0.05$ vs 5-FU).

to one of four groups and subjected to i. p. injection: control (PBS), AOAA (5 mg/kg in PBS, 5 days per week), 5-FU (50 mg/kg in PBS, once a week), and AOAA + 5-FU. D-Luciferin sodium (500 nmol/g body weight) was i. p. administered once every other week for *in vivo* imaging utilizing the IVIS ® Spectrum *in vivo* imaging system (PerkinElmer, USA) and tumor volume was monitored every 3 days as described previously [15]. After 28 days, mice were euthanized, and xenografts were harvested.

2.11. Lentivirus transfection and selection of stable transfection cell lines

Cells were transduced with lentiviruses containing shRNA sequences targeting CBS (shCBS1: Sigma-Aldrich SHCLNV, clone TRCN0000045359; shCBS2: Sigma-Aldrich SHCLNV, clone TRCN0000308284). A non-targeting control shRNA sequence was used as negative control. Colon cancer cells were infected at a MOI of 3 with hexadimethrine bromide (8 μg/ml). Puromycin (4ug/ml) were added 3

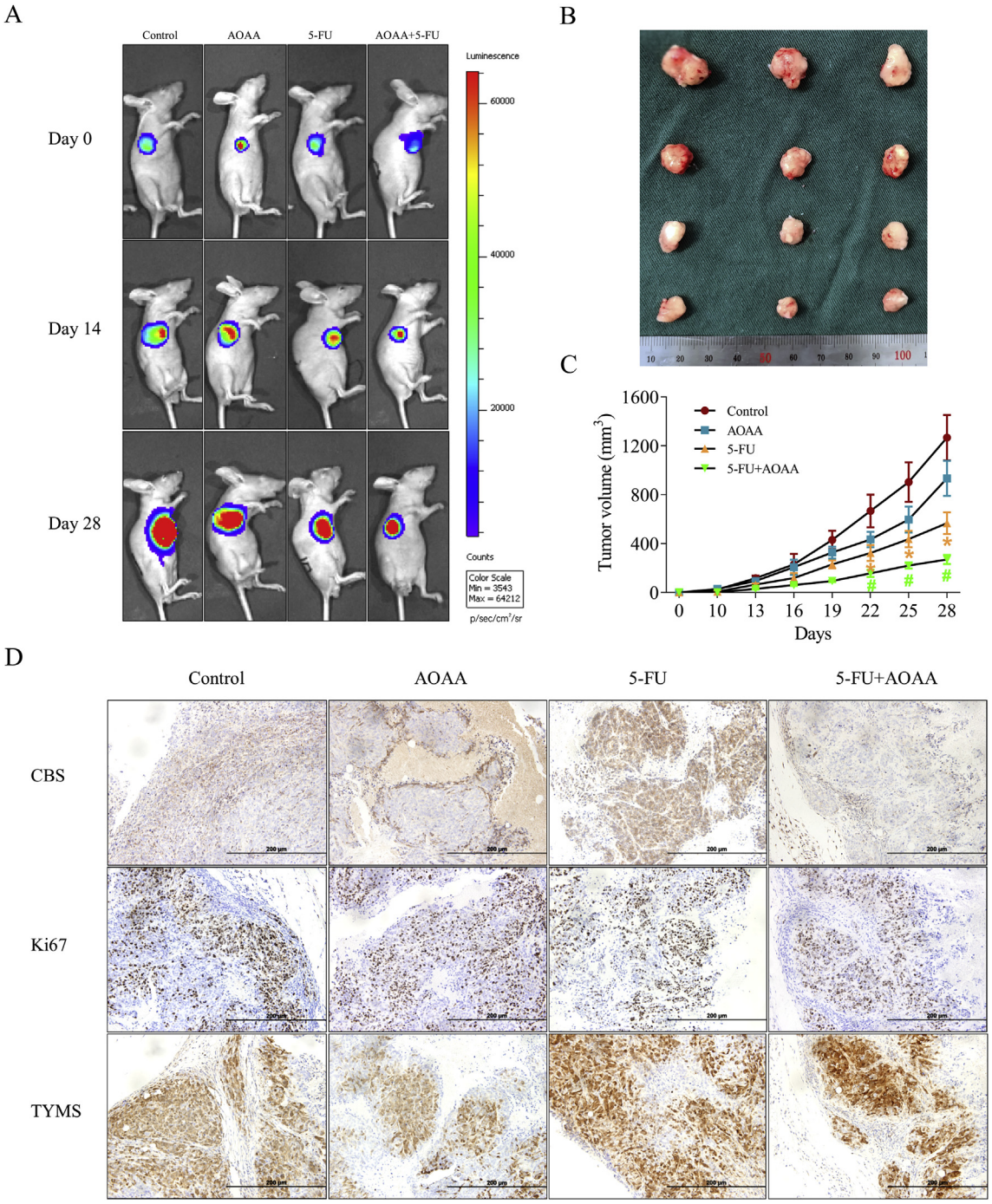

Fig. 5. AOAA sensitizes colon cancer cells to 5-FU *in vivo*. A, B and C, In vivo luciferase assays indicated the synergistic effect between AOAA and 5-FU in inhibiting the proliferation of xenografts. Co-treatment with AOAA further decreased the tumor volumes to 271.8 ± 38.78 mm^3 compared with 569.7 ± 77.14 mm^3 in 5-FU treated group. D, Co-treatment with AOAA abolished the increased expression of CBS induced by 5-FU and co-treatment with AOAA exaggerated the inhibitory effect of 5-FU on the proliferation of tumor cells depicted by decreased Ki67 staining. (*$P < 0.05$ vs Control, # $P < 0.05$ vs 5-FU).

days after transfection and clones with stable transfection were collected.

2.12. Lead sulfide method for detection of H_2S

Lead acetate papers were used to detect H_2S produced by cells cultured in 96 well plates as described previously [16]. Briefly, lead acetate H_2S detection papers (Sigma-Aldrich, USA) was placed above the 96-well plates and incubated 12 h at 37 °C until lead sulfide darkening of the paper occurred. The quantities of H_2S produced by differet wells of cells can be compared by the degreee of blackening of respective areas in the papers.

2.13. RNA sequencing

Total RNA was extracted and purified using a TRIzol reagent kit (Thermofisher, USA) and small RNA libraries were generated from the purified RNA using Illumina's Small RNA v1.5 Sample Preparation kit (Illumina, USA). Briefly, the RNA sample was size fractionated, and RNA of 18–30 nucleotides was isolated and purified (6–8 μl). After 5′

