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Microglia increase CEMIP expression and promote brain metastasis in breast cancer through the JAK2/STAT3 signaling pathway

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Abstract

Objectives: Brain metastasis is the most lethal metastatic site for patients with breast cancer, and the incidence of brain metastasis is increasing every year. Microglia act a pivotal part in promoting the proliferation and metastasis of breast cancer cells in the brain. Therefore, understanding the biological process of brain metastasis in breast cancer is important to improve therapeutic outcomes and prolong the survival of patients.

Materials and Methods: The role of microglia on the prognosis of patients with breast cancer with brain metastasis was verified by immunohistochemistry and the Kaplan–Meier curve. Cell experiments *in vitro* were used to analyze the effect of microglia on cell proliferation, migration and invasion. Knockdown of cell migration-inducing hyaluronan-binding protein (CEMIP) expression and co-culture experiments were carried out to study the mechanism of microglia on the progression of brain metastasis of breast cancer.

Results: We found that microglia may shorten the survival time of patients with breast cancer by regulating the expression of CEMIP in brain metastatic tumors. Co-culture experiments *in vitro* indicated that microglia enhance the proliferation, migration, and invasion abilities of brain metastatic breast cancer cells; however, the knockdown of CEMIP expression suppresses this effect. In addition, we also found that CEMIP expression, increased by microglia, activates the JAK2/STAT3 pathway in brain metastatic breast cancer cells, which induces the secretion of CCL2, IL-6, TGF-β, and VEGF. CCL2 recruits microglia to gather around brain metastases, whereas IL-6, TGF-β, and VEGF induce high CEMIP expression, triggering a positive feedback loop between microglia and brain metastatic breast cancer cells.

Conclusions: Our study proposes a possible mechanism of microglia promoting brain metastasis of breast cancer, indicating that both microglia and CEMIP may be valuable therapeutic targets for patients with breast cancer with brain metastasis.

Keywords: breast cancer; brain metastasis; microglia; CEMIP; JAK2/STAT3 signaling

Introduction

According to statistics, more than 90 % of deaths among patients with breast cancer are attributable to distant metastases [1, 2], with brain metastasis being the most lethal [3–5]. Brain metastasis has become the major factor limiting both the expected survival and quality of life of patients with breast cancer. The median survival time for untreated patients with brain metastasis is 1–2 months, which can be extended to 4–6 months with systematic treatments [6–8]. Therefore, understanding the mechanism of breast cancer brain metastasis and developing a promising target for the treatment of patients with brain metastasis remain urgent necessities at this stage.

Cancer cells, or “seeds”, colonize the brain and successfully metastasize, rendering them inseparable from the support of the special brain microenvironment, or “soil” [5, 9, 10]. This microenvironment consists of various intracranial cells and cytokines, among which microglia are specific macrophages of the central nervous system (CNS) [5, 9]. Sensitive to CNS injury, microglia produce a large number of pro-inflammatory cytokines, adhesion molecules, and cytotoxic substances [11]. The activated microglia aggregate at the edge of brain tumor tissue, mediate immunosuppression and angiogenesis and induce the successful colonization of cancer cells [12–14]. Although studies have confirmed that microglia can promote brain metastasis in patients with breast cancer [9, 12], the exact mechanism remains unclear.

Cell migration-inducing hyaluronan-binding protein (CEMIP, also known as KIAA1199) is a cell migration-inducing protein that was first found to be associated with non-syndromic hearing loss [15–19]. Besides, the overexpression of CEMIP has been proven to be relevant to cell proliferation, adhesion, and invasion in various cancer cells [20–22]. Recent

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studies have shown that CEMIP might act as a "booster" for brain metastases of tumors [15, 16, 23]. In breast cancer, the expression of CEMIP in brain metastases has been found to be higher than that in primary tumors [15], potentially owing to the different brain microenvironment. As microglia are the most essential immune effector cells in the brain microenvironment, it is reasonable to explore the relationship between microglia and CEMIP expression in brain metastasis of breast cancer.

Therefore, immunohistochemical staining was performed in this study to examine the correlation between microglia and CEMIP expression. The results showed that the expression of CEMIP may be mediated by the microglia surrounding the brain metastases. More importantly, the co-culture experiments verified that microglia could increase the expression of CEMIP, which, in turn, induced the progression of brain metastasis of breast cancer via the JAK2/STAT3 pathway. Nevertheless, the knockdown of CEMIP expression inhibited both the activation of the JAK2/STAT3 pathway and the promoting effect of microglia on brain metastasis. In conclusion, our research reveals a possible mechanism by which microglia promote the brain metastasis of breast cancer and suggests a novel therapeutic strategy for the clinical treatment of patients with breast cancer with brain metastasis.

Materials and Methods

Reagents and cell culture

Anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody was purchased from Abcam (ab178846, Shanghai, China). Anti-CCL2 antibody was purchased from Boster (PB0646, Wuhan, China), and the CEMIP polyclonal antibody was purchased from Bioworld (BS71849, Minnesota, USA). Antibodies against JAK2 (3230), p-JAK2 (3771), STAT3 (4904), and p-STAT3 (94994) were purchased from Cell Signaling Technology (Danvers, MA, USA). MDA-MB-231Br and SKBrM3 were kindly provided by the laboratory of the Medical College of Southeast University. MDA-MB-231Br and SKBrM3, known for their high metastatic potential to the brain, were derived from the parental MDA-MB-231 and SKBR-3 cell lines [1, 24]. Microglia cells (HMC3) were purchased from Procell (CL-0620, Wuhan, China). All cells were cultured in Dulbecco's modified Eagle's medium (C11995500BT, Gibco, Carlsbad, USA) supplemented with 10 % fetal bovine serum (FBS; FSP500, Excell Bio, Shanghai, China) and 1 % penicillin–streptomycin (PS; P1400, Solarbio, Beijing, China) and incubated at 37 °C and 5 % CO_2.

