ORIGINAL ARTICLE

miR-125a-5p inhibits cancer stem cells phenotype and epithelial to mesenchymal transition in glioblastoma

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SUMMARY

OBJECTIVE: *Glioblastoma (GBM) is a common type of cancer with high mortality. Epithelial to mesenchymal transition (EMT) plays a vital role in the development of glioblastoma. The aim of this study is to evaluate the role of miR-125a-5p in glioblastoma and in the tumorigenesis of chemotherapeutic drug-resistant cancer stem-like cells in brain glioma.*

METHODS: *The role of miR-125a-5p in the regulation of CSCs, EMT, migration, and invasion in glioblastoma was measured in this study.*

RESULTS: *We showed the roles of miR-125a-5p in the regulation of CSCs, EMT, migration, and invasion in glioblastoma. miR-125a-5p can inhibit the CSCs phenotype and EMT in glioblastoma cells. In addition, its over-expression can significantly regulate CSCs-associated genes and EMT-associated gene expression in glioblastoma cells.*

CONCLUSIONS: *We concluded that miR-125a-5p is one of the key microRNAs regulating CSCs and EMT programs in glioblastoma. The results suggested that miR-125a-5p might be a novel therapy target for glioblastoma.*

KEYWORDS: *Glioblastoma. Neoplastic stem cells. MicroRNAs.*

INTRODUCTION

Glioblastoma (GBM) is one of the most common and lethal primary malignant tumors with a median survival of about one year[1-3]. A subgroup of glioblastoma cells, known as stem cell-like cells in GBM were identified and proved to be responsible for the initiation of glioma and the resistance of GBMs to clinical therapies[4,5]. The epithelial-mesenchymal transition (EMT) and its reverse process, named the mesenchymal-epithelial transition (MET), play crucial roles in embryogenesis[6]. For example, the mesoderm generated by EMTs develops into multiple tissue types and, later in development, mesodermal cells generate epithelial organs, such as the kidney and ovary through MET[7]. The EMT of glioblastoma partly contributes to the development of resistance to chemotherapy or radiotherapy[8,9]. Thus, understanding the mechanism underlying cancer stem cells (CSCs) and EMT in glioblastoma for novel drug targets as well as designing new therapeutic strategies should open an opportunity for cancer treatment.

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miRNAs are small (19–25 nucleotides) noncoding, single-stranded RNAs that control gene expression by targeting mRNA transcripts and leading to their translational repression or degradation[10,11]. Multiple genes regulated by miRNAs play crucial roles in biological processes of tumor progression, including migration, invasion, EMT, and cancer stem cell[12-14]. Recently, a study indicated that miR-125a-5p could inhibit glioblastoma cell proliferation and promote cell differentiation[15].

In this study, we showed the roles of miR-125a-5p in the regulation of CSCs, EMT, migration, and invasion in glioblastoma. miR-125a-5p can inhibit CSCs phenotype and EMT in glioblastoma cells. In addition, its over-expression can significantly regulate CSC–associated genes and EMT-associated gene expression in glioblastoma cells. Thus, we concluded that miR-125a-5p is one of the key microRNAs regulating CSCs and EMT programs in glioblastoma. The results indicate that miR-125a-5p might be a novel target for glioblastoma therapy.

METHODS

Glioblastoma Cell Line

Human glioblastoma U87MG cells were purchased from the MD Anderson Cancer Center (Houston, TX, USA). Complete medium (RPMI 1640 supplement with 10% FCS, Gibco, Grand Island, NY, USA) was used for cell culture, and the cells were maintained in a humidified atmosphere containing 5% CO_2 at 37ºC.

Pre-miR-125a-5p/control miR and Transfection

Pre-miR-125a-5p and control-miR were purchased from Ambion (Austin, TX, USA). A final concentration of 50 nM of pre-miR-125a-5p and its respective negative control (control-miR) were used for each transfection. For transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluence for 24h and then transfected with the transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 6h, the medium was removed and replaced with the normal culture medium for 48h, unless otherwise specified.