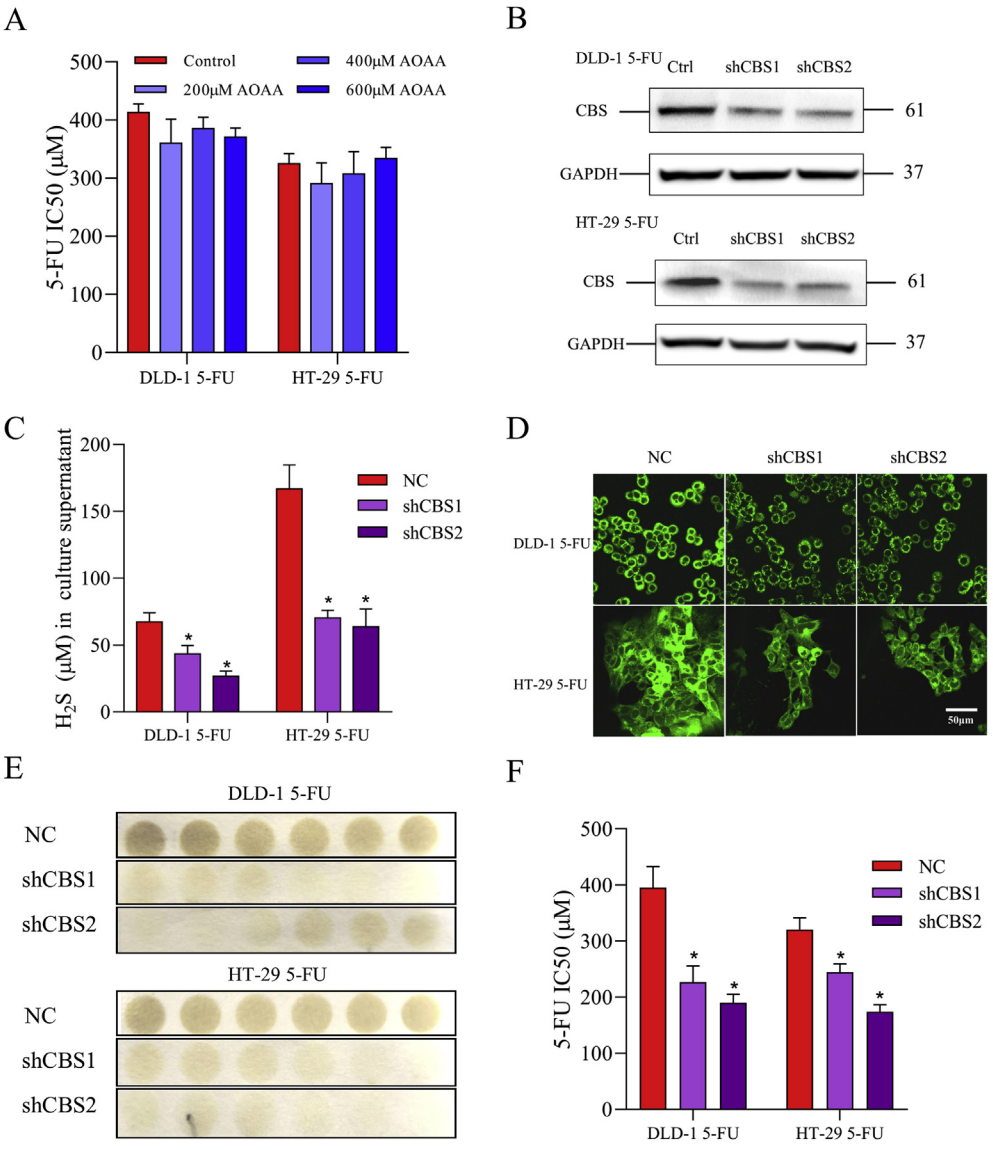

Fig. 6. Decreasing the expression of CBS reversed the acquired resistance to 5-FU. A, AOAA failed to invert the acquired resistance to 5-FU. B, Transfection with shCBS lentiviruses significantly inhibited the expression of CBS. C(sulfur electrode probe), D (AzMC) and E (lead acetate papers), shCBS lentiviruses significantly decreased the production of H_2S in both colon cancer cell lines with acquired resistance to 5-FU. F, Knock down CBS reversed the acquired resistance to 5-FU. shCBS1 decreased the IC50s of 5-FU to 226.7 ± 29.06 μM in DLD-1 5-FU cells and 244.7 ± 14.62 μM in HT-29 5-FU cells. shCBS2 decreased the IC50s of 5-FU to 190 ± 15.28 μM in DLD-1 5-FU cells and 174 ± 12.49 μM in HT-29 5-FU cells. (*$P < 0.05$ vs Control).

and 3′ adaptor ligation, RNA was reverse-transcribed and amplified to generate small RNA libraries. Libraries were sequenced on an Illumina Genome Analyzer II at the Beijing Genomics Institute (BGI).

2.14. Prediction of targeting sites of miR-215-5p

Both *Targetscan* (http://www.targetscan.org/vert_71/) and miRBase (http://www.mirbase.org/) were used to predict the targeting sites of miR-215-5p in the 3′-UTR regions of TYMS and EREG mRNAs.

2.15. Gene expression profiling interactive analysis (GEPIA)

Online analysis of TCGA RNA sequencing data with GEPIA (http://gepia.cancer-pku.cn/) was performed to investigate the correlation between the expression of CBS producing enzymes and EREG, TYMS as described previously [17].