Experimental protocol for co-culture

A 100-μL suspension of microglial cells, at a concentration of 1×10^5 cells/well, was added to the upper chamber of a 6-well Transwell plate, and 600 μL of culture media with FBS were added to the lower chamber. In another Transwell plate, a 600-μL suspension of brain metastatic cells at a concentration of 2×10^5 cells/well was added to the lower chamber, and the upper chamber received 100 μL of culture media with FBS. After overnight incubation, allowing cells to adhere and grow, the original culture media in all Transwell plates were gently removed and replaced with fresh media. Subsequently, the upper chamber with microglia and the lower chamber with brain metastatic cells were combined for co-culture in a single Transwell chamber for 48 h for further experiments.

CEMIP knockdown by siRNA

CEMIP siRNA (ON-TARGET plus SMARTpool Human KIAA1199 L022291-00) and negative control siRNA (ON-TARGET plus Control siRNA Non-Targeting siRNA #1 D-001810-01-05) were purchased from GE Healthcare (Buckinghamshire, England). After co-culturing with microglia, MDA-MB-231Br and SKBrM3 cells were transfected with 100 nM siRNA and immediately used for further experiments after 48 h of treatment.

Cell viability assay

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; CK04, Dojindo, Japan) according to the manufacturer's instructions. Specifically, cells subjected to different experimental conditions were seeded in a 96-well plate at a concentration of 5×10^4 cells/well. After adherence, the cells were transferred to a complete medium containing 10 % CCK-8 reagent and incubated for 2 h. Finally, the absorbance was measured at 450 nm using a microplate reader (Manidoff, Switzerland). Each experiment was repeated three times.

Cell migration assay

The scratch assay was used to evaluate the migration ability of cells. Cells subjected to different treatments were seeded in a 6-well culture plate and incubated overnight until the cell density reached about 90 %. The cell monolayer was scraped with a 10-μL white pipette tip, and the cells were then washed gently with PBS (SH30256.01, HyClone, Logan, USA) at a concentration of 1× with pH values of 7.0–7.2 three times to remove the detached cells. Initial images of the scratched area were captured for reference. Next, the cells were incubated at 37 °C and 5 % CO_2 for 24 h. After incubation, images of the marked area were captured again to observe changes and to calculate the cell migration rate. This experiment was also performed three times.

Cell invasion assay

To assess cell invasion, 50 μL of diluted Matrigel (354234, Corning BioCoat, New York, USA) was added to the bottom of the Transwell upper chamber, ensuring even distribution by gentle shaking. A 100-μL cell suspension was then added to the upper chamber of the Transwell, and 600 μL of cell culture medium was added to the lower chamber. After incubation for 24 h, cells on the upper surface of the filter membrane were gently wiped off with a cotton swab, and the migrated cells on the lower surface were stained with 0.1 % crystal violet (C0121, Beyotime, Shanghai, China) for 30 min. The invasiveness of the cells was determined by counting the cells that had migrated to the lower surface of the filter membrane. Randomly select five

visual fields under an inverted microscope to count the cells and calculate the average value. This experiment was repeated three times.

Western blot analysis

The concentrations of protein samples were determined using a Lowry protein analysis kit (PC0030, Solarbio, Beijing, China). After SDS-PAGE, the proteins were transferred to PVDF membranes (P0012A, Beyotime, Shanghai, China). The membranes were then blocked in triethanolamine-buffered saline with Tween (TBST) containing 5 % fat-free milk (50 mM tris, pH 7.5, 0.15 M NaCl, and 0.05 % Tween 20) for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies. The next day, the membranes were incubated with the secondary antibody (NC-AP132P, Nachuan, Shenzhen, China) for 1 h at room temperature. TBST was used to wash the membranes three times before and after incubation with a secondary antibody. Protein bands were detected by the ECL chemiluminescence method (W1015, Promega, Madison, WI, USA), and gray values were analyzed using ImageJ software (National Cancer Institute, Bethesda, MD, USA). All experiments were repeated three times.

Clinical data collection

The study was approved by the Ethics Committee of the Second Affiliated Hospital of Hainan Medical University (No. LW2023165) and conducted in accordance with the Declaration of Helsinki. This retrospective study included 38 patients with breast cancer who underwent resection of brain metastases from January 2007 to December 2018. All patients provided signed informed consent.

Immunohistochemistry

Fixed paraffin-embedded specimens of brain metastases were obtained from the Pathology Laboratory of the Second Affiliated Hospital of Hainan Medical University. The antibodies were diluted as follows: anti-Iba1 at 1:2000, anti-CEMIP at 1:200, and anti-CCL2 antibody at 1:400. After color development, the results of immunohistochemistry were evaluated by three pathologists who had no access to clinical data. The integrated optical density value analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (version 2.0.0-rc-43/1.50e).

ELISA

The concentrations of CCL-2 (DCP00), IL-6 (D6050), TGF-β (DB100C), and VEGF (DVE00) in different culture supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The concentrations of the samples were estimated using a microplate reader (SpectraMax 190, MD, Changzhou, China) at a wavelength of 450 nm.

Statistical analysis

The correlation between CEMIP expression and immune infiltration in breast cancer was explored using the TCGA dataset on the XIANTAO platform (www.xiantaozi.com). The numerical results of this study are expressed as mean ± SD. SPSS statistical software version 19.0 (IBM, Armonk, NY, USA) and GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) (version 8.0) were used to analyze the data and statistical graphs. Data from multiple groups were evaluated by the *t*-test, whereas survival curves were analyzed using the Kaplan–Meier method and compared using the log-rank test. The chi-square test was used to analyze the relationship between two groups of samples. Use the Cox regression proportional hazards model for multivariate analysis to adjust for clinicopathological variables that may have statistical significance for prognosis in univariate analysis.

