Targeting the MALAT1-miR-206-GLS axis enhances the apoptotic effects of gastric cancer cells by mild hyperthermia treatments

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Research

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Abstract

Background: To explore the molecular mechanisms and modulatory effects of long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in the hyperthermia-induced gastric cancer cell death.

Methods: Combined qRT-PCR and GLS activity detection of glutamine metabolism, we demonstrate the difference of expression of MALAT1 and miR-206 in normal or cancer tissue/ several cell lines and the roles of two in regulation of glutamine, respectively. The relation of MALAT1 and miR-206 was research by bioinformatical analysis and luciferase report. In addition, the molecular regulation between apoptotic effects and MALAT1-miR-206-GLS axis in gastric cancer after mild hyperthermia treatments were discovered by western blotting, luciferase report, quantitative real-time PCR (qRT-PCR), GLS activity detection of glutamine metabolism and cell viability test.

Results: MALAT1 positively associated with GC by promoting glutamine metabolism under short time hyperthermia treatment, it involved in an adaptive reaction, an increased glutamine metabolism rate which could be further overridden by long time hyperthermia exposures. miR-206 could inhibit glutamine metabolism of GC and target 3'UTR of GLS which has oncogenic functions in GC directly. In addition, miR-206-suppresed glutamine metabolism was through direct targeting GLS. LncRNA MALAT1 inhibited miR-206 expression in GC by sponging it as a competing endogenous RNA. In summary, MALAT1-promoted glutamine metabolism was through the miR-206-GLS axis. Furthermore, we demonstrated effective approaches from the MALAT1-miR-206 axis for enhancing the mild hyperthermia-induced cancer death under a short time.

Conclusions: Taken together, the MALAT1-miR-206-glutamine metabolism axis is a new strategy for enhancing the selectivity of hyperthermia treatments specific to GC cells

Background

Gastric cancer (GC), one of the most common malignancies, leads to a high incidence of cancer-related death(1). Currently, surgery is considered the primary approach(2). In addition, radiotherapy, chemotherapy, and the implementation of neoadjuvant therapy are also important treatment strategies with targeting specific signaling pathways(1, 3). Although the 5-year survival rate of early GC improved recently, the precise molecular mechanisms of development and progression of GC are still under investigation.

Hyperthermia is one therapeutic approach against cancer by raising the temperature of the local tumor environment to above normal temperature for a defined period of time(4). Hyperthermia could be applied alone or as an adjunctive with radio- and chemo- therapies(5). Accumulation evidence revealed that above 41 °C, cancer cells exhibit toxicity, resulting in suppression of cancer cell growth and sensitization

to radio- and chemo- therapies(6–8). However, due to the recurrence of malignancies and the side-effects of the existing therapeutic, hyperthermia is not generally accepted as a conventional anti-cancer therapy (9).

The long non-coding RNAs (lncRNAs) are a class of non-coding RNAs which have larger than 200 nucleotidesand play important roles indiverse cancer processes, such as tumorigenesis, metastasis and chemoresistance(10). The MALAT1 is one of human tumor-related lncRNA that promotes the growth and metastasis of multiple cancers(11, 12). Previous studies have demonstrated MALAT1 was frequently upregulated in human cancers by functioning as a potential oncogenic regulator in multiple cancers(13–15). However, the molecular targets of MALAT1 and detailed mechanisms are unclear. MicroRNAs are short endogenous non-coding RNAs (19–23 nucleotides) that regulate gene expression through specific binding to the 3'-untranslated regions (3'-UTRs) of their target mRNAs(16). Similar to lncRNAs, miRNAs play important roles in regulating tumor progresses(17). Studies revealed that miR-206 inhibited proliferation, migration, and invasion of GC(18, 19), suggesting miR-206 acts as a tumor suppressor in GC. Intriguingly, recent discoveries demonstrated that lncRNAs served as competing endogenous RNAs (ceRNA) of miRNAs to downregulate them, leading to modulation of miRNA targetgenes(20). However, whether MALAT1 could interact with miR-206 to regulatethe sensitivity of GC to hyperthermia treatment remains to be elucidated.

For decades, growing evidence demonstrated a tight relation between metabolism reprograming and malignant cancer progresses(21, 22). 1tenance of blood glutamine levels and provides a source of carbon and nitrogen to support biosynthesis of cancer cells(23). Studies suggested that the involvement of glutamine in cancer metabolism is essential as well as glucose metabolism(24). Importantly, dysregulated glutamine metabolism was reported to chemoresistance(23). However, the roles of MALAT1 in glutamine metabolism of GC were currently unknown. In this study, we investigated the roles of MALAT1 in hyperthermia-induced GC cell death. Here in, we provide evidence that MALAT1 was a ceRNA of miR-206. The mRNA target of miR-206 was also identified. In addition, the specific roles of MALAT1 in regulating the cellular glutamine metabolism of GC cells were examined. Our results will contribute to developing novel therapeutic approaches to enhance the cytotoxic effects of hyperthermia treatments against GC.

Materials And Methods

Patient specimen collection

A total of 30 GC patients with no preoperative chemo- or radio- treatments were enrolled in this study. Samples were collected from patients who were diagnosed and underwent primary surgical resection in the Affiliated Cancer Hospital & Institute of Guangzhou Medical University (Guangzhou, China) during 2016-2017. After surgical, specimens were immediately frozen by liquid nitrogen and stored at -80 °C for further analysis. The present study was approved by the Ethics Committee of Affiliated Cancer Hospital &

Institute of Guangzhou Medical University. Before all experiments were carried, the written informed consent was obtained from all patients.

Cell culture and hyperthermia treatments

The human gastric epithelial cell line, GES-1 and GC cell lines, HGC27, SGC-7901, BGC823, MKN-45 and AGS were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) at 37 °C in a humidified, 5% CO_2 atmosphere. Cell culture medium was replenished every 3 days and when the cells reached 80~90% confluence, cells were split at 1:5 for experiments. In a hyperthermia exposure group, cells in an incubator with preheated to 39˚C, 41˚C, 43˚C or 45˚C for 0.5, 1, 2 or 4 h. Cells from control groups were incubated at 37˚C for the same time periods. After hyperthermia exposure, cells were incubated at 37˚C for 8hours prior to experiments.

LncRNA, shRNA and miRNA transfections

For transfections, GC cells were seeded into 24-well plates at $5x10^4$ density for 24 hours, transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA) according to the manufacturer's protocols. Plasmid DNA for overexpression of lncRNA-MALAT1 was constructed according to previous reports (25) and transfected at 2 ug for 48 hours. The shRNA-MALAT1 or empty control was conducted from GenePharma (Shanghai, China). The miR-206 precursor, miR-206 inhibitors or negative control was purchased from RiboBio (Guangzhou, China) and transfected at 50 nM for 48 hours. The overexpression plasmid of GLS was purchased from Origen.com and transfected at 2 ug for 48 hours.

