miR-596-3p suppresses brain metastasis of non-small cell lung cancer by modulating YAP1 and IL-8

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Brain metastasis (BM) frequently occurs in advanced non-small cell lung cancer (NSCLC) and is associated with poor clinical prognosis. Due to the location of metastatic lesions, the surgical resection is limited and the chemotherapy is ineffective because of the existence of the blood brain barrier (BBB). Therefore, it is essential to enhance our understanding about the underlying mechanisms associated with brain metastasis in NSCLC. In the present study, we explored the RNA-Seq data of brain metastasis cells from the GEO database, and extracted RNA collected from primary NSCLC tumors as well as paired brain metastatic lesions followed by microRNA PCR array. Meanwhile, we improved the in vivo model and constructed a cancer stem cell-derived transplantation model of brain metastasis in mice. Our data indicated that the level of miR-596-3p is high in primary NSCLC tumors, but significantly downregulated in the brain metastatic lesion. The prediction target of microRNA suggested that miR-596-3p was considered to modulate two genes essential in the brain invasion process, YAP1 and IL-8 that restrain the invasion of cancer cells and permeability of BBB, respectively. Moreover, in vivo experiments suggested that our model mimics the clinical aspect of NSCLC and improves the success ratio of brain metastasis model. The results demonstrated that miR-596-3p significantly inhibited the capacity of NSCLC cells to metastasize to the brain. Furthermore, these finding elucidated that miR-596-3p exerts a critical role in brain metastasis of NSCLC by modulating the YAP1-IL8 network, and this miRNA axis may provide a potential therapeutic strategy for brain metastasis.

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BACKGROUND

Brain metastases (BM) are a severe clinical issue which tends to occur late in the progression of the primary cancer, and are associated with high morbidity and poor patient prognosis [1, 2]. Lung cancer is the leading cause of cancer-related brain metastases [3]. Non-small cell lung cancer (NSCLC) accounts for 80% of patients with the primary lung cancer and approximately 30%-50% of patients developed brain metastases during the course of their progression [4]. Despite the improvements in the diagnosis and treatment, the median survival for brain metastases patients is about 2–3 and 4–6 months in untreated and treated patients, respectively [5, 6]. Most chemotherapy drugs targeted primary tumors failed to inhibit the occurrence of brain metastatic tumors because of the existence of blood brain barrier (BBB) and the reduced chemosensitivity [7, 8]. Both the ‘seed and soil hypothesis’ and ‘mechanical hypothesis’ have been proposed to the occurrence of brain metastases, but the real underlying mechanisms remain unclear [9, 10].

Increasing evidence was spirited that non protein-coding RNAs are the transcription product for about 60–70% of human DNA [11]. It has been shown that small ncRNAs, microRNAs may act as a survival and prognosis predictor in lung cancer [12]. Studies have suggested that the abnormal expression of microRNAs acts as vital roles in tumor occurrence and tumor development including metastasis [13], and it has been indicated that miR-4270, miR-423-3p, miR-330-3p, miR-423-5p and miR-197 mediate NSCLC brain metastasis [14-17]. Recent works have indicated the capacity to classify primary from secondary brain tumors and microRNA profiles were used to investigate the origin of metastatic lesions in brain [18, 19]. It was found that the formation of brain metastasis lesion from NSCLC was regulated by the altered expression of genes involved in angiogenesis or extracellular matrix invasion [20]. However, tumor metastasis is a complex process and includes of the interactions of tumor cells with the microenvironment in the primary lung tumor tissues and metastatic lesions [9, 20]. Although the critical role of microRNAs in NSCLC has been reported, exploring the underlying molecular mechanism of brain metastasis of NSCLC remains to be elucidated.

In the present study, we extracted RNA from tumor tissues collected from primary NSCLC tumors and paired brain metastatic lesions followed by microRNA profiling, which was closer to the real brain metastasis situation. In addition, our research suggested...
that the miR-596-3p could promote brain metastasis of NSCLC by inducing the expression of two essential genes in the brain metastasis process, YAP1 and IL-8, which together promote the increase in the penetration of tumor cells in the brain and act as a potential therapeutic target for brain metastasis intervention.

**MATERIALS AND METHODS**

**Tissue samples**
The formalin-fixed and paraffin-embedded (FFPE) samples from two groups of patients were studied, including 12 paired tissues (metastatic brain tumors and primary NSCLC samples from the same patient) and 12 primary NSCLC tissues without brain metastasis. All NSCLC patients with brain metastasis only observed the brain metastasis and no metastasis to other organs has been observed. The blood samples were collected from healthy donors (n = 30) and the patients with early stage of NSCLC (n = 30). All FFPE tissues were collected from Harbin Medical University Cancer Hospital between January 2013 to January 2019. All patients provided signed informed consent, and all procedures were performed in accordance with the principles outlined in The Declaration of Helsinki. The study was approved by the Institutional Review Board of Harbin Medical University.

**Generation of brain metastatic sublines PC9-BrM and H1915-BrM**
Brain is the most common site of metastasis from NSCLCs [21]. The NSCLC cell line PC-9 was purchased from Riken Cell Bank (Riken Cell Bank, Tsukuba, Japan) and H1915 was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA), were cultured in RPMI-1640 medium (HyClone, USA) with 10% Fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Sigma, St Louis, MO, USA). We obtained A549, H460 NSCLC cell lines, human brain microvascular endothelial cell (HBMEC) and normal human astrocyte (NHA) from ATCC. All cells were seeded in RPMI-1640 medium (MyCil; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Los Angeles CA, USA) in a 37 °C, 5% CO2 humidified condition.