Results

Microglial gathering and high CEMIP expression led to a poor prognosis in patients with breast cancer with brain metastasis

To analyze the role of microglia and CEMIP expression on the prognosis of patients with breast cancer with brain metastasis, the pathological sections of 38 brain metastasis tissues of patients with breast cancer were examined by immunohistochemical staining with anti-Iba1 and anti-CEMIP antibodies (Figure 1A). Iba1, a specific 17-kDa calcium-binding protein expressed in the microglia of the CNS [25], serves as a reliable marker of microglia [25]. The Kaplan–Meier survival curve showed a negative correlation between the aggregation of microglia around brain metastases tissues and patient prognosis ($p<0.05$; Figure 1B). In addition, a higher expression of CEMIP in brain metastasis tissues was associated with a shorter survival time in patients with breast cancer after the diagnosis of brain metastasis ($p<0.05$; Figure 1C). Evaluation using the Cox regression proportional hazards model suggested that both Iba1 (HR=0.302, p=0.037) and CEMIP expression (HR=0.637, p=0.044) in brain metastases were independent risk factors for survival (Table 1). The above results indicate that both microglia and CEMIP are significantly associated with brain metastasis of breast cancer.

CEMIP expression is closely related to microglial aggregation around brain metastasis tissues in breast cancer

Immune infiltration in tumor microenvironment is a critical factor for the colonization and metastasis of tumor cells. This research analyzed the relationship between CEMIP expression and immune infiltration profiles in breast cancer using the

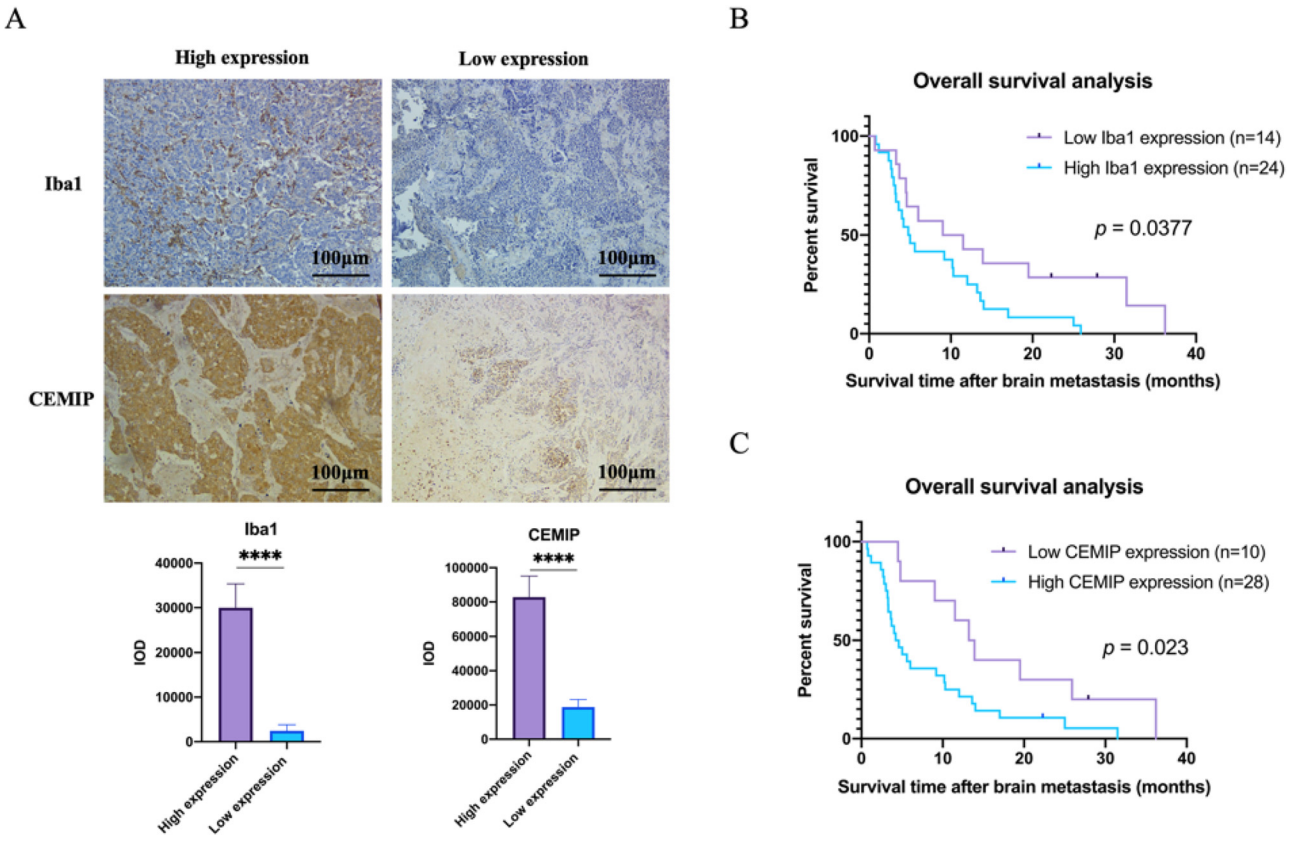

Figure 1: Association of high Iba1 expression and high CEMIP expression with poor overall survival (OS) in patients with breast cancer patients with brain metastasis (A) representative images of immunohistochemical staining for Iba1 and CEMIP in brain metastasis samples from patients with breast cancer (×100). Kaplan–Meier OS curves for brain metastasis tissues from patients with breast cancer with (B) low (n=14) and high (n=24) Iba1 expression (p=0.0377) and (C) low (n=10) and high (n=28) CEMIP expression (p=0.023). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Table 1: Multivariate and univariate analyses of clinicopathological characteristics associated with the overall survival of patients with breast cancer after diagnosis of brain metastasis.

Clinicopathological features	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	p-Value	HR (95 % CI)	p-Value
Tumor stage (T1-2 vs. T3-4)	0.685 (0.351–1.337)	0.265		
Nodal stage (N0-1 vs. N2-3)	1.444 (0.836–2.496)	0.185		
Number of BM (<3 vs. ≥3)	0.566 (0.318–1.008)	0.05		
Radiotherapy (yes or no)	2.741 (1.205–6.232)	**0.039**	2.775 (1.268–6.471)	**0.048**
Iba1 expression (low vs. high)	0.273 (0.126–0.590)	**0.002**	0.302 (0.249–0.628)	**0.037**
CEMIP expression (low vs. high)	0.577 (0.342–0.973)	**0.037**	0.637 (0.389–1.153)	**0.044**

The values less than 0.05 are marked in bold.

