C6-Ceramide Induces Apoptosis in Lung Non-Small Cell Lung Cancer and Suppresses Brain Metastasis by Downregulating The PI3K/AKT/mTOR Signaling Pathway

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Research

Keywords: C6-ceramide, non-small cell lung cancer, apoptosis, brain metastasis, PI3K/AKT/mTOR

DOI: https://doi.org/10.21203/rs.3.rs-139867/v1

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Abstract

Background: The membrane lipid ceramide plays important roles in regulating tumor growth, chemotherapy drug resistance, and apoptosis. However, the mechanisms through which ceramide induces apoptosis are still unclear. In this study, we explored the biological functions and underlying mechanism of C6-ceramide in brain metastasis (BM) arising from non-small cell lung cancer (NSCLC).

Methods: The effects of C6-ceramide on cell apoptosis were studied by flow cytometry. Cell Counting Kit-8 assays, wound-healing assays, and flow cytometry were performed to investigate the biological functions of C6-ceramide. An in vitro blood-brain barrier (BBB) model was constructed, and its effectiveness and availability were tested by evaluating horse radish peroxidase activity and junction-related protein expression. The underlying signaling pathways of C6-ceramide were detected by western blotting, and further verification was performed using pathway inhibitors. RNA sequencing was used to confirm the involvement of C6-ceramide and associated pathways in BM arising from NSCLC.

Results: C6-ceramide induced apoptosis in NSCLC cells, and the optimal working concentration of C6-ceramide was 50 μM. C6-ceramide not only suppressed cell proliferation and migration but also arrested cells at the G1/S transition. An in vitro BBB model constructed using human umbilical vein endothelial cells and human astrocytes cells showed the lowest permeability and the strongest tight junction connections when cells were cocultured for 72 h. Moreover, C6-ceramide suppressed the passage of cells through the BBB model. C6-ceramide at least partially influenced NSCLC biological functions by downregulating the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway, which was further confirmed by rescue assays and RNA sequencing.

Conclusions: C6-ceramide functioned as a tumor suppressor by inducing NSCLC cell apoptosis and BM through the PI3K/AKT/mTOR signal pathway. Thus, this pathway may serve as a novel potential therapeutic target for patients with BM arising from NSCLC.

Background

Primary lung cancer is one of the most common malignant tumors encountered in the clinical setting and the most frequent cause of cancer-related death worldwide [1]. In 2012, the number of global lung cancer deaths was approximately 1.6 million, and it is expected that the number of deaths will reach 3 million by 2035 [2]. Lung cancer can be classified as non-small cell lung cancer (NSCLC) or small cell lung cancer, with NSCLC accounting for approximately 80–85% of cases [3]. The most common distant metastatic site of lung cancer is the brain, with an incidence of 18–65% [4–6]. The occurrence of brain metastasis (BM) has serious effects on patient quality of life. Although various treatments, including surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy, have resulted in improved prognoses in patients with NSCLC, the prognosis remains poor, and the median survival time is only about 13.7 months [7]. Therefore, it is particularly important to elucidate the pathogenesis of BM arising from NSCLC and to improve survival rates in these patients.
The process of BM in NSCLC is very complicated and involves lung tumor growth and shedding to form circulating tumor cells, which migrate to the blood-brain barrier (BBB) through the blood circulation, then passage of the cells through the BBB, and establishment and growth of the new tumors in brain tissue [8, 9]. In recent years, some studies have identified the key molecules involved in this process and their effects on BM. For example, overexpression of matrix metalloproteinases promotes the transfer of NSCLC cells to the brain parenchyma through the vascular system and increases the incidence of BM [10, 11]. In addition, the expression of C-X-C chemokine motif ligand 12 and its receptor C-X-C chemokine receptor 4 (CXCR4) in BM is significantly higher than that in primary lung cancer, and CXCR4 can enhance the adhesion and chemotaxis of lung cancer cells, thereby promoting BM [12–14]. Other protein molecules, including ADAM9 and S100, have also been found to be associated with BM from NSCLC [15, 16]. Despite extensive research on the key molecules of BM in NSCLC, the incidence of BM has not decreased significantly, indicating that the mechanism of BM has not been fully elucidated.