Real-time PCR for miRNA

The total RNA from the cultured cells, with efficient recovery of small RNAs, was isolated using the mirVanamiRNA Isolation Kit (Ambion, Austin, TX, USA). Detection of the mature form of miRNAs was performed using the mirVanaqRT–PCR miRNA Detection Kit, according to the manufacturer's instructions (Ambion, Austin, TX, USA). The U6 small nuclear RNA was used as an internal control.

Sphere Growth

Cells (10^3/ml) in serum-free RPMI1640/1mM Na-pyruvate were seeded on 0.5% agar precoated, 6-well plates. After the first week, half the medium was replaced every third day. Single spheres were picked and counted.

Western Blot Analysis

Western blot analysis was performed as described before. Mainly, after incubation with primary antibody anti-CD133 (1:500; Abcam, Cambridge, MA, USA), anti-body anti-OCT-4 (1:500; Abcam, Cambridge, MA, USA), anti-Nanog (1:500; Abcam, Cambridge, MA, USA), anti-E-Cadherin (1:500; Abcam, Cambridge, MA, USA), anti-Vimentin (1:500; Abcam, Cambridge, MA, USA), anti-MMP2 (1:500; Abcam, Cambridge, MA, USA), anti-MMP6 (1:500; Abcam, Cambridge, MA, USA) and anti-β-actin (1:500; Abcam, Cambridge, MA, USA) overnight at 4ºC, IRDyeTM-800 conjugated anti-rabbit secondary antibodies (Li-COR, Biosciences, Lincoln, NE, USA) were used for 30 min at room temperature. The specific proteins were visualized by Odyssey™ Infrared Imaging System (Gene Company, Lincoln, NE, USA).

Reverse transcription-polymerase chain reaction and real-time for mRNA

Total RNA was isolated from cells or tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 µg of total RNA in a 20 µl reverse transcription (RT) system. Real-time PCR for Vimentin, Fibronectin, and E-Cadherin was done with a Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. The housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the RNA loading control. The PCR primer sequences are as follows:

GAPDH:
Forward-5'-ATTCAACGGCACAGTCAAGG-3',
Reverse-5'-GCAGAAGGGGCGGAGATGA-3';

Vimentin:
Forward-5′-GACAATGCGTCTCTGGCACGTCTT-3′,
Reverse-5′-TCCTCCGCCTCCTGCAGGTTCTT-3′;

Fibronectin:
Forward-5'-TTTTGACAACGGGAAGCATTATCAGATAA-3',
Reverse-5-TGATCAAAACATTTCTCAGCTATTGG-3';

E-Cadherin:
Forward-5′-TCA ACG ATC CTG ACC AGC AGT TCG-3′,
Reverse- 5′-GGT GAA CCA TCA TCT GTG GCG ATG-3′.

Immunofluorescence Analyses

For cell immunofluorescence analyses, cells were plated on glass coverslips in six-well plates and transfected as indicated. After 48h of the transfection, coverslips were stained with the mentioned anti-Vimentin. The Alexa Fluor 488 goat anti-rabbit IgG antibody was used as a secondary antibody (Invitrogen, Carlsbad, CA, USA). Coverslips were counterstained with DAPI (Invitrogen-Molecular Probes, Eugene, Oregon, USA) for visualization of the nuclei. Microscopic analysis was performed with a confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany). Fluorescence intensities were measured in a few viewing areas for 300 cells per coverslip and analyzed using ImageJ 1.37v software (http://rsb.info.nih.gov/ij/index.html).

Wound Healing Assay

It was performed as described before[16].

Statistical Analysis

Data are presented as mean±standard deviation. Student's t-test (two-tailed) was used to compare the two groups ($P<0.05$ was considered significant) unless otherwise indicated (x^2 test).

RESULTS

miR-125a-5p is downregulated in glioblastoma

In an attempt to identify miR-125a-5p expression between glioblastoma tissues and adjacent normal tissues, we performed real-time PCR in glioblastoma tissues versus normal tissues. MicroRNA was isolated from 31 pairs of glioblastoma tissues and adjacent normal tissues. We found that miR-125a-5p was significantly decreased in glioblastoma tissues compared with adjacent normal tissues.

miR-125a-5p inhibits the formation of stem cell-like population and regulates CSCs-associated gene expression in glioblastoma cells

To assess the role of miR-125a-5p in glioblastoma, we transfected U87MG cells with pre-miR-125a-5p or control miR, and then real-time PCR was performed. We found that miR-125a-5p was significantly increased in the cells transfected with pre-miR-125a-5p (Figure1A).