XIANTAO platform. The results showed that among the 24 types of infiltrating immune cells, macrophages have the strongest correlation with CEMIP expression (Figure 2A). It is well-known that microglia are specific macrophages in the brain microenvironment. Therefore, the correlation between microglia and CEMIP expression was analyzed using the results of immunohistochemistry and chi-square test. The results showed that CEMIP expression is closely related to the density of microglia gathered around brain metastasis in patients with breast cancer (Figure 2B). This conclusion was further verified by *in vitro* experiments. Preliminary experiments demonstrated that microglia could promote the expression of CEMIP in breast cancer cell lines (MDA-MB-231 and SKBR-3) (Figure S1). In order to more closely mimic the brain microenvironment, the parental breast cancer cells were replaced with the brain metastatic breast cancer cells (MDA-MB-231Br and SKBrM3) for subsequent experiments. Then, MDA-MB-231Br and SKBrM3 cell lines were co-cultured with microglia for 48 h as one group

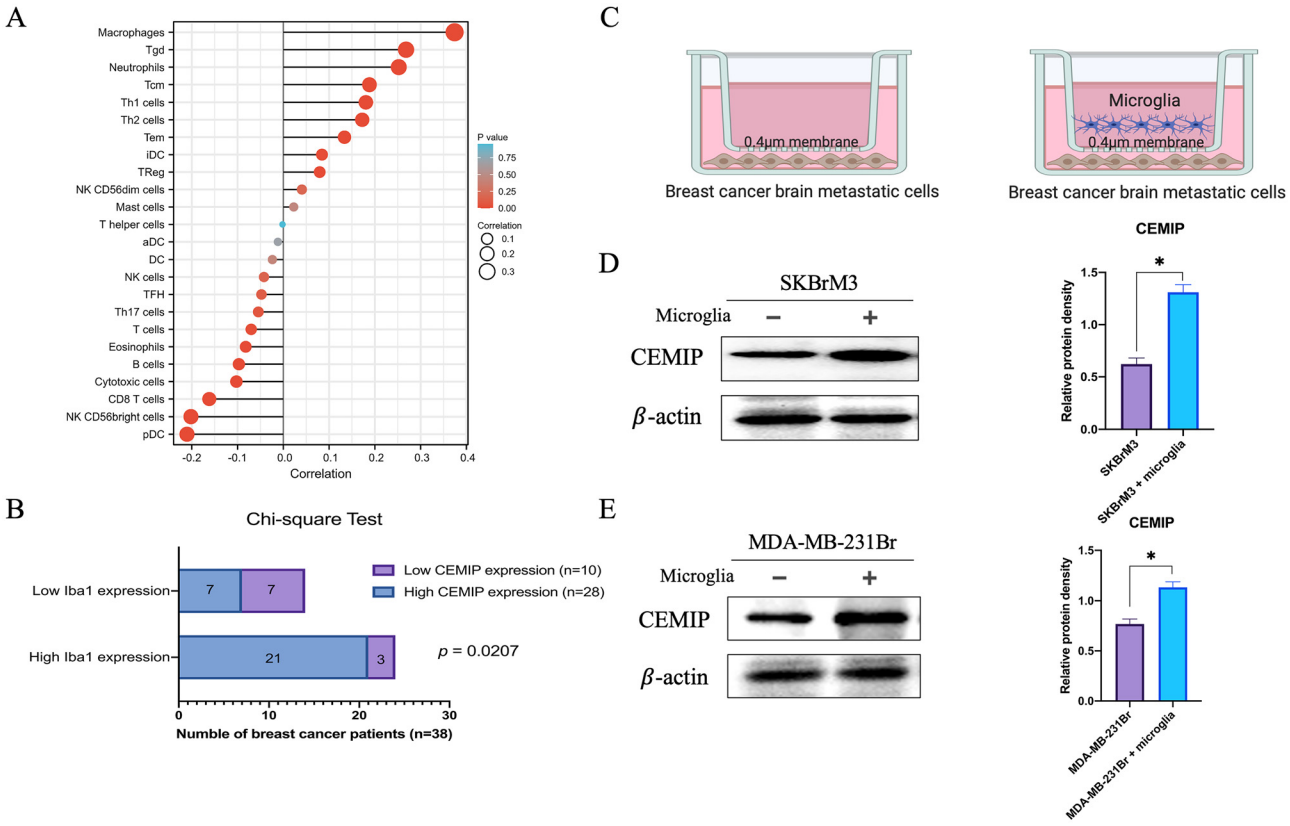

Figure 2: Microglia increase CEMIP expression in brain metastasis of breast cancer. (A) The correlation between CEMIP expression and 24 immune cell types was analyzed using the XIANTAO platform. (B) The chi-square test was used to analyze the relationship between Iba1 expression and CEMIP expression. (C) The procedure of a co-cultured group of microglia and brain metastatic breast cancer cells and the control group of brain metastatic breast cancer cells cultured alone for 48 h. (D and E) Western blot analysis of CEMIP expression in MDA-MB-231Br and SKBrM3 cells. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

and cultured alone as another group (Figure 2C). The results of western blot analysis indicated that both cell lines showed increased CEMIP expression when co-cultured with microglia (Figure 2D and E). This suggests that microglia may promote the progression of brain metastasis in breast cancer by inducing the expression of CEMIP.

Microglia promote the proliferation, migration, and invasion of brain metastatic breast cancer cells by increasing CEMIP expression

To investigate whether the promotion of microglia in brain metastasis of breast cancer has a correlation to the expression of CEMIP, we established different experimental conditions in this study (Figure 3A). Brain metastatic breast cancer cells co-cultured with microglia were then transfected with different siRNAs for subsequent analyses. The results of western blot analysis showed that microglia increased CEMIP expression in MDA-MB-231Br and SKBrM3 cells, and siRNA transfection reduced CEMIP expression (Figure 3B and C). Subsequently, CCK-8 assay, scratch test, and Transwell assay were used to detect the proliferation, migration, and invasion abilities of MDA-MB-231Br and SKBrM3 cells in the three groups. The results showed that co-culture with microglia enhanced the proliferation, migration, and invasion abilities of MDA-MB-231Br and SKBrM3 cells. However, after the knockdown of CEMIP expression by siRNA, the promotional effect of microglia on MDA-MB-231Br and SKBrM3 cells was diminished (Figure 4A–F). In summary, these results indicate that microglia promote the proliferation, migration, and invasion of brain metastatic breast cancer cells, which may be mediated by the high expression of CEMIP.