The membrane lipid ceramide is the core component of the sphingolipid metabolism pathway and plays important roles in regulating cell membrane fluidity and membrane subdomains [17–19]. Ceramide can be produced in response to several stressors, such as ionizing radiation, tumor necrosis factor, and chemotherapeutic agents [20]. Recent studies have shown that ceramide participates in regulating tumor growth, chemotherapy drug resistance, and apoptosis [21, 22]. Additionally, Moro et al. [23] found that the level of ceramide in breast cancer tissue was significantly higher than that in normal tissue; however, ceramide is also significantly associated with the low-invasive phenotype in breast cancer, and patients with high expression of ceramide show improved prognoses. Moreover, a previous study showed that the resistance of breast cancer cells to chemotherapeutic drugs is caused by upregulating UDP-glucosylceramide glucosyltransferase and reducing the level of ceramide, suggesting that ceramide may play important roles in chemotherapy resistance [24]. In a mouse model of liver cancer, liposome C6-ceramide was found to enhance the antitumor immune response of CD8+ T cells through M1 macrophages [25]. Another study found that C6-ceramide inhibits the growth of liver cancer cells by downregulating the activation of the AKT signaling pathway [26]. C6-ceramide is mainly involved in the occurrence and development of cancer; however, the mechanisms through which ceramide modulates BM arising from lung cancer have not been reported.

Activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway is related to various characteristics of cancer, including inhibition of apoptosis, promotion of angiogenesis, enhancement of tumor invasion and metastasis, and insensitivity to antitumor effects [27]. Studies have shown that mutations in the PIK3CA gene encoding the p110α subtype can increase the incidences of liver cancer by 36%, breast cancer by 26%, and colon cancer by 26% [28, 29]. In addition, a small number of mutations in PIK3A have also been found in gliomas [30]. The phosphatase and tension homolog (PTEN) protein encoding PIP3 phosphatase can prevent further transduction of the signaling pathway by phosphorylating PIP3 to PIP2; thus, loss of PTEN plays a key role in enhancing PI3K signaling and promoting cancer development [31, 32]. A previous study also confirmed that PTEN mutation can lead to an increased risk of various cancers, including breast cancer, urogenital tract cancer, and endometrial cancer [33].
AKT acts downstream of PI3K and is one of the members of the AGC protein kinase family, which consists of three homologs (Akt1, Akt2, and Akt3) [34]. Changes in Akt expression levels or activity are closely related to the occurrence and development of various human malignancies [35, 36]. The activation of AKT can downregulate the apoptosis-related proteins BAD and BAX and inhibit cancer cell apoptosis [30]. Furthermore, AKT can phosphorylate Mdm2 to reduce p53-mediated cell death and apoptosis, thereby promoting tumor proliferation [37]. mTOR is located downstream of the AKT signaling pathway and can be a part of mTOR complex (mTORC) 1 or 2. Activation of mTORC2 promotes the phosphorylation of AKT, which plays important roles in tumor proliferation [38]. Studies have shown that mTOR protein is highly expressed in NSCLC and is significantly associated with poor prognosis [39, 40]. However, the roles of the PI3K/AKT pathway in BM arising from NSCLC are still poorly understood.

Accordingly, in this study, we evaluated the mechanisms through which C6-ceramide mediates BM in lung cancer. Furthermore, we successfully constructed a BBB model and examined whether C6-ceramide could inhibit the penetration of NSCLC cells through the BBB. Our findings provided important insights into the effects of C6-ceramide on the metastasis of NSCLC cells to the brain.