Bioinformatical analysis

The Kaplan-Meier survival curves were drawn from the KM plot program (http://kmplot.com/analysis/) according to previous description(26). Prediction of the MALAT1-miR-206interaction was performed by starBase of ENCORI (http://starbase.sysu.edu.cn/) according to previous reports(27). The binding of miR-206 on 3’UTR of GLS was predicted from the TargetScan.org.

RNA extraction and qRT-PCR

Total RNAs were extracted using the Trizol reagent (Invitrogen, Carlsbad, MA, USA) according to manufacturer’s instructions. The quantities of RNA samples were measured using aND-1000 spectrophotometer (NanoDropTechnologies Inc., Wilmington, USA). For detection of lncRNA, one μg of total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For detection of miRNAs, cDNA was synthesized using the TaqMan Advanced miRNA cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Quantitative PCR was performed using the SYBR Green method (Applied Biosystems Inc., Carlsbad, CA,

USA). The thermal cycle was set as follows: 95°C for 1 min and 40 cycles at 95°C for 15 s, 58°C for 20 s, and 72°C for 20 s. Primers used for this study were listed as follows:

MALAT1: Forward:5’-ACTGAATCCACTTCTGTGTAGC-3’

and Reverse: 5’-CGGAAGTAATTCAAGATCAAGAG-3’;

β-actin: Forward: 5’-CTGAGAGGGAAATCGTGCGT-3’

and Reverse: 5’-CCACAGGATTCCATACCCAAGA-3’;

miR-206: Forward: 5’-TGGAATGTAAGGAAGTGTGTGG-3’;

and Reverse 5’-ACACACTTCCTT ACATTCCATT-3’;

U6: Forward: 5’-CTCGCTTCGGCAGCACA-3’

and Reverse: 5’-AACGCTTCAGGAATTTGCGT-3’.

β-actin and U6 were used as an internal control for MALAT1 and miR-206, respectively. The Ct value was measured by the $2^{-\Delta\Delta Ct}$ method. Experiments were performed in triplicate and repeated three times.

Luciferase assay

The wild-type MALAT1 or mutant MALAT1 and 3'-UTR of mutant or wild-type GLS were amplified and cloned into a pGL3 vector. Cells were co-transfected with the wild-type or mutant luciferase vectors and miR-206 or control miRNAs by Lipofectamine 2000 for 48 h. A dual-luciferase reporter gene assay was performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). The relative luciferase activity was normalized to that of Renilla luciferase activity. Experiments were performed in triplicate and repeated three times.

Caspase-3 activity assay

Cells ($5x10^4$) were seeded in 24-well plates for 24 hours. After treatments, cells were collected and the activity of Caspases-3 was measured using a Caspase-3 Activity Assay Kit (Cell Signaling Tech. Danvers, MA, USA) according to the manufacturer’s protocol. Experiments were performed in triplicate and repeated at least three times.

Detection of glutamine metabolism and GLS activity

The glutamine uptake was measured using the Glutamine and Glutamate Determination Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. The activity of GLS and GLS enzyme activity was measured by a Glutaminase Assay Kit (#E-133, Biomedical Research Service Center, Buffalo, NY, USA) according to the manufacturer’s instructions. Briefly, cells were homogenized and mixed with buffers from the kit. The mixture was incubated for 2 h at 37 °C, followed by incubating for another 1

h at 37 °C with new buffers from the kit. The optical density (OD) at 492 nm was determined by a microplate Spectro-photometer. Results were normalized to the cell numbers of each reaction. Experiments were performed and analyzed in triplicate.

Cell viability assay

The cell viability was determined by MTT assay (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Cells (5×10^3) were cultured in 96-well plates and cultured for 24 hours. The next day, medium was refreshed cells were stained with 0.5 mg/ml MTT for 4 hours. Supernatant was discarded and cells were washed by PBS. Then 200 µl of dimethylsulfoxide (DMSO) was added for 2 hours at 37°C to dissolveprecipitates. The optical density (OD) of each well was measured at 490 nm using a spectrometer reader. Relative viability was calculated from the absorbance of hyperthermia treated cells/the absorbance of control cells. Experiments were performed in triplicate and repeated three times.

Western blot

Total proteins were isolated from GC cells using the RIPA buffer (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), with protease inhibitor cocktail (Bio-Rad, Hercules, CA, USA) according to the instructions of the manufacturer. Cells were washed with cold PBS and lysed withlysis buffer on ice. Samples were centrifuged at 10,000×g to collect the supernatant. Protein was quantified by Bradford assay (Biorad, Hercules, CA, USA). Equal amount of total protein (30 mg) of each sample was resolved on a 10% SDS-PAGE geland transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in TBST for 1 hour at room temperature. Membranes were washed with TBST and then incubated with primary antibodies at 1:1000 at 4^oC for overnight. After complete washing by TBST, membranes were then incubated with horseradish peroxidase-linked secondary antibody at 1:3000 at room temperature for 1 hour, followed by visualization using Pierce ECL western blotting kit (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. β-actin was an internal control. Experiments were repeated three times.

Statistical analysis

Statistical analysis was performed using the Prism 6.0 software package (GraphPad Software, Inc.). Data are represented as mean ± standard deviation (SD). Student's t-test was used to compare difference between two groups and the one-way ANOVA was used to compare the continuous variables in multiple groups. Survival rate were analyzed by Kaplan–Meier method from the http://kmplot.com/analysis. Experiments were repeated three times. $p<0.05$ indicates statistical significance.

Results

LncRNA MALAT1 is upregulated in GC and positively associates with glutamine metabolism

Previous studies have revealed the oncogenic roles of MALAT1 in diverse cancers via regulating downstream ceRNA effectors(11, 13, 14). We started to investigate the clinical relevance of MALAT1 in GCs. Expressions of MALAT1 in 30 gastric tumor tissues and their adjacent normal gastric tissues were detected and qRT-PCR results clearly demonstrated that the MALAT1 levels were significantly increased in gastric tumor tissues compared with normal tissues (Fig. 1A). In addition, Kaplan-Meier Plotter analysis illustrated that GC patients with higher MALAT1 expressions associated with lower survival rate (Fig. 1B), suggesting a positive correlation between MALAT1 and GC progresses. Consistently, MALAT1 was significantly upregulated in five GC cells compared with normal gastric epithelial cells, GES-1 (Fig. 1C). Accumulating evidence revealed that glutamine metabolism is dysregulated in diverse cancer cells to support cancer cell progressions, presenting targeting glutamine as a promising approach for cancer therapy(23, 24, 28). Thus, we assessed the roles of MALAT1 in regulating glutamine metabolism of GC cells. AGS and SGC-7091 GC cells were transfected with control or MALAT1 overexpression vector or sh MALAT1 (Fig. 1D). Intriguingly, overexpression of MALAT1 apparently stimulated glutamine uptake, the first step in glutaminolysis through the conversion of glutamine to glutamate by GLS (Fig. 1E). Increased GLS activity was also observed in MALAT1 high expression GC cells (Fig. 1F). On the other way, silencing MALAT1 significantly suppressed glutamine uptake and GLS activity (Fig. 1E, 1F). Taken together, the above results indicated MALAT1 positively associated with GC by promoting glutamine metabolism.