CEMIP promotes brain metastasis of breast cancer through the JAK2/STAT3 pathway

The role of microglia in inducing CEMIP expression, which promotes the development of brain metastasis in

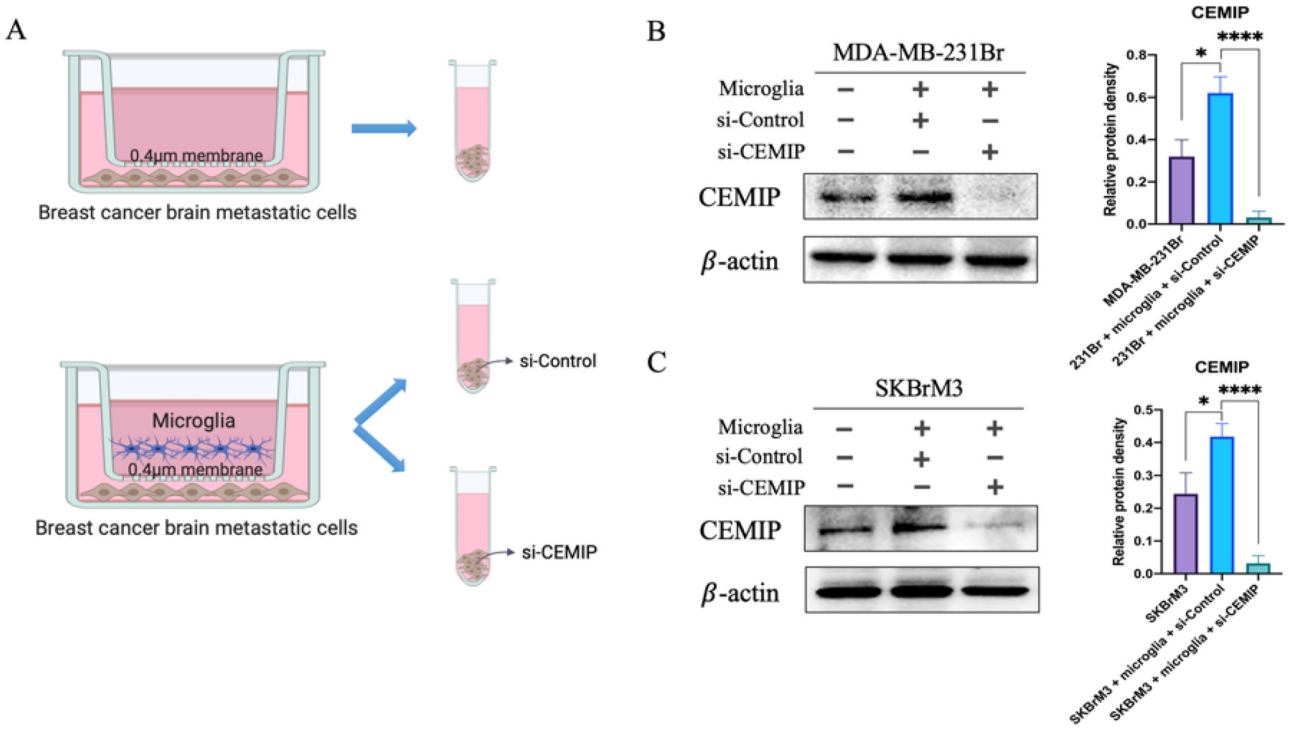

Figure 3: siRNA reduces microglia-induced CEMIP expression in MDA-MB-231Br and SKBrM3 cells. (A) The procedure of knockdown of CEMIP in MDA-MB-231Br and SKBrM3 cells after co-culturing with microglia. (B and C) Western blot analysis of CEMIP expression in MDA-MB-231Br and SKBrM3 cells. The results showed that CEMIP expressions in MDA-MB-231Br and SKBrM3 cells transfected with siRNA were decreased significantly (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

breast cancer, has been explored, but the exact mechanism remains unclear. Studies have claimed that the continuous activation of the JAK2/STAT3 pathway is closely linked to tumor progression [26]. Moreover, the abnormal activation of the JAK2/STAT3 pathway often occurs in brain tissue, producing a large number of inflammatory factors [27], which promotes the proliferation and metastasis of cancer cells. Therefore, western blot analysis was used to detect the expression of the JAK2/STAT3 pathway in MDA-MB-231Br and SKBrM3 cells. The results showed that the activation of the JAK2/STAT3 pathway was more pronounced when MDA-MB-231Br and SKBrM3 cells were co-cultured with microglia. Furthermore, the knockdown of CEMIP inhibited the activation of this pathway (Figure 5A and B). The above results demonstrate that microglia increase CEMIP expression, thereby activating the JAK2/STAT3 signaling pathway. However, the effect of this activation by microglia is weakened when the expression of CEMIP decreases. In summary, microglia-induced CEMIP expression might promote brain metastasis progression in breast cancer via the JAK2/STAT3 signaling pathway.

Microglia recruited by CCL2 can secrete IL-6, TGF-β, and VEGF, promoting brain metastases

As is commonly believed, the JAK2/STAT3 pathway is a classic inflammatory signaling pathway in tumorigenesis and development, which induces the secretion of CCL2 [28]. Therefore, this study used an ELISA kit to detect the secretion levels of CCL2 in the culture supernatant. The results showed that CCL2 secretion was increased in the co-culture group (Figure 6A), suggesting that the JAK2/STAT3 pathway, activated by CEMIP, induces the secretion of CCL2. Previous studies have demonstrated that CCL2 can recruit microglia to inflammatory sites or brain tumors [28]. Accordingly, based on the results of immunohistochemistry analysis, a Kaplan–Meier survival curve was used to examine the association between CCL2 and the survival time after the diagnosis of brain metastasis (Figure 6B and C). A chi-square test was used to verify the relationship between CCL2, microglia, and CEMIP expression (Figure 6D). The results indicated that high CCL2 expression can recruit microglia to gather around brain metastasis sites, subsequently shortening the survival of patients with breast cancer.