**Methods**

**Cell lines and reagents**

PC-9 and H1299 cell lines were obtained from the Cell Bank of Xiangya Medical College of Central South University (Changsha, China). Human umbilical vein endothelial cells (HUVECs) and human brain astrocytes (HAs) were procured from Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences Cell Resource Center (Beijing, China). PC-9 and H1299 cells were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS), and HUVECs were cultured in endothelial cell medium (ScienCell, USA) supplemented with endothelial cell growth factor and 5% FBS. HAs were cultured in astrocyte medium (ScienCell, USA) supplemented with astrocyte growth factors. All cells were cultured in humidified air at 37 °C with 5% CO2. C6-ceramide (N-hexanoyl-D-erythro-sphingosine) was purchased from Avanti Polar Lipids (AL, USA) and dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock. The working concentration of DMSO was always less than 1 µM. The PI3K inhibitor LY294002 was obtained from Sigma (St. Louis, MO, USA) and dissolved in DMSO.

**Apoptosis assay**

H1299 and PC-9 cells were treated with C6-ceramide for 24 h. The cells were then collected with ethylenediaminetetraacetic acid-free trypsin, washed with phosphate-buffered saline (PBS), and stained with Annexin V-FITC and propidium iodide (PI). The samples were detected by flow cytometry and fluorescence microscopy. All experiments were performed in triplicate.

**Cell cycle analysis**

H1299 and PC-9 cells were treated with C6-ceramide. Twenty-four hours later, cells were harvested, washed with ice-cold PBS, and fixed with 70% ice-cold ethanol. The samples were incubated at 4 °C
overnight and then incubated with PI and RNase A for 30 min at 37 °C. Flow cytometry was used to detect the cell cycle distribution of the samples.

**Cell Counting Kit-8 (CCK-8) proliferation assays**

Cells were resuspended in RPMI 1640 medium supplemented with 10% FBS and 50 μM C6-ceramide. Next, 3000 cells were plated in 96-well plates. Cells were washed with PBS and incubated with RPMI 1640 medium for the indicated times (24, 36, 48, or 60 h). Ten microliters of CCK-8 reagent (Beyotime, China) was then added to the culture medium. The samples were incubated at 37 °C for 1 h, and the optional density at 450 nm was measured. These experiments were repeated at least three times.

**Wound healing assays**

PC-9 and H1299 cells were plated into 6-well plates (1 × 10⁶ cells/well). A 200-μL plastic pipette tip was used to scratch the monolayer and create a uniform wound. Then, PBS was used to wash the monolayer, and culture medium with or without C6-ceramide was added to the plates. The distance between the two wound edges of the migrating cell sheets was captured by photographing at 0 and 24 h after injury. All experiments were conducted with three replicates.

**Establishment of stable lung cancer cell lines with green fluorescence**

To generate the PC-9 and H1299 cells showing green fluorescent signals, lentivirus harboring a stable overexpression vector for epidermal growth factor receptor (EGFR) and a CMV promoter-driven and puromycin resistance gene was purchased from Genechem (Shanghai, China). In total, 10000 PC-9 or H1299 cells were seeded in 12-well plates, and then 1 × 10⁸ TU/mL lentivirus and 5 µg/mL polybrene were added to the cell culture medium to facilitate transduction. Cells were incubated in humidified air at 37 °C with 5% CO₂ for 12 h. The culture medium was then refreshed, and cells were cultured for another 2 days. Puromycin was added to select the stable cell lines.

**Construction of the in vitro BBB model**

In order to develop an in vitro BBB model, we cocultured HUVECs and HAs on opposite sides of a 24-well transwell polycarbonate insert (Coring, NY, USA). The transwell insert was first coated with 2% gelatin (Sigma) for 45 min and then placed upside-down, and 100,000 HAs were plated on the bottom side of each insert. The cells were incubated to allow adherence in an incubator at 37 °C with 5% CO₂ and fed with astrocyte medium every 15–30 min. Four hours later, inserts were rolled over and placed in new 24-well plates. One milliliter of astrocyte medium was added to the lower chamber, and HAs were incubated for an additional 24 h. In total, 50000 HUVECs were seeded into the upper chambers of the inserts and incubated for 3 days. The permeability of the BBB model was detected by measuring the expression of tight junction-related proteins and horse radish peroxidase (HRP). For HRP analysis, the culture medium was discarded, and 1 mL RPMI 1640 medium (without red phenol), supplemented with 50 μg/mL HRP, was added to the upper chambers of 24-well plates. Next, 1.5 mL culture medium was added to the lower
chamber. At the indicated times (5, 15, 30, 60, and 120 min), 50 µL culture medium was removed from the lower chambers, and 100 µL 3,3',5,5'-tetramethylbenzidine was added. The medium was then incubated for interaction at room temperature for 30 min, and the reaction was stopped with 1 M H$_2$SO$_4$. The absorbance of the medium was measured at 450 nm, and permeability was calculated according to the following formula: $P_{HRP\%} = \frac{C_{HRP\ lower\ chamber} \times V_{HRP\ lower\ chamber}}{C_{HRP\ upper\ chamber} \times V_{HRP\ upper\ chamber}} \times 100\%$.