2.16. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and analyzed using a Student *t* tests for unpaired data and ANOVA to compare groups whenever required (SPSS 23.0, CA). The correlation between the expression levels of CBS, CSE, MPST and TYMS, EREG were assessed using Pearson rank correlation coefficient. A $P < 0.05$ was used to indicate statistical significance.

3. Results

3.1. Increased expression of CBS in colon cancer tissues

To validate the increased expression of CBS in colon cancer tissues, *Oncomine* database was used to compare the expression of CBS mRNA in colon cancer tissues and normal colon tissues and discrepancies are noticed between studies available. Studies performed by Gaedcke (n = 65) and Graudens (n = 18) revealed significantly increased expression of CBS mRNA in colorectal cancer tissues [18,19]. However, no significant difference was observed in data provided by Ki (Fig. 1A). Then, realtime PCR was performed in 52 colon cancer tissues and paired adjacent normal colon tissues and the results suggested that the expression of CBS mRNA is significantly increased in colon cancer tissues (Fig. 1B). Immunohistochemistry analysis on a tissue microarray (CO 1801, Alenabio) composed of 90 colorectal cancer and paired adjacent normal tissues further validated the increased expression of CBS in colorectal cancer tissues and the increased staining intensity was mainly noticed in colon cancer cells instead of interstitial cells (Fig. 1C, Supplementary Figure 1).