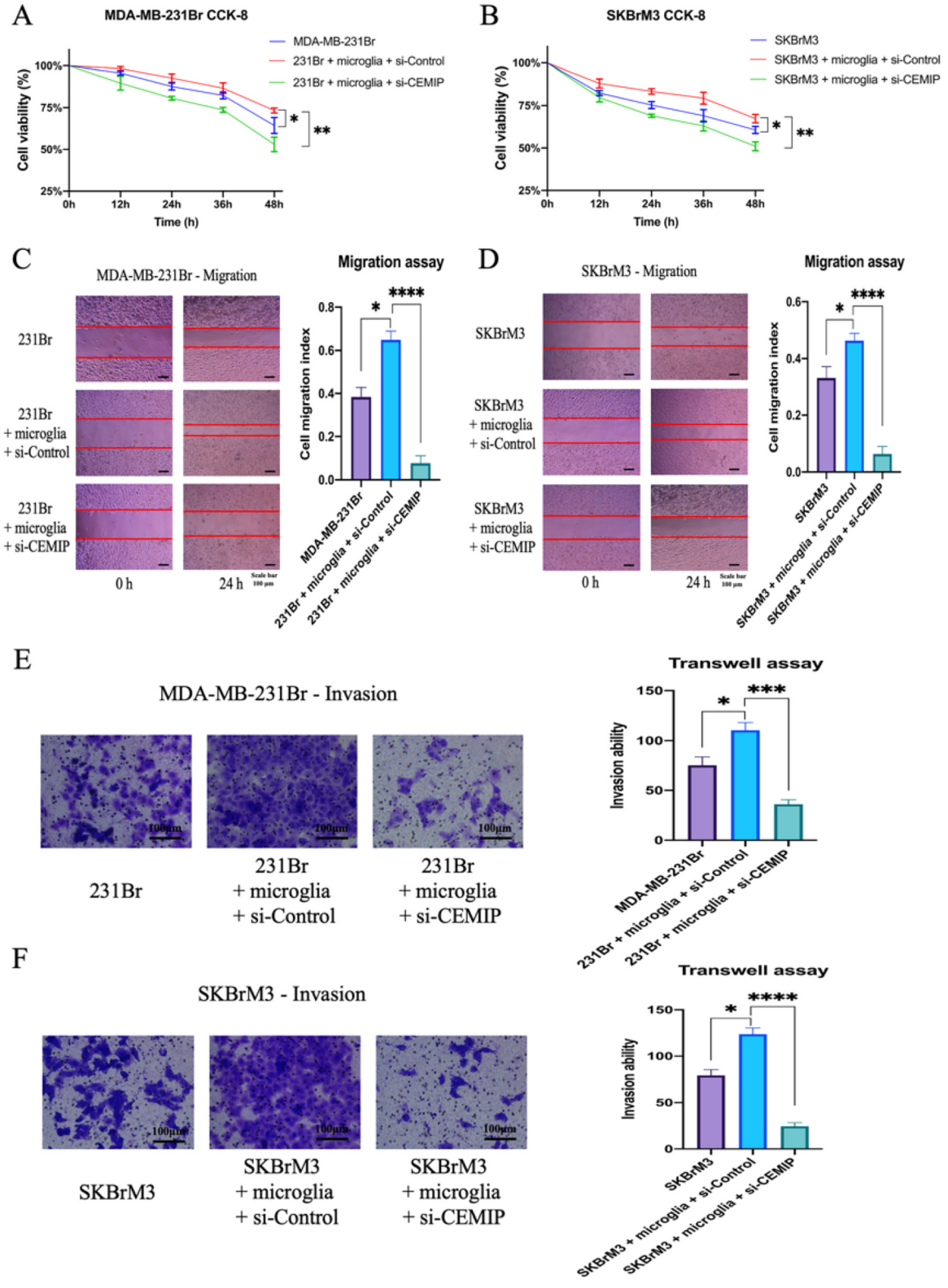

Figure 4: Microglia enhance the proliferation, migration, and invasion abilities of MDA-MB-231Br and SKBrM3 cells, and the knockdown of CEMIP inhibits this promoting effect. Effects of microglia and CEMIP on (A and B) cell viability were detected by CCK-8 assay, (C and D) migration ability was analyzed by scratch test. (E and F) Invasion ability was studied by Transwell assay (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