**Western blotting**

Cells were lysed using Radio Immunoprecipitation Assay lysis buffer (Beyotime), supplemented with protease inhibitor cocktail (Beyotime) and phenylmethylsulfonyl fluoride. The protein concentration was quantified using bicinchoninic acid assays (Beyotime). Next, 20–40 µg protein from cell lysates was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime) and transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). The membranes were incubated with primary antibodies at 4 °C overnight and then with secondary antibodies for 1 h. Finally, the target blots were visualized using ECL chemiluminescent reagent (Meilunbio, China), and data were analyzed with Image Lab Software. The primary antibodies used were as follows: anti-ZO 1 (cat. no. ab96587; Abcam, Cambridge, UK), anti-occludin (cat. no. ab216327; Abcam), anti-claudin 5 (cat. no. ab131259, Abcam), anti-cleaved caspase-3 (cat. no. ab32042; Abcam), anti-caspase-9 (cat. no. ab202068; Abcam), anti-Bax (cat. no. ab32503; Abcam), anti-Bcl-2 (cat. no. ab32124; Abcam), anti-glyceraldehyde 3-phosphate dehydrogenase (rabbit polyclonal antibody; cat. no. 10494-1-AP; Proteintech), anti-AKT1/AKT2/AKT3 (cat. no. ab179463; Abcam), anti-AKT1 (phospho-S473) (cat. no. ab81283; Abcam), anti-PI3K p85 (cat. no. 19H8; Cell Signaling Technology, Danvers, MA, USA), anti-mTOR (cat. no. ab32028; Abcam), and anti-mTOR (phospho-S2448) (cat. no. ab109268; Abcam).

**RNA sequencing**

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the quality of the extracted RNA was assessed using a Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Three micrograms RNA per sample was used as the input material for the RNA sample preparations. Sequencing libraries were constructed using a NEBNext UlarTM RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's instructions. All libraries were sequenced on an Illumina Hiseq platform (Novogene Bioinformatics Technology Co., Ltd., Beijing, China).

**RNA sequencing data analysis**

For quality control of sequencing data, raw data (raw reads) in fastq format were processed through in-house perl scripts, and clean data (clean reads) were obtained. Then, paired-end clean reads were aligned to the reference genome (GRCh38) using Hisat2 (version 2.0.5). FeatureCounts (version 1.5.0) was used to assemble the read numbers mapped to each gene, and then fragments per kilobase of transcript per million mapped reads values for each annotated gene were calculated. Differential expression analysis of read counts for annotated genes was performed by HTSeq-count. Differential expression analysis of PC-9 cells treated with or without C6-ceramide was performed using DESeq2 R package. Genes with an
adjusted P value less than 0.05 and fold change greater than 2 were defined as showing significantly
differential expression. Finally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes
(KEGG) pathway enrichment analyses of differentially expression genes were conducted using the
clusterProfiler R package.

**Statistical analysis**

SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism 8 were used for statistical analyses.
Unpaired *t* test was performed for comparisons of difference between two groups. One-way analysis of
variance with post-hoc Tukey HSD tests was conducted to analyze differences among multiple groups.
The results are presented as means ± standard deviations. For all analyses, results with *p* values less
than 0.05 were considered statistically significant.