In this research, we conducted the in vivo selection experiments to extract metastatic cells from the parental PC9 and H1915 human NSCLC cell lines. The cells were widely used to establish the brain metastatic subcell line. We initially inoculated the mice with PC9 and H1915 cells via the left cardiac ventricle. Tumor development was monitored by weekly

**miRNA microarray profiling**
The primary NSCLC samples and brain metastatic lesions of NSCLC patients were collected from FFPE samples and prepared for 10 μm thickness. Total RNAs were isolated from FFPE samples by employing the miRNeasy FFPE kit (cat.no.217504, Qiagen Group, China) according to the manufacturer’s instruction. RT-qPCR was conducted using SYBR®Premix Ex Taq™II (cat. no. RR820B; Takara, Dalian, China) in the ABI PRISM® 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). U6 was used as an internal reference of miRNA. GAPDH was used as an endogenous control of YAP1.

**Reverse Transcription Quantitative PCR (RT-qPCR)**
Total RNA was extracted from tissues/FFPE samples and cultured cells using TRIzol reagent/the miRNeasy FFPE kit (cat.no.217504, Qiagen Group, China) according to the manufacturer’s instruction. RT-qPCR was conducted using SYBR®Premix Ex Taq™II (cat. no. RR820B; Takara, Dalian, China) in the ABI PRISM® 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA).

**Plasmid transfection**
The tumor cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS at 37 °C in a 5% CO2 condition. siRNAs, pcDNA3.1 and miRNA mimics (50 nM) were purchased from Shanghai GeneChem Co., Ltd.. Cells were transfected with using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA) when the cell confluence was about 70–80%, and cultured at 37 °C for another 4 h. The supernatant medium was removed and added with fresh complete DMEM medium, and subsequent experiments were conducted at 24 h post-transfection.

**Dual-luciferase reporter assay**
The luciferase reporter assay was employed to evaluate the potential binding between miR-200a-3p and YAP1. The psiCHECK2 vector (GeneChem, Inc., Shanghai, China) was used to construct a YAP1 3’-untranslated region (UTR)-containing reporter. The 3’-UTR of YAP1, as well as its wild-type (WT) and mutant (MUT) binding sites with miR-596-3p, were amplified and cloned into luciferase reporter vectors. Human 293T cells were cultured in 6-well plates and the miR-596-3p mimics/miRNA mimic/NC (20 μM) were co-transfected with psiCHECK2-YAP1/IL-8 wild type (WT) or mutant (MUT) by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA) (cat. no. 11668030). The supernatant medium was removed at 4 h post-transfection. After 48 h, the activity of Firefly luciferase was detected using a Dual-Luciferase Reporter Assay System (cat. no. RG027; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturers’ protocol. Renilla luciferase was employed as an internal control.

**Cell proliferation assay**
The proliferation of NSCLC cells was performed by using Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Shanghai, China; CCK-8; cat. no. C0037). PC9-BrM cells were cultured in 96-well plates (2 × 103 cells/well) at

| Gene  | Sequence (5‘-3’) |
|-------|-----------------|
| miR-596-3p-F | AAGCCCTGCCCCGTCCTTCT |
| miR-596-3p-R | GCTGTCACAGATACGGCT |
| YAP1-F | AGACCACTATCAGCCAAAGC |
| YAP1-R | CACAGACTCCAGCCAAG |
| GAPDH-F | CACACCCTCTCACCCTT |
| GAPDH-R | CACACCCTGTTGTGCTGA |
| MMP-2-F | CAGGACATTGTGTTCG |
| MMP-2-R | TGAAAGAAGTGACTGATGCA |
| IL-8-F | CTTGCCAGCTCCTGTA |
| IL-8-R | TCTTTAGACCCTCCTTG |

RT, reverse transcription; miR, microRNA; MMP, matrix metalloproteinase; IL-8, interleukin-8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; YAP1, Yes-associated protein 1.
were cultured on the upper (HBMEC) and bottom (NHA) membrane of a chamber were added with medium supplemented with 20% serum. The non-invading cat. no.3495) containing with serum-free medium. The bottom side was ×100 magnification.

Transmigration assays and matrigel invasion assay
For transmigration assay, the HBMEC cell (1 × 10⁵) and NHA cell (1 × 10⁵) were cultured on the upper (HBMEC) and bottom (NHA) membrane of a transwell chamber (Costar; Corning, Inc.; pore size: 3 μm; cat. no. 3495) and grown to (95%) confluency. Brain metastasis cells (5 × 10⁴) were labeled with Cell Tracker Green (Life Technologies, Carlsbad, CA, USA; cat.no. C7025) and were added with serum-free medium in the top inserts. The transfected cells were washed twice with PBS and collected by adding Cell Death and Disease          (2022) 13:699

Western blotting
The transfected cells were washed twice with PBS and collected by adding Thermo scientific RIPA buffer (Pierce, USA; cat.no.89900) with protease inhibitors. The PIERCE BCA protein assay kit was used to measure the protein concentration. The PVDF (Millipore, Billerica, MA, USA) membranes were blocked with 5% BSA at room temperature for 1 h and incubated separately with relative primary antibody. Then they were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1 000; Proteintech Group, Inc., Wuhan, China; cat.no.10494-1-AP) for 1 h. The relative protein expression levels on the PVDF membrane were detected with the ECL system with ChemiDocTM MP Imaging System and analyzed by Image Lab software V3.0 (Bio-Rad Laboratories, Inc.).