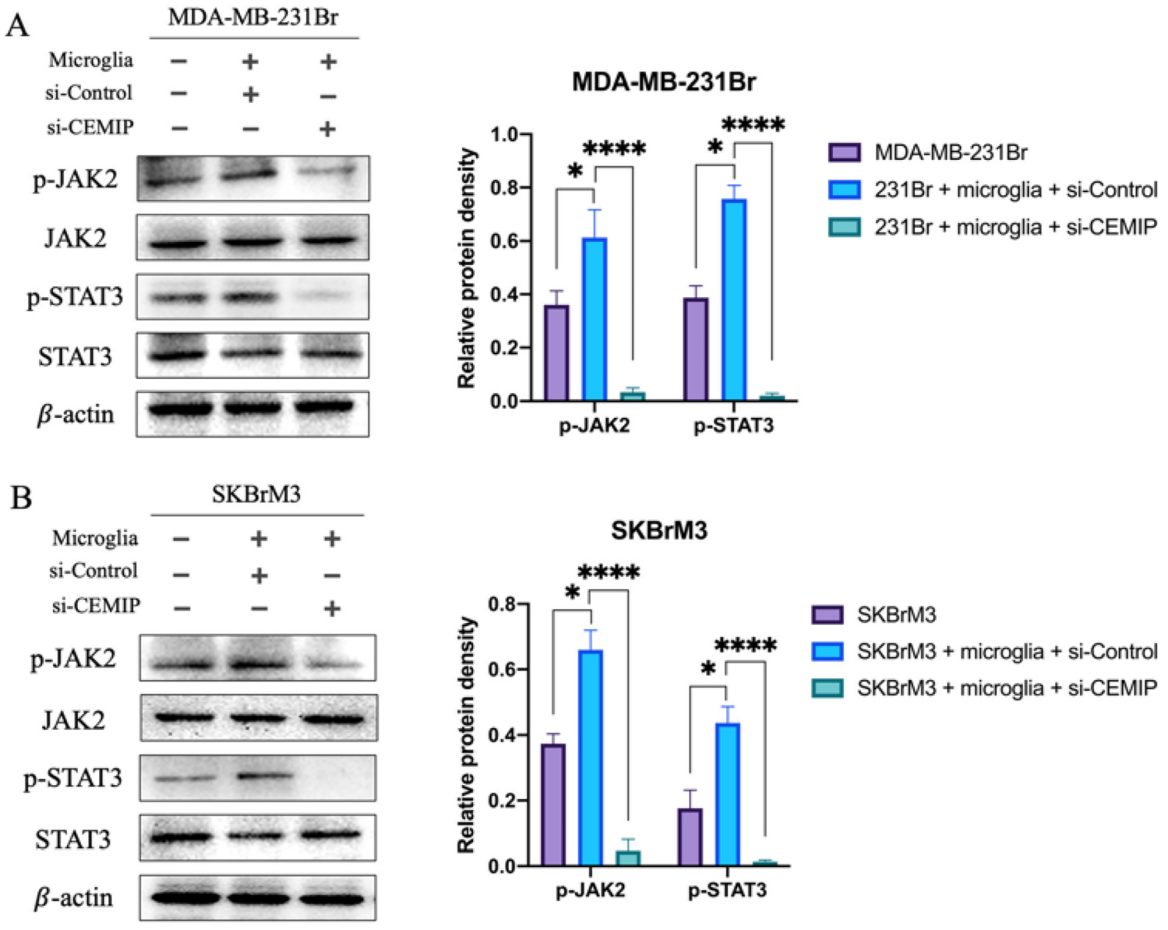

Figure 5: Microglia increase the expression of CEMIP, which activates the JAK2/STAT3 pathway in brain metastatic breast cancer cells, and knockdown of CEMIP inhibits this activation. (A and B) The expressions of JAK2 and STAT3 were detected by western blot analysis in MDA-MB-231Br and SKBrM3 cells. The results showed that the expressions were increased in the co-cultured group, and knockdown of CEMIP significantly reduced these expressions (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Microglia, known to secrete various cytokines, play an important role in the brain metastasis microenvironment. They release TGF-β, IL-6, and VEGF, which are involved in local immune suppression and angiogenesis around tumors, thereby enhancing the proliferation and infiltration of tumor cells [29–31]. Therefore, the secretion levels of IL-6, TGF-β, and VEGF in the culture supernatant were also detected in this study. The results implied that IL-6, TGF-β, and VEGF were predominantly secreted in the microglia and co-culture groups (Figure 6E), indicating that they were mainly secreted by microglia and not tumor cells.

In summary, microglia increase the expression of CEMIP in brain metastatic breast cancer cells, which, in turn, activates the JAK2/STAT3 signaling pathway and promotes the secretion of CCL2, IL-6, TGF-β, and VEGF, establishing a positive feedback loop that induces the progression of breast cancer brain metastasis (Figure 7).

Discussion

The occurrence of brain metastasis is an end-stage event in patients with breast cancer, which predicts a poor prognosis [7]. The development of effective treatments for brain metastasis is restricted by the limitations of our understanding of the molecular mechanisms underlying breast cancer brain metastasis. Among the factors that determine whether breast cancer cells can be successfully colonized in the brain to culminate in brain metastasis, the brain microenvironment emerges as a crucial element [9]. In the brain microenvironment, microglia, as the most representative innate immune cells, are widely distributed in the CNS [29]. Microglia are known as the key players in brain metastasis and brain microenvironment remodeling [9, 15], and they are closely linked with tumor cell growth and angiogenesis [13, 30, 31]. Microglia are very sensitive and can be activated even by slight