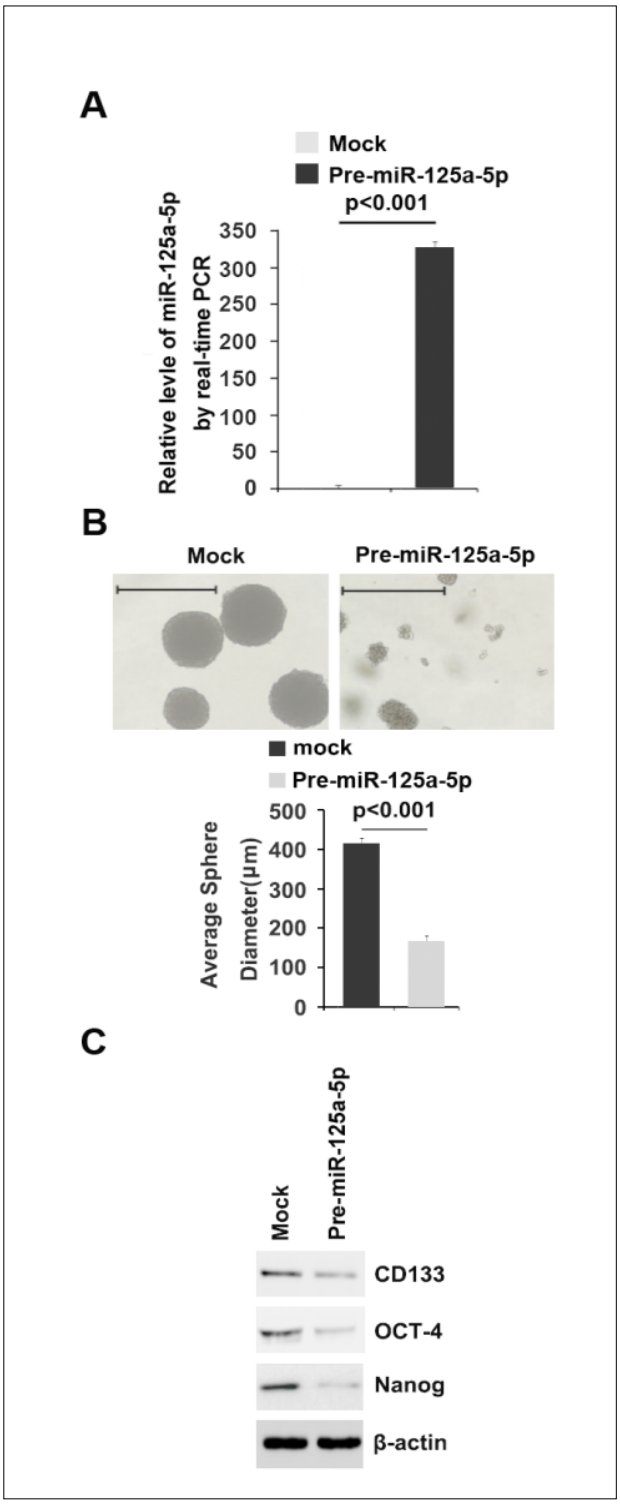
To determine whether miR-125a-5p could affect stem-like cell characteristics, we performed a sphere-forming assay to assess the capacity of CSC or CSC-like cells to self-renewal in this study. We found that the formation of spheres was decreased by miR-125a-5p in U87MG cells (Figure1B). We also performed western blot to detect whether CSCs markers - CD133, OCT-4, and Nanog were affected by miR-125a-5p in the cells. The results showed that CD133, OCT-4, and Nanog protein were significantly decreased by miR-125a-5p in U87MG cells (Figure1C).

miR-125a-5p inhibits epithelial-mesenchymal transition (EMT) in glioblastoma cells

Increased formation of stem cell-like population can result in EMT of cancer cells[17]. Thus, miR-125a-5p could inhibit EMT in glioblastoma cells. To determine whether U87MG cells with stem-like cell characteristics phenotype could have changed the MET, we transfected U87MG cells with pre-miR-125a-5p and then observed that its overexpression caused significant changes in the cell's morphology (MET, mesenchymal to epithelial transition) (Figure2A).