Immunofluorescence analysis
Cells were fixed with cold ethanol for 20 min and incubated with 0.1% Triton x-100 for 5 min at room temperature. The slides were washed with PBS and blocked with 5% BSA in PBS. Then cells were added with rabbit anti-human IL-8 (Table 2) and incubated for 1 h at 4°C. After that samples were incubated with Rhodamine (TRITC)-conjugated Goat Anti-Rabbit IgG secondary antibody (1:1 000, Proteintech Group, Inc., China; cat.no. SA00007-2). The nuclei were stained with 4’-6-diamidino-2-phenylindole (DAPI) for 15 min. Fluorescence images were captured by a fluorescent microscope (Nikon Corporation).

Transendothelial electrical resistance (TEER) assay
BBB function of the in vitro BBB model was explored by the TEER assay. The resistance values (Ω) was measured in confluent HBMEC monolayers by employing an EVOM™ Epithelial Voltameter (World Precision Instruments, Sarasota, FL, USA). The HBMEC cells (5 × 10⁴) and NHA cells (5 × 10⁴) were placed in both membrane of the upper insert and grown to confluency. Conditioned medium (CM) from PC9-BrM and PC9-BrM-miR-596-3p were seeded into the upper insert and TEER was detected every 12 h.

ELISA assay
ELISA assay was performed to measure the level of MMP-2 in PC9-BrM cells by human MMP-2 pre-coated ELISA kit (Beijing Solarbio Science & Technology Co., Ltd., China; cat.no.SEKH-0253) in accordance with the manufacturer’s instruction. Data was measured using Tecan microplate reader (Infinite F50; Tecan Group, Ltd., USA).

Cytokine array
Cytokine array in PC9-BrM transfected with miR-596-3p conditioned medium (CM) was conducted using Cytokine Antibody Array Kit 1 kit from Abcam (Abcam, Cambridge, United Kingdom; cat.no. Ab133998). After blocking the array membranes for 30 min, the membranes were incubated with 1 mL of CM at room temperature for 1.5 h. Then the membrane was washed with buffer, and fixed with primary biotin-conjugated antibody to the membrane and incubated for another 2 h. Then the horseradish peroxidase-conjugated streptavidin was fixed with the membrane, we added the detection buffer on the membrane, employing Image Lab software V3.0 (Bio-Rad Laboratories, Inc., USA). The data was measured by the Image J software.

Sphere formation assay
Brain metastatic PC9-BrM cells were cultured in DMEM medium supplemented with 2% B27 supplement, 0.04% bovine serum albumin (BSA), 20 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF) (Cat.no.P5453and P5552; Beyotime Institute of Biotechnology, Shanghai, China). Cells were then added in 96-well low attachment plates (Corning Life Sciences, Bedford, MA, USA; cat.no.CLS3474). The tumor spheres may be observed under the microscope in a week. For passage culture, PC9-BrM cells were added in low attachment 10 cm dish.

Cancer stem cells isolation
MACS method was employed to collect cancer stem cells. Then, 5 × 10⁵ cells were fixed in 1 ml MACS buffer and added with DNase for 10 min at 37°C. Cells were then suspended in 100 ml of MACS buffer and subsequently incubated with biotin-conjugated anti-CD24, APC-conjugated antiCD44 and biotin-conjugated anti-ESA antibodies followed by incubation with anti-biotin antibody and anti-APC antibodies at room temperature for 10 min. CD24, CD44⁺ and ESA⁻ fractions were collected by serial passages through magnetic columns according to the manufacturer’s protocol.

Animal experiments
To establish the brain metastasis NSCLC cell line, female BALB/c nude mice (4 weeks old) were purchased from Vital River Laboratory Animal Technology (Beijing, China). The mice were placed in an anesthesia induction box and anesthetized with isoflurane (induced concentration, 3–4%) for 2–3 min and maintained anesthetized using at 1-1.5% isoflurane. The nude mice were inoculated with 5 × 10⁶ luciferase-labeled PC9/H1915 cells in PBS (100 µL) into left cardiac ventricle. After 65-75 days, the brain metastasis of cancer cells was examined by Bruker In Vivo system. Then all the mice were sacrificed using CO₂ (30% volume displacement per min) and the brain were removed in sterile conditions. Subsequently the brain was minced and cultured in RPMI-1640 medium. This selection was performed twice.
To obtain a highly and specific brain metastatic mice model, we developed a cancer stem cell (CSCs) derived brain metastasis model in mice. Then the nude mice ($n=10$) were injected with luciferase-labeled PC9-BrM cell or PC9-BrM/CSCs in PBS ($5 \times 10^5$, 100 µL) into left cardiac ventricle. The mice were placed in an anesthesia induction box and anesthetized with isoflurane (induced concentration, 3–4%) for 2–3 min and maintained anesthetized using at 1–1.5% isoflurane. The PC9-BrM/CSCs cells (luciferase-labeled) were fixed in 0.1 mL BD Growth Factor Reduced Matrigel (Becton Dickinson Company, Tokyo, Japan; cat.no.354230) with DMEM medium (5:3). Firefly luciferase-labeled cells were generated by lentivirus infection (GeneChem, Shanghai, China).