MALAT1 and glutamine metabolism are induced under short time and mild hyperthermia, but suppressed in long time and high temperature

To assess whether the MALAT1-modulated glutamine metabolism is correlated with hyperthermia treatment, we treated GC cells with normal and gradually increased mild hyperthermia by maintaining at 37˚C (control group) or hyperthermia groups at 39˚C, 41˚C, 43˚C or 45˚C for four intervals (0.5, 1, 2and 4 h), respectively. Expressions of MALAT1 from treated cells were then analyzed by qRT-PCR. As depicted in Fig. 2A, MALAT1 expression was shortly induced in the slight hyperthermia group (41˚C and 43˚C) at short time exposures (30 min). However, cells with short time exposure under relative higher temperature (45˚C) showed downregulation of MALAT1. Moreover, we found cells with relative long-time exposures (1–4 hours), expressions of MALAT1 were significantly decreased under hyperthermia conditions (Fig. 2B-2D). Consistently, we detected the glutamine uptake and GLS activities were induced at short and mild hyperthermia exposure (41˚C and 43˚C) (Fig. 2E, 2F). After 1 hour, the induced glutamine uptake and GLS activities were significantly decreased (Fig. 2E, 2F). These results indicated under short time hyperthermia treatment, MALAT1 involved in an adaptive reaction, an increased glutamine metabolism rate which could be further overridden by long time hyperthermia exposures.

MALAT1 sponges miR-206 as a ceRNA in GC cells

We next explore the molecular mechanisms for the MALAT1-regulated glutamine metabolism under hyperthermia. Recent studies have demonstrated lncRNAs act as sponges to form a lncRNA-miRNA interaction which induces miRNA degradation to affect downstream target mRNA expressions(20). Therefore, we performed bioinformatics analysis through the online lncRNA-miRNA interaction prediction program, starBase (http://starbase.sysu.edu.cn/index.php) and observed a putative binding site on MALAT1 for miR-206 (Fig. 3A), indicating MALAT1 could inhibit miR-206 expression via direct sponging to the seeding region of miR-206. MiR-206a has been reported to be negatively correlated with multiple cancer progresses(18, 19), suggesting miR-206 might be a ceRNA partner of MALAT1. From the analysis of MALAT1 and miR-206 expressions from GC tissues, we found a significantly negative correlation between them: the higher MALAT1 expressions accompanied with lower miR-206 levels in gastric tumor tissues. Expectedly, overexpression of MALAT1 effectively inhibited miR-206 levels in GC cells, AGS and SGC-7091 (Fig. 3C). Meanwhile significant miR-206 upregulation by silencing MALAT1 was observed in GC cells (Fig. 3D). To validate the direct binding of MALAT1 on miR-206, we conducted binding site nucleotides mutation on MALAT1 (Fig. 3A). We then cloned the MALAT1 WT and Mut constructs into luciferase vector and co-transfected with miR-206 precursor into AGS cells. A luciferase assay showed that miR-206 significantly decreased the luciferase activity of WT MALAT1 but not the binding site mutant MALAT1 (Fig. 3E). Taken together, the above results strongly demonstrated lncRNA MALAT1 inhibited miR-206 expression in GC by sponging it as a competing endogenous RNA.

miR-206 is downregulated and shows invert expression pattern with MALAT1 in GC

To characterize the tumor suppressive roles of miR-206 in glutamine metabolism of GCs, we compared the expressions of miR-206 in GCs and normal gastric tissues. As we expected, miR-206 was significantly downregulated in 30 gastric tumor tissues (Fig. 4A). In addition, Kaplan-Meier Plotter analysis clearly reflected that GC patients with higher miR-206 expressions associated with higher survival rate (Fig. 4B). Subsequently, we detected miR-206 was significantly downregulated in five GC cells compared with normal gastric epithelial cells, GES-1 (Fig. 4C). Furthermore, overexpression of miR-206 (Fig. 4D) apparently suppressed glutamine uptake and GLS activity in GC cells (Fig. 4E, 4F). In summary, these results indicated miR-206could inhibit glutamine metabolism of GC.

miR-206 suppresses glutamine metabolism under hyperthermia through direct targeting GLS

The above results demonstrated a negative regulation of miR-206 by MALAT1. In order to assess the roles of miR-206 in glutamine metabolism and the hyperthermia-induced cell death, we next examined the miR-206 expressions under 37˚C, 39˚C, 41˚C, 43˚C or 45˚C for four intervals (0.5, 1, 2 and 4 h), respectively. In contrast to MALAT1 expression patterns, miR-206 was shortly suppressed (30 min) in the slight hyperthermia group (41˚C and 43˚C) (Fig. 5A). Moreover, cells with short time exposure under relative higher temperature (45˚C) showed induction of miR-206 (Fig. 5A). We observed cells with relative

long-time exposures (1–4 hours), expressions of miR-206 were significantly elevated under hyperthermia conditions (Fig. 5B-5D). We next asked whether the long-time hyperthermia-suppressed glutamine metabolism was through miR-206 induction. miR-206 inhibitor or negative control was transfected into AGS cells, followed by treatments under normal and hyperthermia. As we expected, inhibition of miR-206 significantly overcame the hyperthermia (41^{o}C and 43^{o}C)-suppressed glutamine uptake and GLS activity (Fig. 5E, 5F).

Combined with the above MALAT1 expressions under hyperthermia, these results indicated under hyperthermic conditions, miR-206 was induced due to MALAT1 suppression, resulting in impaired glutamine metabolism.