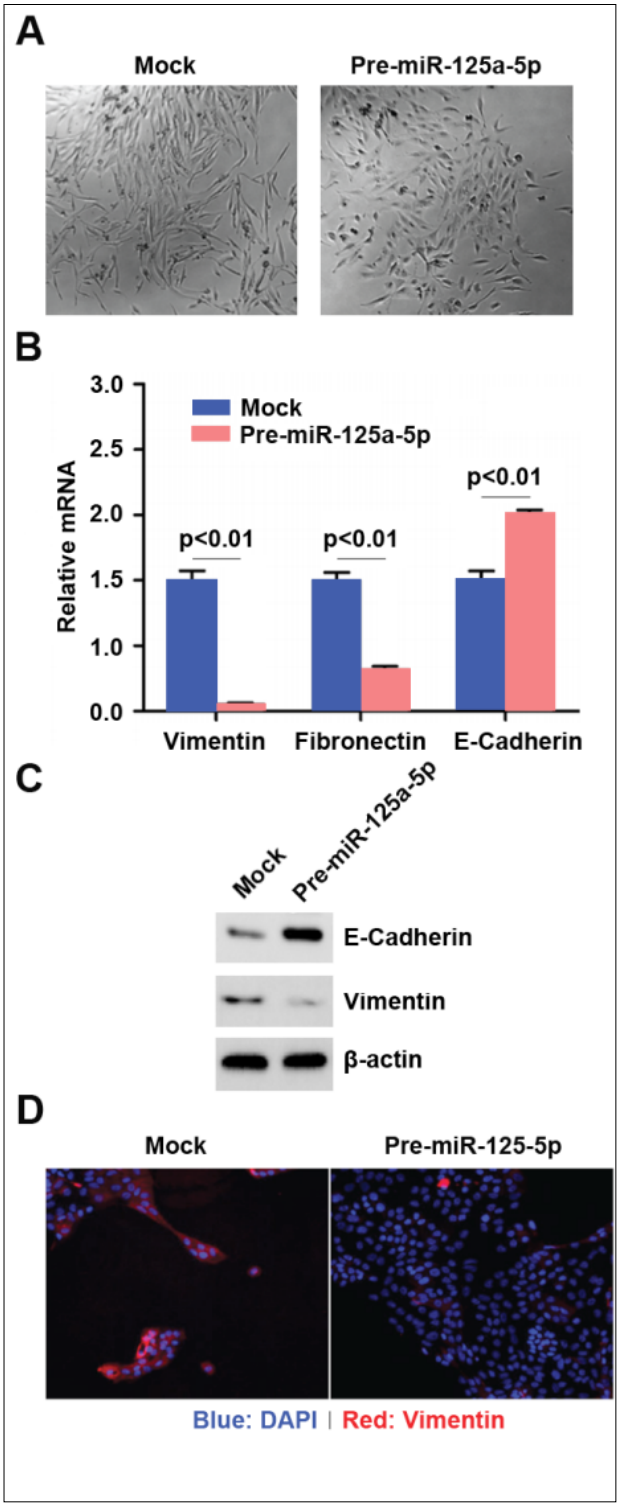
To further verify that the changes in cell morphology were caused by the MET expression levels of epithelial and mesenchymal markers were compared in U87MG cells transfected with pre-miR-125a-5p with the cells transfected with control miR. The results of real-time PCR revealed that the E-cadherin mRNA (epithelial marker) was induced, and Vimentin as well as Fibronectin mRNA (mesenchymal markers) were suppressed by miR-125a-5p in U87MG cells (Figure2B). To further analyze whether miR-125a-5p could affect E-cadherin and Vimentin, we performed western blotting to detect their expression in the cells transfected with pre-miR-125-5p. The results demonstrated that E-cadherin was promoted, and Vimentin was suppressed by miR-125a-5p (Figure2C). In order to further show the effect of miR-125a-5p on Vimentin protein, we performed

FIGURE1. MIR-125A-5P INHIBITS THE FORMATION OF STEM CELL-LIKE POPULATION AND REGULATES CSCS-ASSOCIATED GENES EXPRESSION IN GLIOBLASTOMA CELLS