The brain metastasis development was detected and the bioluminescence imaging (photons/s/cm²/sr) was measured using Bruker In-Vivo FX PRO Imaging System (Bruker corporation, Germany). After 50 days or present the signs of morbidity, all mice were sacrificed using CO₂ (30% volume displacement per min) and the brain were removed and fixed in 4% paraformaldehyde. The histologically analysis was conducted by H-E staining. Approximately 100 sections (10 mm) were prepared and stained with hematoxylin and eosin. All applicable international, national and/or institutional guidelines or the care and use of animals were followed. These procedures were performed in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of Harbin Medical University.

**Statistical analysis**

All data were presented as mean ± standard error of the mean of at least three independent experiments. Statistical analysis was analyzed using SPSS 20.0 (IBM Corp.). Comparisons between groups were examined by One-way ANOVA with post hoc Tukey’s test or Student’s t-test. The overall survival was assessed with Kaplan–Meier plots and log-rank test using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was regarded as statistically significant.

**RESULTS**

Establishment of brain metastasis NSCLC cell lines

To investigate the effect of miR-596-3p on the tumor metastasis process, we employed PC9 cell, a NSCLC cell line isolated from lymph node-derived human lung adenocarcinoma cell lines [24] and the H1915 cells, which was probably derived from a lung cancer patient with brain metastasis according to the description in the ATCC [25] to develop a new brain high-metastatic cell line. The PC9 and H1915 cells were firstly inoculated into BALB/c nude mice by intracardiac injections to isolate sub-populations of cells. The selection process was conducted twice, and we collected the cell lines as PC9-BrM and H1915-BrM. The brain metastasized cancer cells were explored by in vivo bioluminescence imaging system (Bruker In Vivo). The bioluminescence image of mice with the PC9-BrM and H1915-BrM cell brain metastasis. The protein level of EMT-related biomarkers (E-cadherin and Vimentin) was measured between parental cell and brain metastasis cells. The protein level of EMT-related biomarkers (E-cadherin and Vimentin) was examined by the western blot assay. The invasive ability of PC9/H1915 and PC9-BrM/H1915-BrM cells were validated by a Transwell assay.
yielding brain metastatic derivative cell populations (PC9-BrM and H1915-BrM), which displayed an increased capacity of brain metastatic compared with the primary cell line [26]. We identified that PC9-BrM and H1915-BrM cells presented morphological characters of the epithelial-mesenchymal transition (EMT) such as elongated and fibroblast-like morphology (Fig. 1C). We then detected the expression of EMT-related biomarkers, the results indicated that PC9-BrM and H1915-BrM cells undergo EMT process (Fig. 1D). Subsequently, we investigated the invasive capacity of PC9-BrM and H1915-BrM cells and the results suggested a higher invasiveness compared with parental NSCLC cells than parental PPC9 and H1915 cells (Fig. 1E). These results revealed that PC9-BrM and H1915-BrM cells presented mesenchymal features and significantly elevated the invasiveness.

The expression of miR-596-3p is decreased in brain metastatic lesions of NSCLC cancer patients

In order to investigate the key microRNAs that are differentially expressed in the progression process of brain metastasis, we collected the microRNA data in NSCLC cell line (PC-9), Brain metastatic derivative cells (PC-9 BrM) and PC-9 parental cell by extracting the expression profiling data from the Gene Expression Omnibus from the GEO database (GSE83132). The edge R package of R software was used to determine differentially expressed miRNA with significant differences (fold change > 2 and false discovery rate (FDR) < 0.01) based on the Benjamini–Hochberg method [27]. It turned out that a series of miRNAs was altered in PC-9 BrM compared with that in PC-9 parental cells and top 20 differentially expressed miRNAs were displayed (Fig. 2A, B).

In this study, we also extracted the total RNA from the clinical specimens of primary tumors and paired metastatic tumors in the brain, followed by the Affymatrix GeneChip miRNA 1.0 Array. These data showed that the expression of 10 miRNAs were significantly increased while ten miRNAs were decreased in brain metastatic lesions (Fig. 2C, fold change (FC) > 2 and false discovery rate (FDR) < 0.01). By overlapping these data, we found that miR-596-3p was significantly downregulated in the metastatic brain tumors and brain metastasis cells compared to the primary NSCLC tissues/cells. To confirm this observation, we subsequently explored the primary tumors from NSCLC patients with or without brain metastasis and also in tumor from brain metastatic lesions. As shown in Fig. 2D, the expression of miR-596-3p was significantly reduced in the brain metastatic lesions, indicating a potential role of miR-596-3p in brain metastasis.

Integrative analysis for the identification of genes involved in NSCLC

Given the lack of early diagnosis and effective therapies of NSCLC at the early stages, it is vital to explore the underlying mechanism. Hence, we designed a comprehensive, cross-platform integrated analysis of NSCLC. We examined the level of miR-596-3p from the blood of healthy donors and early stage NSCLC patients. Notably, a significantly higher level of miR-596-3p was determined in the circulation of healthy donors (n = 30), the patients with early stage of NSCLC (n = 30) and brain metastasis patients (n = 30) (Fig. 3A).