It is widely studied that miRNAs function through direct binding to 3'UTR of their target gene to regulate gene expressions(16). We then searched the potential targets of miR-206 through the online miRNA databases TargetScan. We found the 3'-UTR of human glutaminase (GLS), an enzyme which catalyzes the convert of glutamine to glutamate(23, 28), contains a putative binding region of miR-206 (Fig. 6A). For examining whether miR-206 could downregulate GLS expression, we transfected the control miRNAs or miR-206 precursor into AGS cells. Overexpression of miR-206 clearly suppressed the GLS protein expressions in GC cells (Fig. 6B). To examine whether miR-206 could directly bind to the 3-UTR region of GLS, luciferase reporter assays were performed by co-transfecting the WT-GLS 3'UTR or nucleotides mutant GLS 3'UTR with control miRNA or miR-206 precursor into AGS cells. Expectedly, miR-206 significantly decreased the luciferase activity of wild type GLS 3'-UTR but could not affect that of the binding site mutant GLS 3'-UTR (Fig. 6C). Consistent results from Spearman's correlation analysis elucidated a negative correlation between miR-206 and GLS in gastric tumors (Fig. 6D). Importantly, bioinformatics analysis revealed GLS is negatively correlated with survival rate of GC patients (Fig. 6E). Taken together, the above results strongly demonstrated miR-206 could directly target 3'UTR of GLS which has oncogenic functions in GC.

To test whether miR-206 inhibited glutamine metabolism through targeting GLS, we performed rescue experiments by transfecting GC cells with control, miR-206 precursor alone or miR-206 plus GLS overexpression plasmid. Western blot results demonstrated co-transfection of miR-206 and GLS successfully restored the GLS protein expressions in miR-206 overexpressing GC cells (Fig. 6E). Consequently, the glutamine uptake was significantly recovered in GLS rescued cells (Fig. 6F), suggesting the miR-206-suppresed glutamine metabolism was through direct targeting GLS.

The MALAT1-promoted glutamine metabolism is through the miR-206-GLS axis

We next asked whether the MALAT1-mediated glutamine metabolism was through the miR-206-GLS pathway. GC cells were transfected with control shRNA, MALAT1 shRNA or MALAT1 shRNA plus anti-miR-206. Silencing endogenous MALAT1 apparently induced miR-206 expressions and suppressed GLS expressions (Fig. 7A, 7B). In addition, inhibition of miR-206 in the MALAT1 silenced cells recovered the GLS protein levels (Fig. 7A, 7B), suggesting MALAT1 upregulated GLS through directly sponging miR-206

in GC cells. As we expectedly, inhibiting miR-206 in MALAT1 silenced cells apparently restored glutamine uptake and GLS activity (Fig. 7C, 7D). Taken together, these results demonstrated the MALAT1-promoted glutamine metabolism was through the miR-206-GLS axis.

Targeting the MALAT1-miR206 axis enhances the anti-cancer effects of mild hyperthermia treatments

The above results illustrated a MALAT1-mediated glucose metabolism under hyperthermic conditions. A rapidly growing area of research in hyperthermia revealed its clinical applications for anti-cancer treatment. Therefore, we assessed the clinical relevance of the MALAT1-miR-206 axis in the hyperthermia-induced cancer cell death. GCs were transfected with control shRNA, MALAT1 shRNA or MALAT1 shRNA plus miR-206 inhibitor, followed by exposing to 37^{o}C, 41^{o}C and 43^{o}C for a short time (30 min). In the control group, cells were slightly affected by short time hyperthermia treatments. However, cells with endogenous MALAT1 silencing showed synergistically inhibitory effects under hyperthermia conditions (Fig. 8A). Expectedly, these cells with further miR-206 inhibition exhibited rescued cell viability under mild hyperthermia (Fig. 8A). Consistent results from Caspase-3 activity assay showed the Caspase-3 was significantly activated in MALAT1 silenced GC cell under 41^{o}C and 43^{o}C and such phenomenon was overridden by miR-206 inhibition (Fig. 8B). To examine whether miR-206, the downstream effector of MALAT1 could directly be response to the hyperthermia-induced cell apoptosis, GCs were transfected with control, miR-206 or miR-206 plus GLS, followed by exposing to 37^{o}C, 41^{o}C and 43^{o}C for a short time (30 min). Similar to the above results (Fig. 8A), cells from the control group showed slightly decreased viabilities by short time hyperthermia treatments. Moreover, cells with exogenous miR-206 overexpression showed synergistically inhibitory effects under hyperthermia conditions (Fig. 8C). Restoration of GLS in miR-206 overexpressing cells rescued the inhibitory effects by mild hyperthermia (Fig. 8C). Consistently, we observed Caspase-3 was significantly activated in miR-206 overexpressed GC cell under 41^{o}C and 43^{o}C and such activation was contradicted by GLS restoration (Fig. 8D), suggesting the MALAT1-miR-206 mediated hyperthermia sensitivity in a Caspase-dependent manner. In summary, these results demonstrated effective approaches from the MALAT1-miR-206 axis for enhancing the mild hyperthermia-induced cancer death under a short time.

Discussion

The long non-coding RNA MALAT1 has been demonstrated to regulate diverse cancer processes, including chemo- and radio- sensitivities(11, 12). As a potential oncogenic lncRNA, MALAT1 was known to be upregulated in multiple cancers(13–15). This study reports the upregulation of MALAT1 in GC tissues and cells. Moreover, Kaplan-Meier Plotter analysis illustrated that GC patients with higher MALAT1 expressions associated with lower survival rate, validating a positive correlation between MALAT1 and GC progresses. Meanwhile, we showed miR-206, which was significantly downregulated in GC tissues and cells, was positively correlated with survival rate of GC patients.

Accumulating studies revealed that lncRNAs function as competing endogenous RNAs of miRNAs to suppress target miRNA expressions, resulting in de-repression of the targets of miRNAs(20). Here we demonstrated MALAT1 directly bond to seeding region of miR-206, which targeted GLS mRNA to subsequently promote the glutamine metabolism of GC cells. Rescue experiments verified that the miR-206-inhibited glutamine metabolism was through direct targeting GLS. These results are consistent with previous functional study of MALAT1 and miR-206 in GCs and present MALAT1-miR-206-GLS as a therapeutic target against GC.

It was widely known that glucose metabolism was the central focus on the study of cancer metabolism due to Otto Warburg's pioneering work on glycolysis(29). Glutamine, as a conditionally essential amino acid, plays important roles under catabolic stressed conditions through dynamic glutamine consumption(23, 24, 28). Recently, the roles of glutamine in cancer progressions were appreciated due to important energy-generating and biosynthetic functions regulated by glutamine metabolism. Moreover, studies demonstrated that inhibiting glutaminolysis of cancer cell has the therapeutic potential to effectively target cancer cells(23, 24). We report here under short and mild hyperthermia treatment, the MALAT1 expression and glutamine metabolism were induced since high temperature will stimulate an increased cellular metabolism rate. However, under short (30 min) and mild hyperthermia, we did not detect the cell death, suggesting an adaptive response occurred in GC cells. Consequently, under longer hyperthermia exposure (1–4 hours), cancer cells displayed apparently apoptosis. These results suggest intense heating of GC cells effectively induced apoptosis. However, our study also focused on limiting the overheating on local normal tissues to improve the efficacy of hyperthermia application.