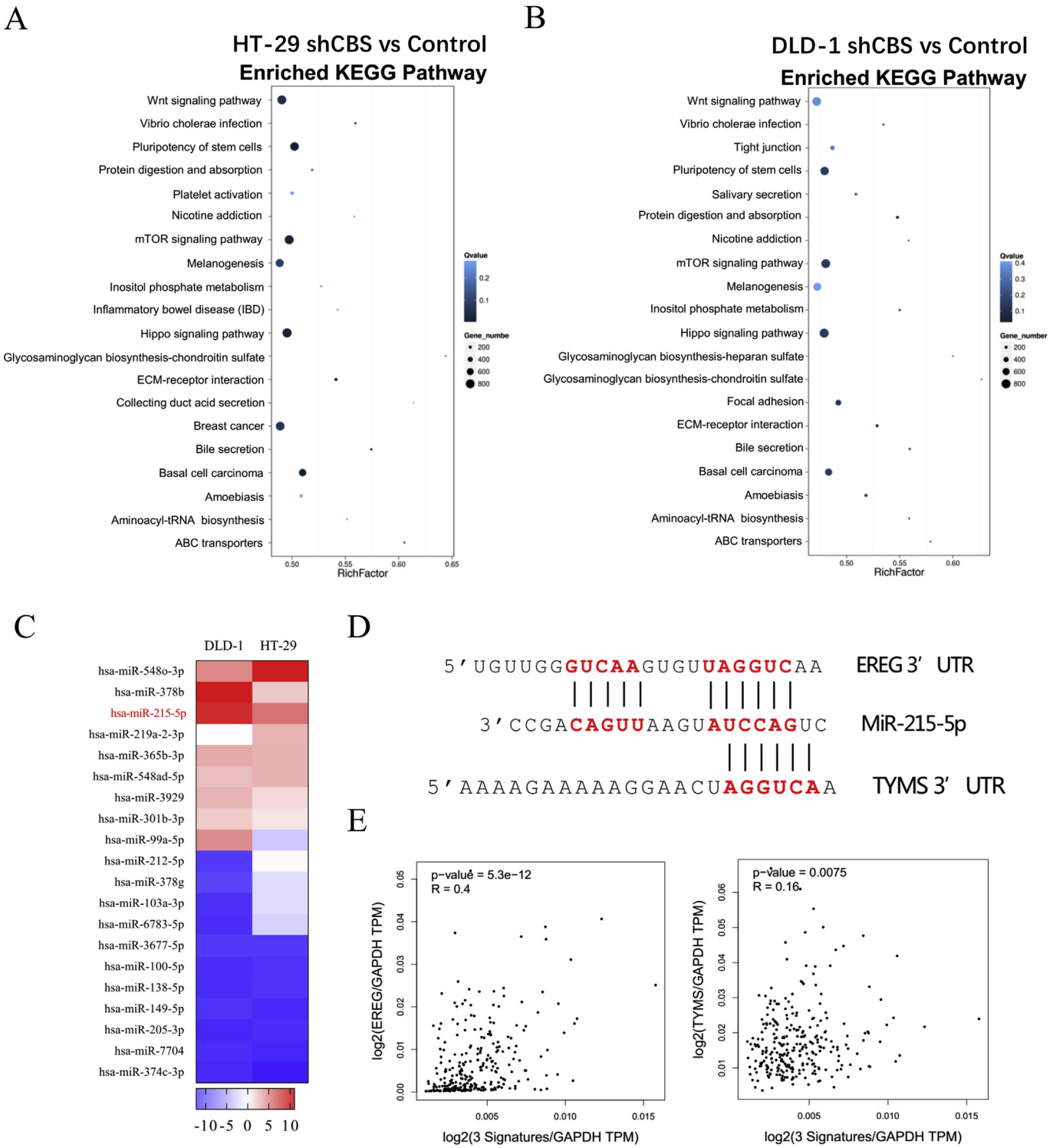

Fig. 7. RNA sequencing and in silico analysis of microRNAs involved in the effect of CBS-H_2S axis. A and B, KEGG analysis suggested that plethora of resistance and stemness related pathways were significantly altered by knocking down CBS in both colon cancer cell lines with acquired resistance to 5-FU. C, miR-215-5p was revealed as one of the miRNAs with most significantly altered expression levels. Knock down CBS induced 9.2-fold increase of miR-215-5p in DLD-1 cells and 6.6-fold increase in HT-29 cells. D, Predicted targeting sites of miR-215-5p in the 3′UTR regions of both EREG and TYMS mRNAs. E, GEPIA analysis revealed positive correlations between these 3 signatures of endogenous production of H_2S and TYMS, EREG.

3.2. The inhibitory effect of AOAA on the production of H_2S and proliferation in colon cancer cells

Aminooxyacetic acid is a general inhibitor of pyridoxal phosphate (PLP)-dependent enzymes, including CBS, and has been widely used as an inhibitor of production of H_2S in multiple studies [20,21]. HT-29 and DLD-1 cells were incubated with increasing doses of AOAA for 48 h and the level of Hydrogen sulfide in the culture supernatant collected form each group was measured. The results suggest that AOAA, added at 200 μM, significantly inhibited the production of H_2S and the effect of AOAA intensifies with the increasement of doses (Fig. 2A). Fluorescence analysis performed with a fluorescent H_2S probe, AzMC, further