In addition, we investigated the level of miR-596-3p in PC9, H1915, A549, H460, PC9-BrM and H1915-BrM cells. The latter two cell lines were generated from PC9 and H1915 cells through multiple rounds of in vivo selections of clones that specifically metastasized to the brain [26]. We detected that the expression of miR-596-3p was significantly decreased in PC9-BrM and H1915-BrM cells compared to the normal lung cell line (Beas-2B) and parental cells, indicating that miR-596-3p is associated with in the progression of brain metastasis (Fig. 3B).
The miR-596-3p was subjected to GO analyses to identify main functions, and the data were collected from GEO database (https://www.ncbi.nlm.nih.gov/geo/). GO analysis showed that miR-596-3p was related to the development of NSCLC and may affect cell migration and invasion (Fig. 3C). KEGG pathway analysis demonstrated that signaling pathways involved in ECM receptor interaction, cell cycle, Ribosome pathway and p53 signaling pathway were enriched (Fig. 3D). We also performed GSEA (Gene set enrichment analysis) for the patients with brain metastasis (n = 13) and patients without any distant metastasis (n = 72) by using top 100 most predicted miR-596-3p target genes based on StarBase V2.0 analysis. Notably, the expressions of miR-596-3p target genes were significantly enriched in the patients with brain metastasis, which further confirms our study that the miR-596-3p is involved in the development of brain metastasis by modulating groups of target genes (Fig. 3E). All these results indicated that miR-596-3p genes are highly likely related to the occurrence of cancer.

Genes of similar categories cluster together
In order to investigate the molecular mechanisms of NSCLC, the present study re-analyzed the gene expression profiles of GSE11969. The interactive web tool, GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r) was applied to screen and identify the differentially expressed genes (DEGs) between normal lung tissue and NSCLC samples. The metastasis related genes which were associated with miR-596-3p (top 100 most predicted) in GSE11969 were investigated. Gene expression levels were correlated pairwise for all candidate genes to identify the potentially common regulatory mechanisms based on concordant or discordant expression patterns (Fig. 3F). We observed strong inter-gene correlations among a majority of cytokine/chemokine...
genes (interleukins, ILs) and matrix degrading enzymes (metallo-proteinases). Therefore, the findings of the present study may provide further understanding of NSCLC development and lead to an improved diagnosis of NSCLC.

miR-596-3p suppresses the expression of YAP1 gene

To investigate the role of miR-596-3p in brain metastasis, we detected the potential target genes in four database (Starbase V2.0, TargetScan, miRanda and miRDB) and identified that six genes were overlapping with a criterion of \( P \)-value < 0.01 (Fig. 4A). Then the candidate genes were determined in PC9-BrM cells transfected with miR-596-3p or miR-NC by the qRT-PCR assay. The result showed that YAP1 was significantly reduced in transfected PC9-BrM cells (red dots, Fig. 4B, C and Additional Fig. 1A). The potential binding site of YAP1 3'UTR and miR-596-3p are displayed in Fig. 4D. To determine the effect of miR-596-3p on the expression of YAP1, we increased the level of miR-596-3p in PC9-BrM and H1915-BrM cells. The data indicated that YAP1 was upregulated in PC9-BrM and H1915-BrM cells compared with the parental cells and increased the level of miR-596-3p significantly reduced the YAP1 expression (Fig. 4E). However, decreased of miR-596-3p significantly enhanced YAP1 expression in PC9 and H1915 cells (Fig. 4F).

To further verify these results, we co-transfected miR-596-3p mimics or miR-NC with YAP1-WT or YAP1-MUT in 293 T cell.
Luciferase reporter assays suggested that the reporter activity was decreased after co-transfection of the YAP1-WT and miR-596-3p mimics, while the cells co-transfected with YAP1-MUT and miR-596-3p mimics or miR-NC did not limit the luciferase activity (Fig. 4G). Moreover, we explored the relationship between miR-596-3p and YAP1 in clinical brain metastatic lesions by RT-qPCR assay, and the result showed that miR-596-3p was negatively correlated with YAP1 in brain metastatic samples (Fig. 4H). We also performed meta-analysis of the YAP1 expression by collecting the patients with or without brain metastasis from a combined GEO databases (GSE8894, GSE11969, GSE68465, GSE37745 and GSE13213) and provided evidence that YAP1 was significantly increased in patient with brain metastasis (Fig. 4I). Then YAP1 was subject to GO analyses to identify main functions, and the result showed that YAP1 was related to the process of metastasis and may affect GAP junction and diapedesis (Fig. 4J).

miR-596-3p suppresses tranendothelial cell migration by blocking YAP1-induced MMP-2 expression

To further explore the role of miR-596-3p and YAP1 on NSCLC brain metastasis, we detected the tranendothelial cell migration capacity of brain metastasis cells by using an in vitro blood-brain barrier (BBB) model, which was established by employing the transwell chamber coated with human brain endothelial cells (HBMEC) and normal human astrocytes (NHA) (Fig. 5A). Then we detected that miR-596-3p significantly inhibited the transmigratory capacity of PC9-BrM cells, while increase of YAP1 may restrain the suppressive effect of miR-596-3p (Fig. 5B). In this research, we also measured the effect of YAP1 on the tranendothelial cell migration capacity of PC9 and PC9-BrM cells by using the in vitro BBB model. The result showed that overexpression of YAP1 may promote the transmigratory capacity of PC9-BrM cells than PC9 parental cells (Fig. 5C). Moreover, we conducted the transwell
miR-596-3p suppresses brain metastasis in a cancer stem cell derived brain metastasis model

To identify microRNAs which are specific to metastatic CSCs, we first isolated CSCs population using well established markers, CD24+, CD44+ and ESA+, from NSCLC brain metastasis cells. These cells were explored for their tumor initiating ability by injecting them into mice. The data of our limiting dilution analysis suggested that the isolated CSCs (CD24+/CD44+/ESA+) population has significantly stronger ability of generating tumors compared to non-stem cell population. We then determined the metastatic ability of these cells by implanting CSCs into mice via intracardiac injection.