The hyperthermia approach against cancer cells has been controversial since high temperature burns the malignant cells but also damages the healthy cell due to missing the selectivity. Although the heated tumor is forced to elevate cellular metabolism in the short period of hyperthermia as an adaptive process, when the intensive heating lasted longer, tumor will quickly deflate from nutrients, impair essential cellular processes, and undergo apoptosis(9). Importantly, we found a MALAT1-miR-206-GLS axis which is response to the hyperthermia-promoted cell adaptation and death. Our data clearly demonstrated that either inhibition of the hyperthermia-induced MALAT1 or overexpression of miR-206 during the early (30 min) mild hyperthermia (41 ^{o}C) treatment could significantly induce GC cells.

Conclusions

In summary, we proposed a new strategy for enhancing the selectivity of hyperthermia treatments specific to GC cells by targeting the MALAT1-miR-206-glutamine metabolism axis. This study will contribute to the development of new chemotherapeutic agents that enhance hyperthermia cancer treatment.

Abbreviations

Metastasis-associated lung adenocarcinoma transcript 1 MALAT1

Glutaminase	GLS
Gastric cancer	GC
Long non-coding RNAs	lncRNAs
Competing endogenous RNAs	ceRNA

Declarations

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Consent for publication: All the authors in this paper consent to publication of the work.

Authors' contributions:

Hui-juan Shi and Kejun Li designed all experiments. Kejun Li, Gaojie Liu and Jinxin Feng carried out the experiments. Hui-juan Shi & Xiangliang Zhang analyzed and interpreted the results. Yanlin Feng was responsible to statistical analysis. Xiangliang Zhang wrote and edited the manuscript.

Conflict of Interest

The Authors declare that they have no conflicts of interest.

Availability of data and materials: Not applicable

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Figures

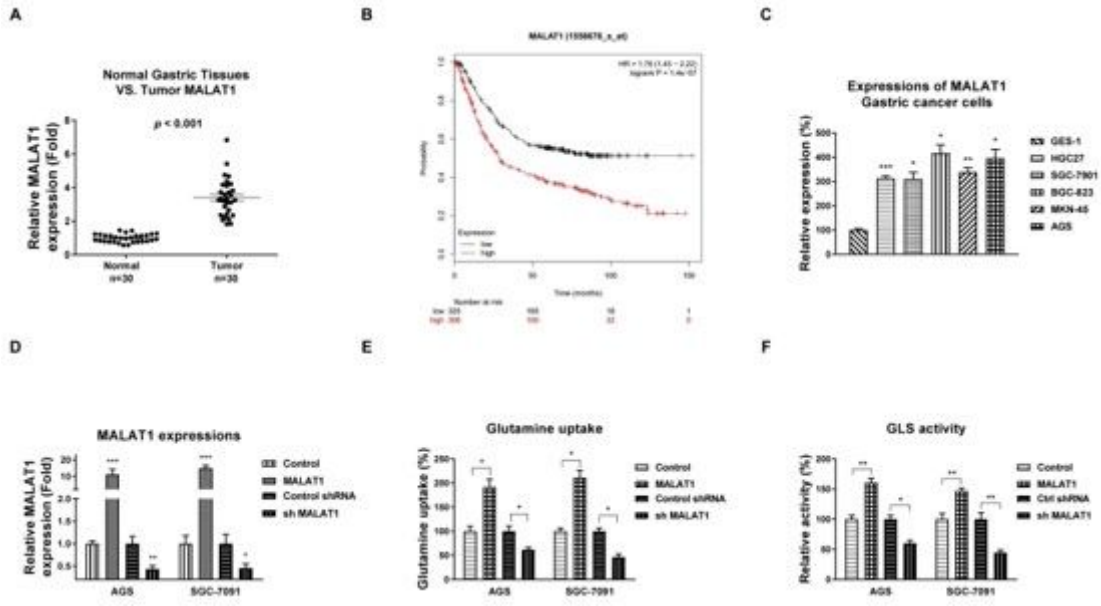

Figure 2

MALAT1 expression was positively associated with gastric cancer patient prognosis. (A) Expressions of MALAT1 in 30 pairs of gastric cancer tissues and paired normal gastric tissues were detected by qRT-PCR. (B) Kaplan-Meier Plotter analysis indicated that gastric cancer patients with higher MALAT1 expressions associated with lower survival rate. (C) Expressions of MALAT1 in normal gastric epithelial cell, GES-1 and five gastric cancer cell lines were detected by qRT-PCR. (D) AGS and SGC-7091 cells were transfected with control, MALAT1 overexpression plasmid or control shRNA, MALAT1 shRNA for 48 hours, the expressions of MALAT1 were detected by qRT-PCR. (E) The above transfected cells were subjected to measurements of glutamine uptake and (F) GLS activity. Bars and error Bars represent means±SD of three independent experiments.*, p<0.05; **, p<0.01; ***, p<0.001.