A. Real-time PCR for miR-125a-5p in U87MG cells transfected with pre-miR-125a-5p or control miR (mock). U6 was a loading control. n=3. **B.** Sphere growth for U87MG cells transfected with pre-miR-125a-5p and control miR (mock). Bars=1000μm. n=3. **C.** Western Blot for CD133, OCT-4, and Nanog in U87MG cells transfected with pre-miR-125a-5p and control miR (mock). β-actin was a loading control. n=3

FIGURE 2. MIR-125A-5P INHIBITS EPITHELIAL-MESENCHYMAL TRANSITION (EMT) IN GLIOBLASTOMA CELLS



A. Images for U87MG cells transfected with pre-miR-125a-5p and control miR (mock). n=3. **B.** Real-time RT–PCR for Vimentin, Fibronectin, and E-cadherin mRNA in U87MG cells transfected with pre-miR-125a-5p or control miR (mock). GAPDH was a loading control. n=3. **C.** Western Blot for E-cadherin and Vimentin in U87MG cells transfected with pre-miR-125a-5p and control miR (mock). β-actin was a loading control. n=3. **D.** Immunofluorescence analyses for Vimentin protein in U87MG cells transfected with pre-miR-125a-5p and control miR(mock). n=3

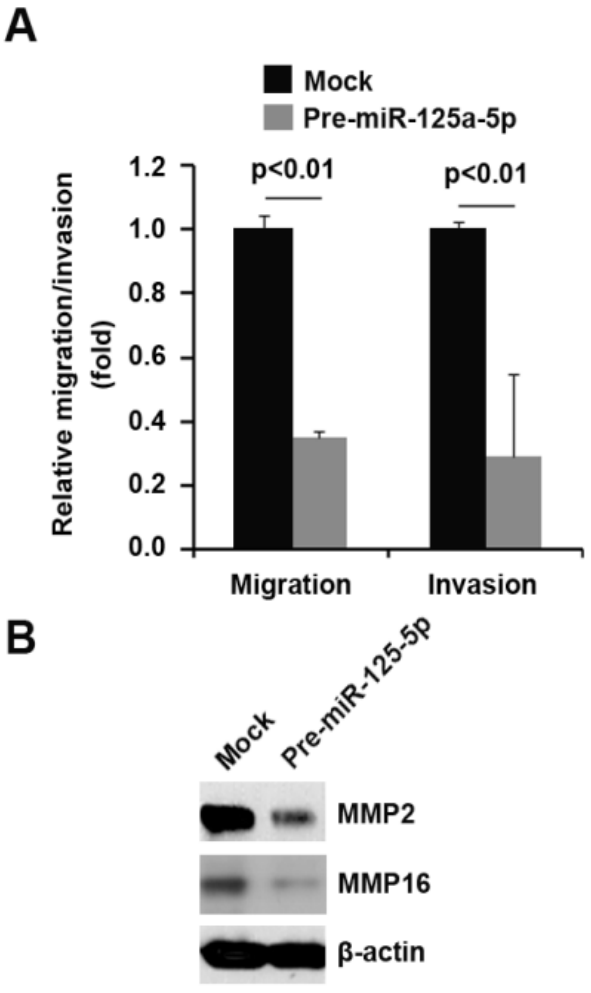
immunofluorescence analysis. Consistent with the results of western blot, we found that Vimentin expression was inhibited in the cells transfected with miR-125a-5p (Figure2D).

miR-125a-5p can suppress migration and invasion as well as attenuate MMP2 and MMP16 protein in U87MG cells

EMT is not only associated with tumor stem-like cell characteristics but also provides those cells with a distinct advantage for migration and invasion[18]. Thus, we reasoned that miR-125a-5p could also affect invasion and migration in U87MG cells. To identify this reason, we performed an invasion and migration assay. We found that miR-125a-5p overexpression could suppress migration and invasion in U87MG cells (Figure3A). In addition, we found that MMP2 and MMP16 protein can be inhibited by miR-125-5p (Figure3B).