**Results**

**C6-ceramide promoted apoptosis in NSCLC cells**

Previous studies have reported that C6-ceramide plays key roles in the progression of cancers, particularly
by promoting apoptosis in cancer cells [23, 25]. Therefore, to investigate the roles of C6-ceramide in
NSCLC cells, we performed dose-climbing apoptosis assays in PC-9 cells, which harbored an EGFR
mutation at exon L858R and had the potential for brain metastasis. The results showed that there were
no significant differences in apoptosis rates between the control group treated with DMSO and the blank
group for PC-9 cells (*p > 0.05; Fig. S1), suggesting that the dissolving agent DMSO has no significant
effect on apoptosis in PC-9 cells. However, compared with the control group, PC-9 cells treated with 1 µM
C6-ceramide showed significantly increased apoptosis (*p < 0.01). Moreover, the proportion of apoptotic
cells increased as the concentration of C6-ceramide increased; the proportion of apoptotic cells was
49.71% ± 2.02% when the concentration of added C6-ceramide was 50 µM. Therefore, we used 50 µM as
the working concentration of C6 ceramide in subsequent experiments.

A previous study showed that H1299 cells were susceptible to BM [41]. Thus, we also used H1299 cells to
explore the functions of C6-ceramide. Apoptosis assays were performed using flow cytometry and
fluorescence microscopy analyses, and the results revealed that 50 µM C6-ceramide increased apoptosis
rates in both H1299 and PC-9 cells when compared with that in the control group (both *p < 0.001; Fig. 1a, b), with apoptosis rates of approximately 50%. Moreover, in cells treated with 50 µM C6-ceramide for 24 h,
the morphology became round, and cells shrunk in size compared with control cells. The connections
between cells disappeared, and the cytoplasm of some cells was concentrated (red arrow, Fig. 1c); these
morphological changes were consistent with early apoptosis. In addition, some cells showed “bubbling”
and debris in the cytoplasm (blue arrow, Fig. 1c), further confirming the occurrence of changes related to
late apoptosis. To further verify the effects of C6-ceramide on NSCLC cell apoptosis, we used western
blotting to detect the expression levels of apoptosis-related proteins, including Bcl-2, Bax, cleaved-
caspase3, and caspase9. The results showed that after PC-9 and H1299 cells were treated with 50 µM
C6-ceramide, the expression level of the anti-apoptosis protein Bcl-2 was decreased, whereas those of
Bax, cleaved-caspase3, and caspase9 were increased (Fig. 1d). Taken together, these findings indicated that C6-ceramide promoted apoptosis in NSCLC cells.

**C6-ceramide suppressed NSCLC cell proliferation, migration, and BM in vitro**

To investigate the biological functions of C6-ceramide in NSCLC cells, we performed numerous assays. First, we evaluated the effects of C6-ceramide on NSCLC cell proliferation in vitro. CCK-8 assays showed that C6-ceramide significantly suppressed cell proliferation compared with that in control cells ($p < 0.001$; Fig. 2a). Additionally, wound healing assays showed that the migratory capabilities of both H1299 and PC-9 cells were markedly decreased by C6-ceramide ($p = 0.003$ and $p = 0.004$, respectively, Fig. 2b) when compared with that in cells treated with DMSO. In addition, cell cycle assays revealed that C6-ceramide induced G1 phase arrest, indicating that this compound modulated the cell cycle ($p < 0.01$; Fig. 2c).

To explore the effects of C6-ceramide on BM in NSCLC cells, we constructed an in vitro BBB model consisting of HUVECs and HAs seeded in 24-well transwell polycarbonate inserts. The permeability of the BBB was detected by evaluating HRP and the expression levels of tight junction-related proteins. The results showed that the HRP permeability of the BBB model was lowest when HUVECs and HAs were cocultured for 72 h (Fig. S2a). Furthermore, the expression levels of the tight junction proteins ZO-1, occludin, and claudin-5 were the highest after HUVECs were incubated for 72 h (Fig. S2b). These results suggested that the BBB model constructed using HUVECs and HAs could achieve an ideal tight junction state and showed the lowest permeability when cells were cocultured for 72 h; these conditions were used in subsequent experiments.