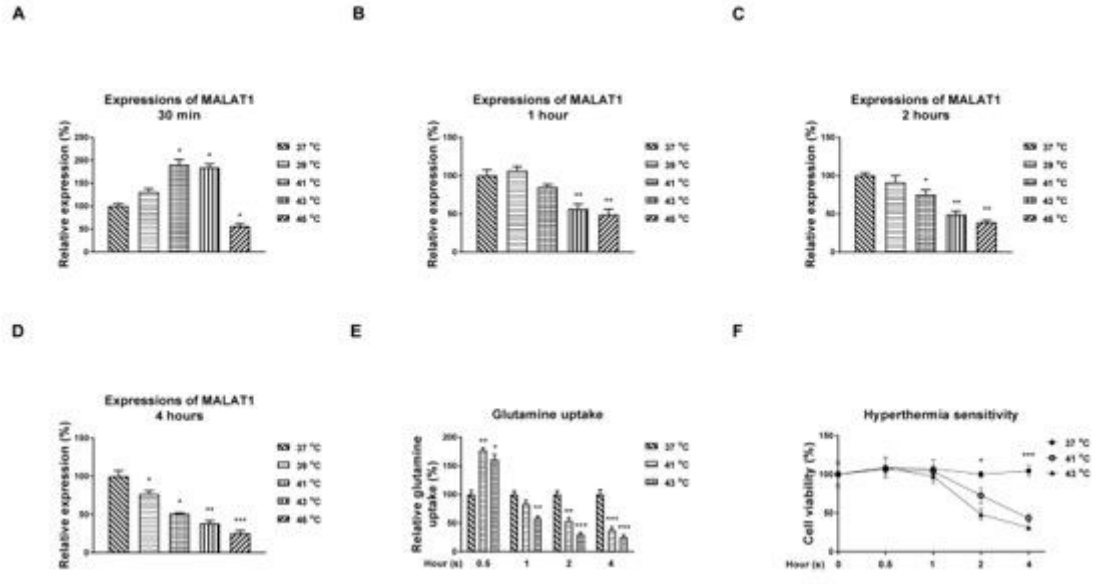

Figure 4

Hyperthermia exposure regulates MALAT1 expressions and promotes cell death in gastric cancer cells. (A) Detection of MALAT1 expressions by qRT-PCR following normal (37oC) or hyperthermia exposure (39oC, 41oC, 43oC and 45oC) for 30 min, (B) 1 hour, (C) 2 hours, and (D) 4 hours. (E) AGS cells were treated with normal (37oC) or hyperthermia exposure (39oC, 41oC, 43oC and 45oC) for 0.5, 1, 2, or 4 hours. The glutamine uptake and (D) Cell viability were measured. Bars and error Bars represent means ± SD of three independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001.

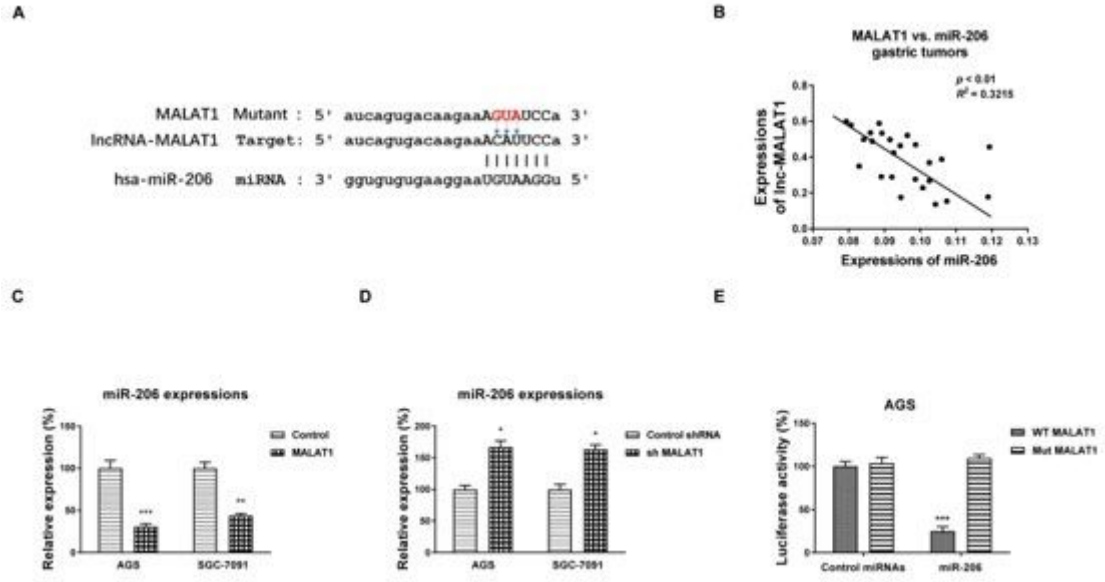

Figure 6

MALAT1 inhibits miR-206 through sponging it. (A) Prediction of binding sequence of MALAT1 on miR-206 from starBase. (B) Negative correlation between miR-206 and MALAT1 was found in gastric cancer tissues. (C) AGS and SGC-7091 cells were transfected with control or MALAT1 overexpression plasmid for 48 hours, expressions of miR-206 were detected by qRT-PCR. (D) AGS and SGC-7091 cells were transfected with control shRNA or MALAT1 shRNA for 48 hours, expressions of miR-206 were detected by qRT-PCR. (E) Luciferase assay showed miR-206 bond to the wild-type MALAT1 but not binding site mutant MALAT1. Bars and error Bars represent means ± SD of three independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001.

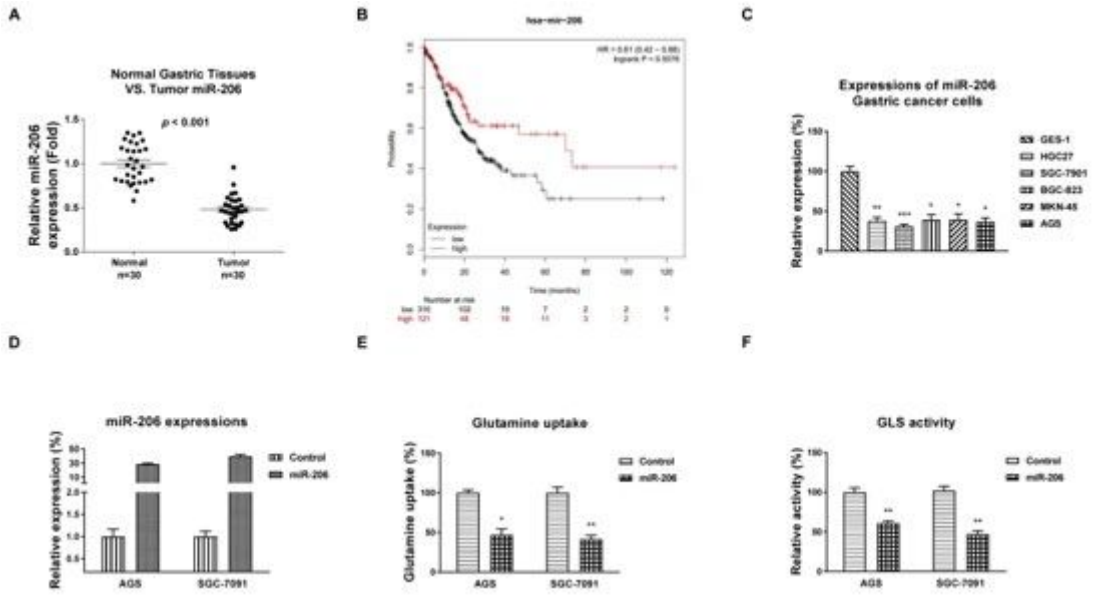

Figure 8

miR-206 negatively correlates with gastric cancers and suppresses glutamine metabolism. (A) Expressions of miR-206 in 30 pairs of gastric cancer tissues and paired normal gastric tissues were detected by qRT-PCR. (B) Kaplan-Meier Plotter analysis indicated that gastric cancer patients with higher miR-206 expressions associated with better survival rate. (C) Expressions of miR-206 in normal gastric epithelial cell, GES-1and five gastric cancer cell lines were detected by qRT-PCR. (D) AGS and SGC-7091 cells were transfected with control miRNAs or miR-206 precursor for 48 hours; the expressions of miR-206 were detected by qRT-PCR. (E) The above transfected cells were subjected to measurements of glutamine uptake and (F) GLS activity. Bars and error Bars represent means ± SD of three independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001.