DISCUSSION

Evidence has shown that altered patterns of miRNA expression correlate with the progression of glioblastoma[19]. The behavior of miRNAs is complex because they regulate hundreds of targets, resulting in the downregulation of numerous target genes, including oncogenes and tumor suppressors[20]. MicroRNAs play crucial roles in glioblastoma stem cell-like phenotypes and EMT[21]. The dynamic nature of cancer stem cells and EMT that underlie angiogenesis, metastasis, proliferation, migration, and invasion, has profound implications for glioblastoma therapy[22]. Thus, the molecular knowledge of CSCs and EMT in glioblastoma



A. Invasion and migration assays for U87MG cells transfected with pre-miR-125a-5p and control miR (mock). n=3. **B.** Western Blot for MMP2 and MMP16 in U87MG cells transfected with pre-miR-125a-5p and control miR (mock). β-actin was a loading control. n=3

FIGURE 3. MIR-125A-5P CAN SUPPRESS MIGRATION AND INVASION AS WELL AS ATTENUATE MMP2 AND MMP16 PROTEIN IN U87MG CELLS

is important because it will be helpful in the discovery of novel drug targets as well as in the design of new therapeutic strategies for improving the treatment of glioblastoma.

miR-125a-5p has been identified as a tumor suppressor gene in various cancers[15,23]. More recently, it has been reported that miRNA-125a-5p can inhibit glioblastoma cell proliferation and promote cell differentiation[15]. Consistent with these results, we showed that miR-125a-5p was downregulated in glioblastoma tissues. CD133 has been proposed as a stem cell marker, and it can affect clinical outcomes in glioblastoma[24]. More than 50% of Oct4-positive cells expressed the putative CSC markers CD133[25]. NANOG can promote gliomasphere clonogenicity, CD133(+) stem cell behavior, and proliferation[26]. In this study, we found that the overexpression of miR-125a-5p inhibited the formation of stem cell-like population and CD133, OCT-4, and Nanog protein expression. Molecular connections between the EMT program and the stem-cell state are beginning to emerge[27]. We showed that miR-125a-5p also suppressed EMT in glioblastoma cells, indicating that miR-125a-5p is one of many connections between cancer stem cells and EMT in glioblastoma.

CD133-positive tumor cells and EMT confer glioma radioresistance, chemotherapeutic-drug resistance, and it could be the source of tumor recurrence after radiation. Targeting CD133 in cancer stem cells may overcome this radioresistance and chemotherapeutic-drug resistance as well as provide a therapeutic model for malignant brain cancers[28]. Thus, we reason that miR-125a-5p may play an important role in glioma radioresistance and chemotherapeutic-drug resistance.

DISCLOSURE

The authors report no conflicts of interest in this work.

Author Contributions

Conceptualization, Xi-De Zhu; formal analysis, Zhen-Juan Gao; writing — original draft preparation, Xi-De Zhu; writing — review and editing, Xi-De Zhu; supervision, Qi Pang; funding acquisition, Guo-Dong Zheng

RESUMO

OBJETIVO: *O glioblastoma (GBM) é um câncer comum e de alta mortalidade. A transição epitélio-mesênquima (EMT) desempenha um papel vital no desenvolvimento do glioblastoma. O objetivo deste estudo é avaliar o papel do miR-125a-5p no glioblastoma e a tumorigênese de células-troco cancerígenas resistentes a medicamentos quimioterápicos em gliomas cerebrais.*

METODOLOGIA: *Os papéis do miR-125a-5p na regulação de células-tronco cancerígenas, EMT, migração e invasão do glioblastoma foram medidos neste estudo.*

RESULTADOS: *Mostramos a função do miR-125a-5p na regulação das células-tronco cancerígenas, EMT, migração e invasão do glioblastoma. O miR-125a-5p pode inibir o fenótipo e a EMT de células-tronco cancerígenas em células de glioblastoma. Além disso, a sua superexpressão pode regular de forma significante genes associados às células-tronco cancerígenas e a expressão de genes associados à EMT em células de glioblastoma.*

CONCLUSÕES: *Concluímos que o miR-125a-5p é um dos principais microRNAs na regulação de células-tronco cancerígenas e programas de EMT em glioblastomas, e os resultados sugerem que o miR-125a-5p pode ser um novo alvo terapêutico em casos de glioblastoma.*

PALAVRAS-CHAVE: *Glioblastoma. Células-tronco neoplásicas. MicroRNAs.*

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