Subsequently the effect of miR-596-3p in metastatic ability of brain metastatic cells in vivo was examined. To obtain a high success rate of brain metastatic mice model, CSCs were collected from PC9-BrM and they were inoculated into nude mice through intracardiac injection (1 x 10^6, suspended with Matrigel), followed by monitoring brain metastasis with Bruker In-Vivo system for 90 days. To further explore the role of miR-596-3p in the stemness of PC9-BrM/CSCs cells. We investigated the level and the self-renewal ability of miR-596-3p in PC9-BrM/CSCs cells. Then the western blot assays demonstrated that miR-596-3p decreased the level of specific stemness markers, including Bmi-1, Oct-4, Sox-2 and Nanog (Additional Figure-1C).

As shown in Fig. 7A, B, the PC9-BrM/CSCs showed higher rate of brain metastasis and less brain metastasis-free survival time compared with PC9-BrM parental cells. We next examined the effect of miR-596-3p in metastatic ability of CSCs in vivo. CSCs were prepared from PC9-BrM that was transfected with lentivirus carrying with or without miR-596-3p. Then we determined the specificity of miR-596-3p expression in BrM/CSCs and non-stem cell population. The generality of this observation was further confirmed by examining the expression of miR-596-3p in PC9 and H1915 cell lines, and their metastatic variants. Then we identified that the expression of miR-596-3p was significantly reduced in the brain metastatic variants of these cells in a CSCs-specific manner (Additional Fig. 1B).

Bioluminescent (BLI) image right after the CSCs inoculation demonstrated distribution of CSCs cells in the whole body suggesting a successful intracardiac injection. After eight weeks, metastatic tumor observed in the brain was prominent in all mice that received CSCs transfected with miR-NC only. On the other hand, mice that injected of CSCs carrying miR-596-3p displayed significantly less metastatic lesions in the brain, suggesting that miR-596-3p may suppress the brain metastasis capacity of PC9-BrM/CSCs (Fig. 7C, D). H&E staining indicated the lesion of microscopic metastasis in the brains of 6/10 mice receiving injections of PC9-BrM/CSCs-miR-596-3p. Ten sections of each brain were employed to calculate the number of micro-metastasis lesions. Mice transfected with miR-596-3p CSCs showed significant decrease in the formation of micrometastatic lesion (Fig. 7E, F). Representative bioluminescent images showed lung cancer cells preferentially metastasis to the brain and bone. We observed miR-596-3p/CSCs group mice to have decreased brain metastasis (3/10), but increased bone metastasis (7/10) compared to the miR-NC/CSCs group (brain metastasis, 6/10 and bone metastasis, 4/10). These results suggest that miR-596-3p promotes brain metastasis of circulating breast cancer cells.

**DISCUSSION**

Metastatic colonization of the brain is the most common and detrimental stage for the lifetime prevalence approaching 25% in NSCLC patients [1]. Brain metastasis may cause significant neurologic diseases, cognitive disorder and emotional difficulties [2]. However, the molecular mechanisms underlying the process of brain metastasis remains obscure. The classical hypothesis of “seed and soil” suggests that metastatic tumor cells could develop
microenvironment for their growth and survival through complex interactions. Meanwhile, accumulating evidence implies that epigenetic regulation plays a vital role in tumorigenesis and metastasis [32, 33]. In this research, we evaluated the miRNAs from tumor tissues collected from primary NSCLC tumors and paired brain metastatic lesions followed by microRNA profiling, and the paired tissues study (lung cancer and brain metastasis) was the first time used in the process of brain metastasis.

It has been indicated that tumor cells often metastasize to preferred organs and are partly restricted by microRNAs [34]. In the brain metastasis condition, the decrease of miR-596-3p in brain NSCLC samples indicated that the miRNA may be involved in "brain-seeking" metastatic potential, which may be involved in the progression of BrM from primary NSCLC. This hypothesis was supported by our observation that miR-596-3p is downregulated in both primary lung cancer specimens and brain metastasis samples. Wei et al. demonstrated that miR-330-3p promotes brain metastasis and EMT process in non-small cell lung cancer [15]. Singh et al. have recently shown that blocking the STAT3-miR-21 axis may form a strong notion for a potential therapeutic strategy in patients with lung-to-brain metastasis [35]. Sun et al. [14] have suggested that miR-4270 downregulation and miR-423-3p upregulation was associated with an increased risk of brain metastasis in NSCLC patients and these miRNAs might be useful prognostic and clinical treatment targets. More recently, it was found that miR-95-3p is decreased in brain metastatic tissues compared with lung adenocarcinoma and may represent a critical role in conferring brain metastatic potential to lung cancer cells [36]. However, the biological function of miR-596-3p has not been sufficiently examined. To our knowledge, there is no previous

| Gene     | Fold change |
|----------|-------------|
| IL-8     | 5.7         |
| TGF-β    | 3.2         |
| IFN-γ    | 2.8         |
| TIMP-1   | 2.5         |
| MIF      | 2.4         |
| IL-1β    | 2.1         |
| EGF      | 1.9         |