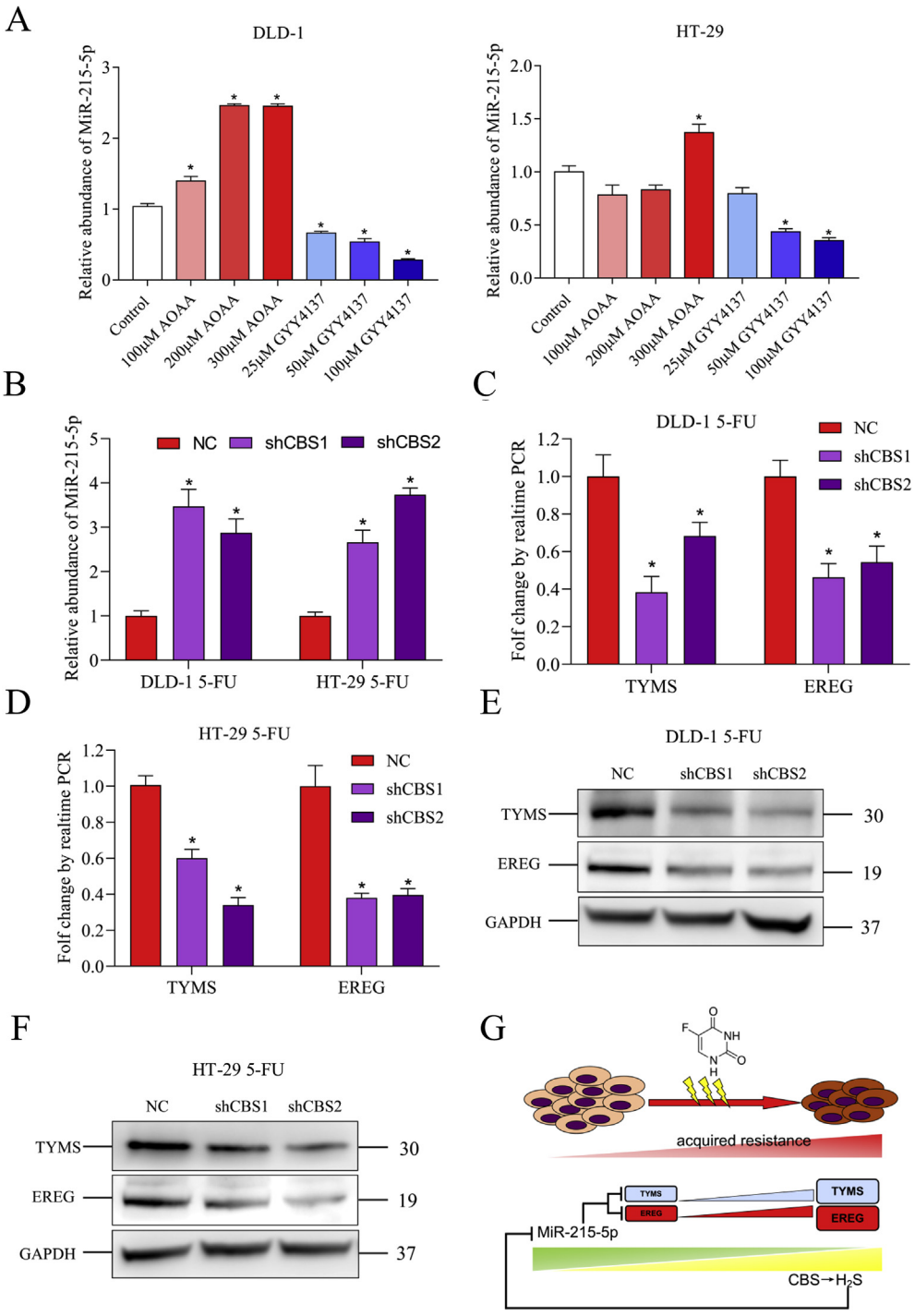

Fig. 8. H_2S increased the expression of TYMS and EREG via inhibiting miR-215-5p. A, GYY4137 significantly inhibited the expression of miR-215-5p in a dose-dependent manner and AOAA significantly increased the expression of miR-215-5p in both cell lines. 300 μM AOAA induced 2.4-fold increase of miR-215-5p in DLD-1 cells and 1.4-fold increase in HT-29 cells. 100 μM GYY4137 induced 0.32-fold decrease of miR-215-5p in DLD-1 cells and 0.42-fold decrease in HT-29 cells. B, Knock down CBS utilizing shCBS lentiviruses significantly increased the expression of miR-215-5p in both colon cancer cell lines with acquired resistance to 5-FU. C and D, Knock down CBS significantly decreased the expression of both EREG and TYMS mRNAs in both colon cancer cell lines with acquired resistance to 5-FU. E and F, Knock down CBS significantly decreased the expression of both EREG and TYMS proteins in both colon cancer cell lines with acquired resistance to 5-FU. G, Schematic overview depicting the role of CBS-H_2S axis in acquired resistance to 5-FU in colon cancer.