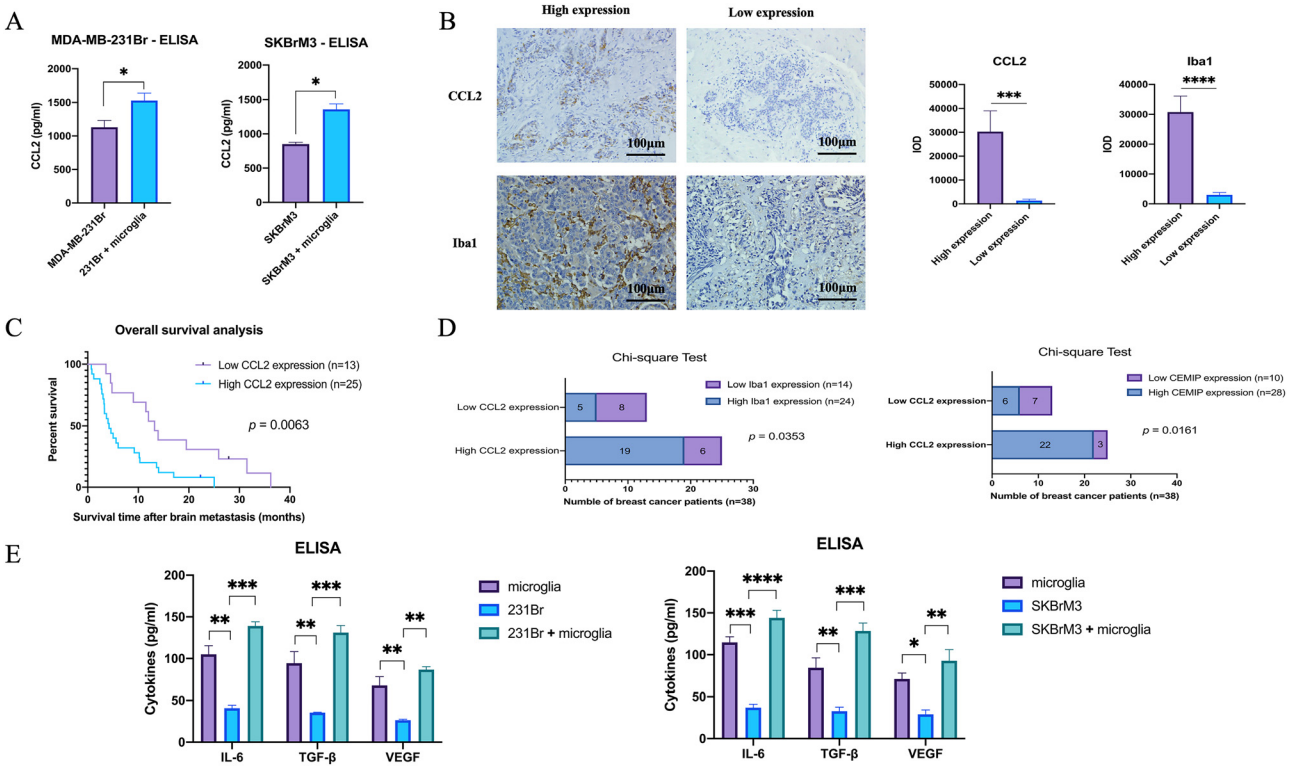

Figure 6: Role of CCL2 in microglial recruitment and potential impact on CEMIP expression in brain metastases. (A) Secretion levels of downstream cytokine CCL2 in different groups assessed by ELISA. (B) Representative images of immunohistochemical staining for CCL2 and Iba1 in brain metastasis samples of patients with breast cancer (×200). (C) Kaplan–Meier OS curve for brain metastasis tissues with low (n=13) and high (n=25) CCL2 expression (p=0.0063). (D) Correlations between CCL2 and Iba1/CEMIP expression verified by chi-square test. (E) Secretion levels of IL-6, TGF-β, and VEGF assessed by ELISA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

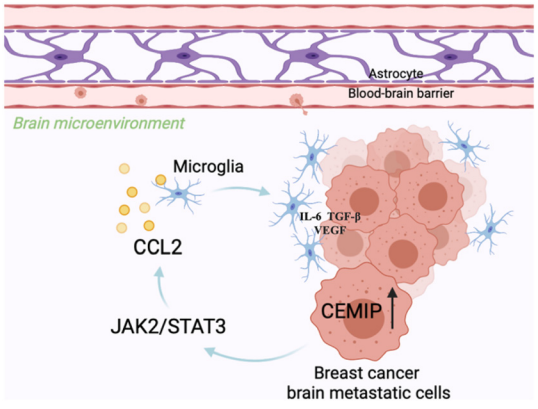

Figure 7: Proposed model for the mechanism involving microglia and CEMIP in the progression of brain metastasis and the positive feedback loop between brain metastatic breast cancer cells and microglia.

stimulation or CNS injury [32]. The activated microglia gather around tumor cells to produce a tumor-promoting effect [33, 34]. In this study, we found that patients with breast cancer with high Iba1 expression at brain metastasis sites had poor survival (p=0.0377). This indicates that microglia may promote the progression of brain metastasis in patients with breast cancer.

Extensive evidence indicates that the abnormal expression of CEMIP has a relation to tumor metastasis and progression [16–18, 35]; high CEMIP expression, in particular, promotes brain metastasis [20, 36]. In alignment with previous perspectives, our research demonstrates that high expression of CEMIP in brain metastases results in a short survival time in patients with breast cancer (p=0.023). In addition, our study demonstrates that both microglia (HR=0.302, p=0.037) and CEMIP expression (HR=0.637, p=0.044) are independent risk factors for the prognosis of brain metastasis in these patients. More importantly, our findings prove that CEMIP expression in brain metastases is correlated with the density of microglia around brain metastases (p=0.0207), suggesting the plausible role of microglia in inducing CEMIP expression in brain metastases of breast cancer.

We also found that microglia enhanced the proliferation, migration, and invasion abilities of brain metastatic breast cancer cells. This promoting effect of microglia is mitigated when CEMIP expression in brain metastatic breast cancer cells is reduced. It suggests that microglia may promote brain

metastasis of breast cancer by increasing the expression of CEMIP. Although it is widely acknowledged that elevated CEMIP levels can transmit or even amplify signals to promote tumor progression by linking with the cytokine pathway [16], the exact mechanism remains unclear. Our research proved that the increase in CEMIP expression, induced by microglia, activates the JAK2/STAT3 pathway. Moreover, the downstream cytokine CCL2 recruits microglia to gather around the brain metastases, whereas IL-6, TGF-β, and VEGF, in turn, cause high CEMIP expression in brain metastases, forming a positive feedback loop to promote the development of brain metastasis in breast cancer. However, a limitation of this study is that the mechanism is not verified *in vivo*, which needs to be explored in further research.

In conclusion, our study highlights the roles of microglia and CEMIP in brain metastasis of breast cancer, underscoring their potential as biomarkers for the progression of this condition. An in-depth understanding of the mechanism of brain metastasis may provide a new opportunity for the targeted treatment and improvement of prognosis in patients with breast cancer with brain metastasis.

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Research ethics: Approval of the research protocol by an Institutional Reviewer Board: The study conformed to the provisions of the Declaration of Helsinki, and was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Hainan Medical University (No. LW2023165).

Informed consent: All patients in the research have signed informed consent.

Author contributions: Qi Qin conceived and designed the experiments. Qi Qin, Chaoying Wang, Yongfu Li, and Qiuyu Mo conducted and analyzed the data. Qi Qin wrote this manuscript. All authors read and approved the final manuscript.

Competing interests: The authors have no conflict of interest.

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Data availability: All data presented in this study are available from the corresponding author upon reasonable request.

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