Fig. 6 miR-596-3p suppresses brain metastasis by targeting IL-8. A Conditioned medium from PC9-BrM cells that were transfected with miR-596-3p/miR-NC were conducted by cytokine array (Abcam) analysis. Fold change (FC) was examined by using Image J software. B PC9-BrM and H1915-BrM cells were transfected with miR-596-3p/miR-NC, and the expression of IL-8 was determined by qRT-PCR assay. C PC9-BrM cell was transfected with miR-596-3p, and the level of IL-8 was detected by immunofluorescence assay. D PC9-BrM cells was treated with miR-596-3p inhibitor, miR-inhibitor NC or miR inhibitor + YAP1, and the level of IL-8 was detected by qRT-PCR. E Analysis of the luciferase activity of 293 T cell co-transfected with miR-596-3p mimics/miR-NC and the luciferase reporter psi-CHECK2-YAP1-WT/MUT. F PC9 parental cells were cultured on the upper side of a transwell chamber by adding with various doses of recombinant IL-8, and the number of invaded cells was calculated after 24 h. G PC9 cells with increased IL-8 were added into the transwell insert, and the number of invaded cells was calculated after 24 h. H HBMEC cells were added with conditioned medium from PC9-BrM, 231BrM+miR-596-3p and recombinant IL-8, the transendothelial electrical resistance was detected by EVOM2 epithelial voltohmmeter.
study about the role of miR-596-3p in lung cancer. These results showed that the microRNA network may exert as a vital role in the metastatic process of lung cancer.

In this study, we explored microRNA profiling on a cohort of 12 pairs of primary samples and brain metastatic tumor tissues from NSCLC patients for the first time in study of NSCLC brain metastasis and investigated the differentially expressed miRNAs in GEO database. By overlapping two set of data, we detected that the miR-596-3p is involved in the formation of brain metastasis by targeting YAP1-induced MMP-2 secretion which regulate the permeability of BBB. Our research further validated that brain metastasis is a highly dynamic multi-stage process and involved a series of key factors which are affected by at least one or few microRNAs. YAP1 acts as an oncogene which promotes the tumor progression by targeting various signaling pathways [37]. Hu et al. detected that overexpression of YAP1 was correlated with ARHGAP29, which may cause cytoskeletal rearrangement and then promote the progression and metastasis in gastric cancer [38]. And overexpression of YAP1 significantly enhanced the brain metastatic ability in less brain metastatic potential NSCLC cell line, indicating that YAP1 may exert the post-transcriptional regulatory in the process of YAP1-mediated brain metastasis. We also noticed that the number of patients included in this study was a significant limitation, but since additional well-annotated samples were not readily available to us. We wish that our study will help other researcher to independently validate our findings in their specimen collections.

Metastasis is a multi-step process which include the intravasation into vessel, survival in the circulation, extravasation and establish colonization in brain parenchyma [39, 40]. In this study, we detected that miR-596-3p may reduce the secretion of IL-8 which lead to a suppression of BBB permeability of cancer cells into the brain. Previous studies have indicated that pro-inflammatory cytokines such as TGF-β and IL-8 may impede the penetration of BBB by limiting the cell-cell adhesion proteins [41, 42]. Then we detected the role of miR-596-3p on IL-8 using the luciferase reporter assay, however the effect is not statistically significant, indicating that the effect is indirect. In this study, we have shown that miR-596-3p mediated IL-8 plays a critical role in brain metastasis of NSCLC cells.

Fig. 7 miR-596-3p suppresses brain metastasis in vivo. A PC9-BrM transfected with miR-596-3p/miR-NC were grown as tumor-spheres in tumor sphere media. A hallmark of cancer stem cells (CSCs), was validated through assessment of secondary sphere-forming capacity. B Kaplan–Meier analysis for brain metastasis-free survival of mice that were injected with PC9-BrM/PC9-BrM-CSCs. C PC9-BrM stem cells transfected with miR-596-3p/miR-NC were intracardially injected into nude mice (n = 8) followed by monitoring tumor growth by detecting the total bioluminescence in the brain. The line chart indicated the tumor BLI value. D Kaplan–Meier analysis was performed for brain metastasis-free survival of mice. E The brain histology image of mice injected with miR-596-3p/miR-NC in PC9-BrM-CSCs. F Evaluation of micrometastatic lesions in mice brain.
How miR-596-3p modulates IL-8 expression is an intriguing question. When we tested the effect of miR-596-3p on TNFα using a 3’UTR reporter plasmid, we did not see any effect of miR-596-3p (Fig. 7E), suggesting that the effect is indirect. We then detected the transcription factors that potentially control IL-8 expression by conducting the human transcription factor ChIP sequence data which was released by the ENCODE project [46, 47]. We found several such factors; however, none of them were potential targets of miR-596-3p based on our Targetscan analysis. Therefore, the underlying mechanism of miR-596-3p modulating IL-8 expression is unknown, which may be involved in the regulatory network of brain metastasis process.