validated the inhibitory effect of AOAA (200 μM) on the production of H_2S in both cell lines (Fig. 2B). Besides, *in vitro* assays suggested that AOAA significantly inhibited the enzymatic production of H_2S, depicting a inhibitory effect of AOAA on the enzymatic activity of CBS (Fig. 2C). The effect of AOAA on the proliferation of colon cancer cells was further investigated by CCK-8 assays and the results suggest that AOAA significantly inhibited the proliferation of colon cancer cells at concentrations above 400–500 μM (Fig. 2D).

3.3. AOAA sensitizes colon cancer cells to 5-FU

HT-29 and DLD-1 cells were incubated with increasing doses of 5-FU with or without 200 μM AOAA and the IC50s of 5-FU were calculated. The results suggest that AOAA significantly decreased the IC50s of 5-FU in both cell lines (Fig. 3A). Colony formation assays were further performed with one third of the IC50s of 5-FU in both cell lines and the results suggest that AOAA significantly sensitizes both cell lines to 5-FU, featured by decreased relative colony numbers (Fig. 3B). AOAA alone, added at 200 μM, has no significant effect on the relative colony numbers.

3.4. Increased cell cycle arrest, apoptosis, bioenergetic dysfunction and oxidative stress induced by co-treatment with AOAA and 5-FU

Cell cycle arrest is one of the mechanisms underlying the effect of 5-FU, featured by accumulation of cells in S phase. Flowcytometry

analysis were performed and the results suggest that 5-FU significantly increased the proportion of cells in S phase. Co-treatment with AOAA further increased the accumulation of cells in S phase compared with cells incubated with 5-FU alone (Fig. 4A and B). The apoptosis elicited by 5-FU has been attributed to the release of cytochrome C from mitochondria and the consequent activation of apoptosis cascade [22]. The release of cytochrome C from the membranes of mitochondria is regulated by the Bcl-2 family, including Bcl-2 and Bax. Bcl-2 inhibits apoptosis by attenuating the release of cytochrome C. On the contrary, Bax propagates apoptosis by increasing the release of cytochrome C. 5-FU induces apoptosis by upregulating Bax as well as downregulating Bcl-2. Our results suggest that co-treatment with AOAA further exaggerated the changes of expression of Bcl-2 and Bax induced by 5-FU (Fig. 4C and D). As expected, co-treatment with AOAA significantly increases the cytoplastic distribution of cytochrome C (Fig. 4 E and F).

The bioenergetic dysfunction associated with mitochondrial damage is an important mechanism underlying the chemotherapeutic effect of 5-FU [23]. Both bioenergetic dysfunction and oxidative stress is vital markers of mitochondrial injuries. Co-treatment with AOAA further expanded the bioenergetic dysfunction induced by 5-FU in both cell lines, featured by decreased ATP levels (Fig. 4G). Increased production of ROS has also been related with the anti-tumor effect of 5-FU [24]. Our results suggest that AOAA also exaggerated the oxidative stress induced by 5-FU (Fig. 4H). These results suggest that the exaggerated mitochondrial injuries might be one of the mechanisms underlying the sensitizing effect of AOAA on the anti-tumor effect of 5-FU.

3.5. AOAA sensitizes colon cancer cells to 5-FU in vivo

In vivo luciferase assays were performed utilizing DLD-1 cell lines with stable overexpression of luciferase and the results suggested that co-treatment with AOAA significantly exaggerated the inhibitory effect of 5-FU on the proliferation of tumor xenografts (Fig. 5A). Representative xenograft tumors collected from different groups at the experiment end points were illustrated in Fig. 5B. The mean tumor volume was used to construct a tumor growth curve to evaluate the therapeutic efficacy of AOAA and 5-FU and the results were in accordance with luciferase assays (Fig. 5C). Immunohistochemical assays were performed and the results suggested that chemotherapy with 5-FU significantly increased the expression of CBS in tumor xenografts, and intriguingly, co-treatment with AOAA abolished the effect of 5-FU on the expression of CBS (Fig. 5D). As expected, the proliferation of tumor cells was significantly inhibited by 5-FU, depicted by decreased intensity of Ki67 staining and co-treatment with AOAA exaggerated the inhibitory effect of 5-FU on the proliferation of tumor cells. The expression of TYMS, the molecular target of 5-FU, was also analyzed and surprisingly, AOAA significantly inhibited the expression of TYMS, depicting the potential correlation between H_2S and TYMS, which was further investigated in colon cancer cells with acquired resistance to 5-FU in the following experiments.