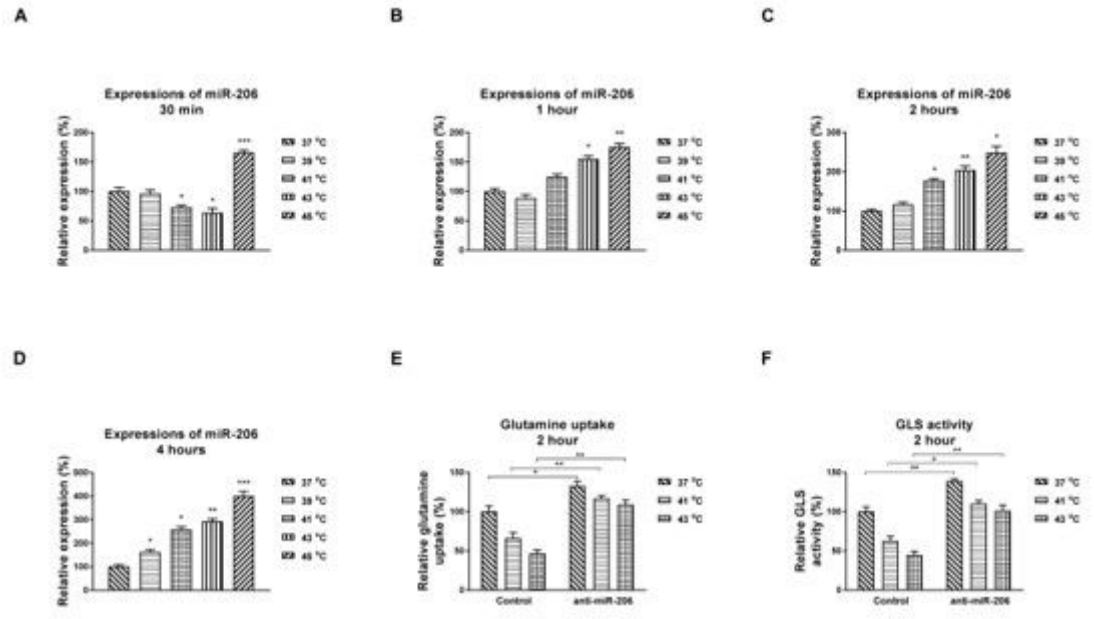

Figure 9

The hyperthermia-suppressed glutamine metabolism is through the induction of miR-206. (A) Detection of miR-206 expressions by qRT-PCR following normal (37oC) or hyperthermia exposure (39oC, 41oC, 43oC and 45oC) for 30 min, (B) 1 hour, (C) 2 hours, and (D) 4 hours. (E) AGS cells were transfected with control antisense or miR-206 antisense for 48 hours, cells were then treated with normal (37oC) or hyperthermia exposure (41oC and 43oC) for 2 hours. The glutamine uptake and (F) GLS activity were measured. Bars and error Bars represent means ± SD of three independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001.

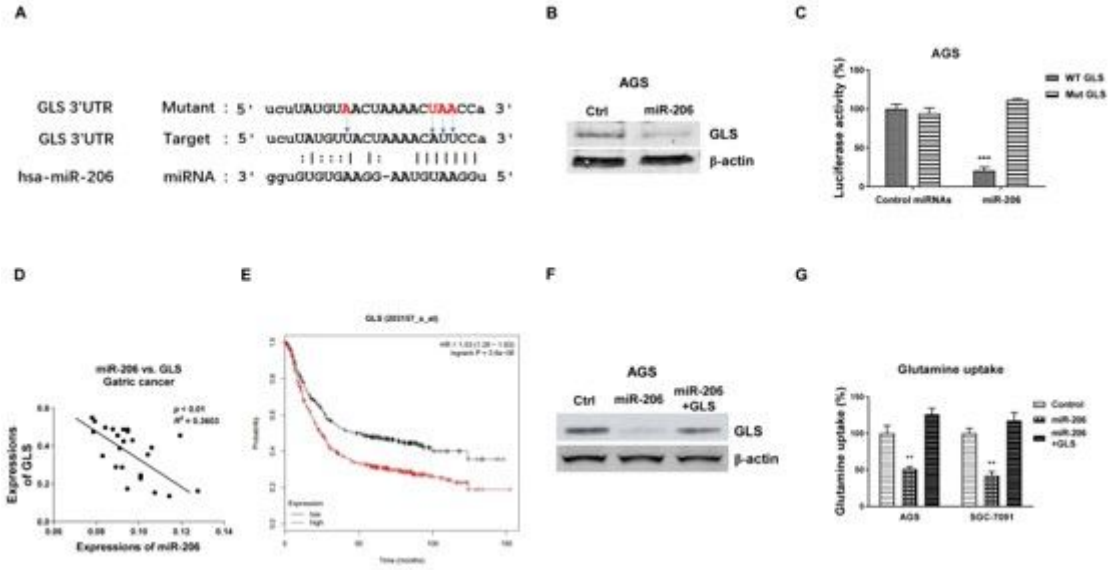

Figure 12

miR-206 directly targets 3'UTR of GLS mRNA. (A) Bioinformatics analysis predicted that 3'UTR of GLS mRNA harbored miR-206 binding sites. (B) AGS cells were transfected with control miRNAs or miR-206 precursor for 48 hours. The protein expressions of GLS were detected by Western blot. β-actin was an internal control. (C) AGS cells were co-transfected with control miRNAs or miR-206 precursor and wild type 3'UTR or binding site mutant 3'UTR of GLS for 48 hours. Luciferase activity was determined. (D) The negative correlation between miR-206 and GLS mRNA expressions was analyzed in gastric cancer tissues. (E) Kaplan-Meier Plotter analysis indicated that gastric cancer patients with higher GLS expressions associated with lower survival rate. (F) AGS cells were transfected with control miRNAs, miR-206 precursor or miR-206 plus GLS overexpression plasmid for 48 hours, the expressions of GLS were detected by Western blot. (G) AGS and SGC-7091 cells were transfected with control miRNAs, miR-206 precursor or miR-206 plus GLS overexpression plasmid for 48 hours, the glutamine uptake was measured. Bars and error Bars represent means ± SD of three independent experiments. **, p<0.01; ***, p<0.001.