Xenograft models constructed by intracardiac injection of human tumor cells into immunodeficient mice have been regarded as useful tools for the study of metastasis. However, the success rate of xenograft tumor model established by anchorage-dependent cell is not high. Recently, cancer stem-like cells (CSCs) are considered to play a role in metastatic progression of cancer and that these cells may exist in tumor mass even at an early stage. These findings led us to construct a NSCLC/CSCs derived brain metastasis model. In addition, the formation and success rate of brain metastases model is higher than that of anchorage-dependent cell intracardiac injection models. The CSCs derived model may screen the cell population of high proliferation rate and self-renewal, which may better form the brain metastasis model. The model formation cycle and successful rate may also be improved. This brain metastasis model acquired the same character of NSCLC patients and the feature of brain metastatic potential. And the CSCs derived brain metastasis model acquired the same brain metastasis capacity compared with the anchorage-dependent cell model, which was confirmed in this study. Therefore, our cancer stem cell derived model of NSCLC metastasis provided an effective strategy for the study of NSCLC development.

In conclusion, our study suggested that miR-596-3p/YAP1 pathway may provide a promising therapeutic strategy for brain metastasis of NSCLC, which may mitigate brain metastasis development and behavior, leading to an improvement of prognosis for patients with metastatic brain cancer.

**DATA AVAILABILITY**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**REFERENCES**

1. Eichler AF, Loeffler JS. Multidisciplinary management of brain metastases. Oncologist. 2007;12:884–98.
2. Soffetti R, Ruda R, Mutani R. Management of brain metastases. J Neurol. 2002;249:1357–69.
3. Schouten LJ, Rutten J, Huveneers HA, Twijnstra A. Incidence of brain metastases in a cohort of patients with carcinoma of the breast, colon, kidney, and lung and melanoma. Cancer. 2002;94:2698–705.
4. Hanna N, Shepherd FA, Fossa SV, Pereira JR, De Marinis F, von Pawel J, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol. 2002;20:1599–79.
5. Villa S, Weber DC, Morettes C, Manes A, Combescurale C, Jove J, et al. Validation of the new Graded Prognostic Assessment scale for brain metastases: a multicenter prospective study. Radiat Oncol. 2011;6:1–8.
6. Ge M, Zhuang Y, Zhou X, Huang R, Liang X, Zhang Q. High probability and frequency of EGFR mutations in non-small-cell lung cancer with brain metastasis. J Neurooncol. 2011;103:413–8.
7. Steeg PS, Camphausen KA, Smith QR. Brain metastases as preventive and therapeutic targets. Nat Rev Cancer. 2011;11:352–63.
8. Arslan C, Dizdar O, Altundag K. Systemic treatment in breast-cancer patients with brain metastasis. Expert Opin Pharmacother. 2011;12:839–46.
9. Xiao J, Chen L, Mao GS, Gao WY, Lu M, He XJ, et al. Neurorerestorative clinical application standards for the culture and quality control of olfactory ensheathing cells. J Neurorestoratology. 2017;5:175–9.
40. Izraely S, Sagis-Assif O, Klein A, Meshel T, Tsarfaty G, Pasmanik-Chor M, et al. The metastatic microenvironment: brain-residing melanoma metastasis and dormant micrometastasis. Int J Cancer. 2012;131:1071–82.

41. Yu H, Huang X, Ma Y, Gao M, Wang O, Gao T, et al. Interleukin-8 regulates endothelial permeability by down-regulation of tight junction but not dependent on integrins induced focal adhesions. Int J Biol Sci 2013;9:966–79.

42. Ganatra D, Vasavada A, Vidya N, Gajjar D, Rajkumar S. Trichostatin A restores expression of adherens and tight junction proteins during transforming growth factor β-mediated epithelial-to-mesenchymal transition. J Ophthalmic Vis Res. 2018;13:274–83.

43. Wang W, Lv S, Zhou Y, Fu J, Li C, Liu P. Tumor necrosis factor-alpha affects blood-brain barrier permeability in acetaminophen-induced acute liver failure. Eur J Gastroenterol Hepatol. 2011;23:552–8.

44. Yang GY, Gong C, Qin Z, Liu XH, Lorris Betz A. Tumor necrosis factor alpha expression produces increased blood-brain barrier permeability following temporary focal cerebral ischemia in mice. Brain Res Mol Brain Res. 1999;69:135–43.

45. Saija A, Princi P, Lanza M, Scalese M, Aramnejad E, De Sarro A. Systemic cytokine administration can affect blood-brain barrier permeability in the rat. Life Sci. 1995;56:775–84.

46. Gerstein MB, Kundaje A, Hanishar M, Landt SG, Yan KK, Cheng C, et al. Architecture of the human regulatory network derived from ENCODE data. Nature. 2012;489:91–100.

47. Wang XY, Sun N, Meng XQ, Chen M, Jiang CL, Cai JQ. Review of clinical nerve repair strategies for neurorestoration of central nervous system tumor damage. J Neurorestoratology. 2020;8:172–81.

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AUTHOR CONTRIBUTIONS
C. L., H. Z., Y. H., H. J. and J. X. designed and performed the experiments, analyzed and interpreted data, and C. L. and H. L. wrote the manuscript; Y. H., S. A. and Y. W. provided technical and material support, and T. S., H. J. and G. S. acquisition and analyzed data; X. X., T. F. and Y. W. edited the manuscript; P. L. and X. G. designed experiments and critically reviewed the paper.

COMPETING INTERESTS
The authors declare no competing interests.

ETHICS
Animal procedures were approved via the Institutional Animal Care and Use Committee of Harbin Medical University and were processed in accordance with the guideline of Animal Care and Use Committee of the National Institutes of Health.

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