3.6. Knock down CBS reversed the acquired resistance to 5-FU

Increased expression of CBS has been validated in colon cancer cells with acquired resistance to 5-FU. Based on previous reports and the results mentioned above, we set out to investigate the potential sensitization effect of inhibiting endogenous production of H_2S in colon cancer cells with acquired resistance to 5-FU. Surprisingly, AOAA failed to reverse the resistance to 5-FU in both colon cancer cell lines with acquired resistance to 5-FU, even at the highest doses of 600 μM (Fig. 6A). Considering the common multidrug resistance in cells with chemoresistance, the cells with acquired resistance to 5-FU may also acquire resistance to AOAA. Thus, lentiviruses with shCBS sequences targeting the mRNA of CBS was used in the following experiments.

Incubation with both lentiviruses with different targeting sequences against CBS mRNA significantly decreased the expression of CBS in both colon cancer cell lines with acquired resistance to 5-FU (Fig. 6B). The concentrations of endogenously produced H_2S in the culture supernatants were measured with a specific sulfur ion electrode and the results suggested that both lentiviruses significantly decreased the concentrations of H_2S in both cell lines (Fig. 6C). Fluorescence analysis performed with AzMC further validated the inhibitory effect of shCBS lentiviruses on the production of H_2S in both cell lines (Fig. 6D). Besides, lead acetate paper was further used to measure the H_2S secreted by cells and the results were in accordance with electrode and fluorescence tests, depicting the inhibitory effect of shCBS lentiviruses on the production of H_2S in both cell lines (Fig. 6E). Strikingly, knock down CBS significantly reversed the acquired resistance to 5-FU in both cell lines, featured by decreased IC50s of 5-FU (Fig. 6F).

3.7. CBS derived H_2S significantly inhibited the miR-215-5p-EREG/TYMS axis in both colon cancer cell lines with acquired resistance to 5-FU

To further investigate the mechanisms underlying the reversion of acquired resistance to 5-FU by decreasing the expression of CBS, miRNA sequencings were performed after knocking down the expression of CBS utilizing shCBS lentiviruses in both colon cancer cell lines. Differentially expressed miRNAs were analyzed by KEGG enrichment and the results suggested that plethora of resistance and stemness related pathways were significantly altered by decreasing the expression of CBS in both colon cancer cell lines (Fig. 7A and B). What's intriguing is that miR-215-5p was revealed as one of the miRNAs with most significantly altered expression levels (Fig. 7C). Multiple previous studies have validated miR-215-5p as a pivotal tumor suppressor in colon cancer and therefore following experiments mainly focused on the potential connection between CBS-H_2S axis and miR-215-5p. Online prediction tools (*Targetscan, miRbase and PicTar*) suggest that epiregulin (EREG), an activator of EGFR signaling, and thymidylate synthetase (TYMS), a key target of 5-FU, are both potential targets of miR-215-5p (Fig. 7D). GEPIA analysis of TCGA colon cancer RNA sequencing database was further used to analyze the correlation between the expression of 3 signatures of H_2S producing enzymes (CBS, CSE and MPST) and TYMS, EREG. Positive correlations were revealed between these 3 signatures and TYMS ($R = 0.4$, $P < 0.01$) and EREG ($R = 0.16$, $P < 0.01$) (Fig. 7E). The correlation has been further tested in both breast and esophageal carcinoma. The positive correlation between 3 signatures of H_2S producing enzymes and TYMS holds true for both types of cancer and positive correlation between 3 signatures and EREG is also revealed in breast cancer. However, no significant correlation exists between EREG and 3 signatures in esophageal carcinoma. (Supplementary Fig. 1). Together, these results suggested that endogenous H_2S might be involved in the development of acquired resistance to 5-FU in colon cancer cells by regulating the expression of both TYMS and EREG via miR-215-5p.

3.8. H_2S increased the expression of TYMS and EREG via inhibiting miR-215-5p

Both parental colon cancer cell lines were incubated with increasing doses of AOAA and GYY4137, which has been widely used as a slow releasing donor of H_2S [25]. The results suggested that GYY4137 significantly inhibited the expression of miR-215-5p in a dose-dependent manner in both cell lines (Fig. 8A). AOAA significantly increased the expression of miR-215-5p in both cell lines, with a relatively inconspicuous dose-dependent manner (Fig. 8A). What's more interesting is that decreasing the expression of CBS utilizing shCBS lentiviruses significantly increased the expression of miR-215-5p in both colon cancer cell lines with acquired resistance to 5-FU (Fig. 8B). Considering that both the mRNAs of EREG and TYMS are direct targets of miR-215-5p, the effect of decreasing the expression of CBS on the expression of both EREG and TYMS was further investigated. Intriguingly, decreasing the expression of CBS significantly decreased the expression of both EREG

and TYMS at both mRNA and proteins levels in both colon cancer cell lines with acquired resistance to 5-FU (Fig. 8C, D, E, F). Considering the role of TYMS as a direct target of 5-FU and the important role of EREG in EGFR signaling, our results provide a novel connection between the endogenous H_2S and the acquired resistance to 5-FU in colon cancer cells through miR-215-5p mediated down-regulation of both EREG and TYMS (Fig. 8G).