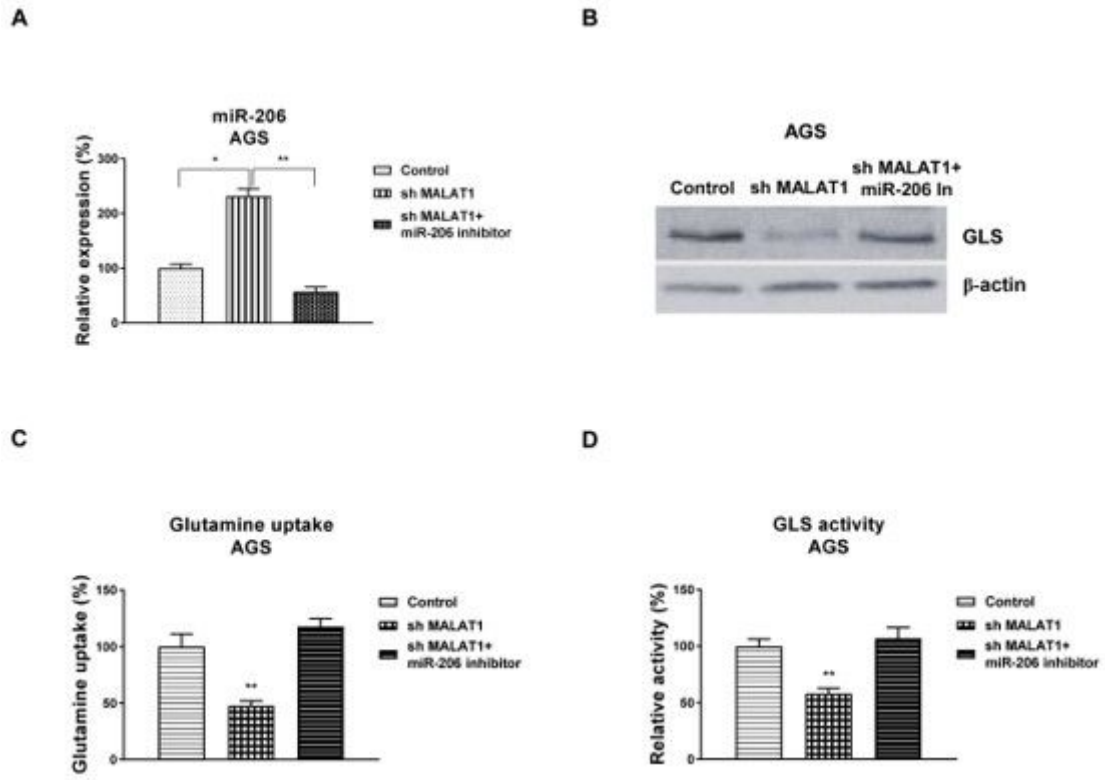

Figure 14

The hyperthermia-suppressed glutamine metabolism is through the MALAT1-miR-206-GLS axis. (A) AGS cells were transfected with control, MALAT1 shRNA or MALAT1 shRNA plus miR-206 inhibitor for 48 hours, the miR-206 expressions and (B) GLS expressions were measured by qRT-PCR and Western blot, respectively. (C) The glutamine uptake and (D) GLS activity from the above cells were measured. Bars and error Bars represent means ± SD of three independent experiments. *, $p<0.05$; **, $p<0.01$.

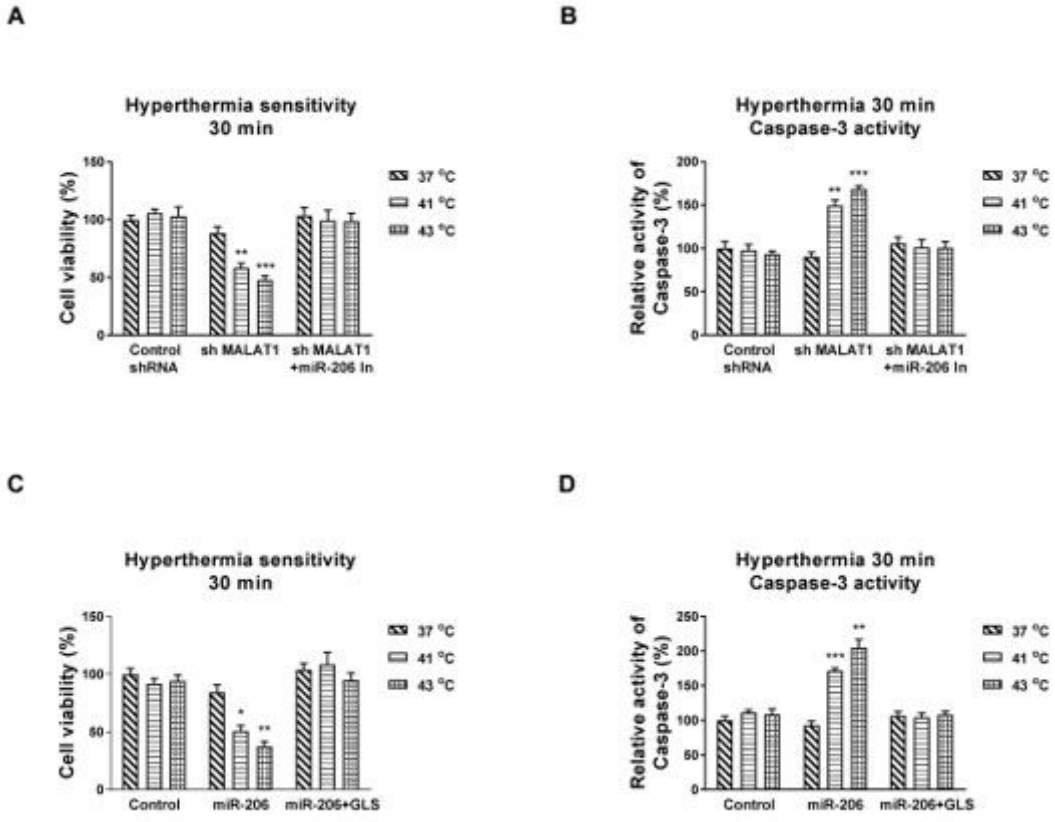

Figure 16

Targeting the MALAT1-miR-206 axis increases the anti-cancer effects of hyperthermia treatments. (A) AGS cells were transfected with control, MALAT1 shRNA or MALAT1 shRNA plus miR-206 antisense for 48 hours, cells were exposed to normal (37oC) or mild hyperthermia (41oC or 43oC) for 30 min, the cell viability and (B) Caspase-3 activity were measured by MTT assay and Caspase-3 activity assay, respectively. (C) AGS cells were transfected with control miRNAs, miR-206 precursor or miR-206 plus GLS overexpression plasmid for 48 hours, cells were exposed to normal (37oC) or hyperthermia exposure (41oC and 43oC) for 30 min, the cell viability and (B) Caspase-3 activity were measured by MTT assay and Caspase-3 activity assay, respectively. Bars and error Bars represent means ± SD of three independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.