4. Discussion

As the third confirmed gaseous transmitters, endogenous H_2S has been reported to play an important role in plethora of physiological and pathological processes [26,27]. Increased expression of the producers of H_2S, including CBS and CSE, has been validated in multiple types of cancer tissues, including colorectal cancer, ovarian cancer, renal cancer and breast cancer [28–30]. Recently, upregulating the expression of CBS reprogramed the metabolic profiles of normal colon epithelial cells towards features of colon cancer cells, depicting the pivotal role of CBS and H_2S in the pathogenesis of colon cancer [31]. Studies suggested that increased production of H_2S induced by amino acid restriction could lend protection against ischemic injuries via interacting with SQR by donating electrons to the ETC via coenzyme Q, thus potentially serving as a source of electrons during ischemia [32]. Considering the common ischemic and hypoxic microenvironment of solid tumors, increased production of H_2S might be an alternative energy supply. Moreover, by a paracrine action on peritumor/intratumor vascular endothelial cells, H_2S has been reported to promote tumor angiogenesis [8]. Recently, increased expression of two major H_2S-generating enzymes (CBS and 3-MST) and production of H_2S has been revealed in colon cancer cells with acquired resistance to 5-FU [33]. Targeting the endogenous production of H_2S by inhibiting the increased expression of CBS might be a promising approach in tackling the acquired resistance to 5-FU in colon cancer cells.

In this study, series of experiments were performed in both parental colon cancer cell lines and cell lines with acquired resistance to 5-FU both *in vitro* and *in vivo* to investigate the effect of inhibiting H_2S synthesis on the sensitization of colon cancer cells to 5-FU. Firstly, increased expression of CBS and CSE was validated in TCGA online database and colon cancer tissue microarrays. (Fig. 1). Herein, inhibiting the enzymatic activity of CBS with AOAA significantly exaggerated the inhibitory effect of 5-FU on the proliferation of colon cancer cells and a synergistic effect was revealed between AOAA and 5-FU (Figs. 2 and 3). Increased cell cycle arrest, apoptosis, bioenergetic dysfunction and oxidative stress induced by co-treatment with AOAA and 5-FU (Fig. 4). The synergistic effect between AOAA and 5-FU was further validated *in vivo* and the inhibitory effect of AOAA on the expression of TYMS, a major target of 5-FU, was revealed (Fig. 5). Albeit no significant effect of AOAA on reserving the acquired resistance to 5-FU was observed, decreasing the expression of CBS with lentiviruses significantly reversed the acquired resistance to 5-FU (Fig. 6). MicroRNA sequencings indicated that multiple resistance associated signaling pathways were modified by decreasing the expression of CBS and miR-215-5p was significantly increased in cell lines with decreased expression of CBS (Fig. 7 A, B and C). Both EREG and TYMS were predicted to be the direct targets of miR-215-5p (Fig. 7D). Online analysis of TCGA database utilizing GEPIA depicted a positive correlation between the expression of major producers of H_2S (CBS, CSE and MPST) and EREG, TYMS in colon cancer tissues (Fig. 7E). Further experiments revealed the inhibitory effect of H_2S on the expression of miR-215-5p in parental colon cancer cell lines and decreasing the expression of CBS with shCBS lentiviruses significantly increased the expression of miR-215-5p in both cell lines with acquired resistance to 5-FU (Fig. 8A–D). Finally, knock down CBS significantly decreased the expression of EREG and TYMS in both mRNA and protein levels in cell lines with acquired resistance to 5-FU (Fig. 8 E and F). The schematic overview of this study was illustrated in Fig. 8G, we hypothesized that the increased production of H_2S could increase the expression of both TYMS and EREG via inhibiting the expression of miR-215-5p and delineating the exact molecular interaction between H_2S and miR-215-5p will be the focus of our future work.

Together, these results suggested that inhibiting H_2S synthesis by decreasing the expression of CBS might be a potential target for reversing the acquired resistance to 5-FU in colon cancer cell lines and inhibiting the miR-215-5p-EREG/TYMS axis might be one of the mechanisms underlying the effect of targeting CBS.

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Conflicts of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.09.006.

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