In order to verify the effects of C6-ceramide on the ability of NSCLC cells to penetrate the BBB, we transfected H1299 and PC-9 cells with enhanced green fluorescent protein and conducted experiments using the constructed BBB model. The results showed that when H1299 and PC-9 cells were treated with C6-ceramide, the number of cells that passed through the BBB model was significantly decreased compared with that in the control group ($p < 0.001$; Fig. 2d). Collectively, these results indicated that C6-ceramide suppressed the proliferation, migration, and BM of NSCLC cells.

**C6-ceramide inhibited BM by downregulating the PI3K/AKT/mTOR signaling pathway**

C6-ceramide suppressed the proliferation, migration, and BM of NSCLC cells by promoting cell apoptosis; however, the specific mechanisms were still unclear. Activation of the PI3K/AKT/mTOR signaling pathway is related to various characteristics of cancer, including inhibition of cell apoptosis [27]. Therefore, we used western blotting to detect the expression levels of proteins related to the PI3K/AKT/mTOR signaling pathway in NSCLC cells treated with C6-ceramide. The results showed that the levels of phospho-AKT (Ser473) and phospho-mTOR (Ser2448) were significantly decreased in cells treated with C6-ceramide ($p < 0.05$; Fig. 3), whereas the protein expression levels of total PI3K, AKT, and ...
mTOR did not change significantly. These results suggested that C6-ceramide promoted NSCLC apoptosis, inhibited cell proliferation, and blocked BM by downregulating the PI3K/AKT/mTOR signaling pathway.

**LY294002 further enhanced the apoptotic effects of C6-ceramide**

LY294002 is a specific PI3K inhibitor, and a previous study reported that NSCLC cells treated with 20 µM LY294002 for 24 h showed obvious downregulation of the PI3K/AKT/mTOR signaling pathway [42]. To further verify the relationship between the PI3K/AKT/mTOR signaling pathway and the pro-apoptotic effects of C6-ceramide in NSCLC cells, we treated H1299 and PC-9 cells with 20 µM LY294002 for 24 h and then detected the expression levels of apoptosis-related proteins. The results in Fig. 4a showed that the expression of Bcl-2 was significantly lower in cells treated with C6-ceramide and LY294002 than in cells treated with C6-ceramide only \( (p < 0.01) \). The opposite changes were observed for BAX, cleaved-caspase3, and caspase9. In addition, LY294002 further suppressed C6-ceramide-induced cell passage through the in vitro BBB model (Fig. 4b). These results suggested that the PI3K inhibitor LY294002 could enhance the pro-apoptotic effects of C6-ceramide and block BM arising from NSCLC cells. Taken together, our findings supported that C6-ceramide suppressed the progression of NSCLC cells partly by downregulating the PI3K/AKT/mTOR signaling pathway.

**RNA sequencing verified the effects of C6-ceramide on related pathways and gene functions**

In order to verify the above experimental findings at the mRNA level, we further adopted RNA-Seq to analyze PC-9 cells treated with C6-ceramide and a matched control group. In total, 700 genes were expressed in cells treated with C6-ceramide compared with that in cells in the control group (Fig. 5a). In addition, from DESeq2 analysis with an adjusted \( p \) value less than 0.05 and a |log\(_{2}\)FoldChange| more than 2, we found 465 differentially expressed genes, including 131 upregulated genes and 334 downregulated genes, between the two groups (Fig. 5b). Then, to further improve our understanding of the interactions and key genes among all 131 upregulated genes, we used the String database. The results demonstrated that five genes, including ITGAX, NOX3, EIF2AK3, DDIT3, and HERPUD1, were highly correlated with other genes, indicating that these genes may be the core genes in cells treated with C6-ceramide (Fig. 5c). Moreover, we conducted DAVID GO and KEGG pathway analyses to enrich related pathways based on the 465 differentially expressed genes. The GO analysis showed that the molecular functions of C6-ceramide mainly included “mitotic nuclear division”, “chromosome segregation”, and “DNA replication” (Fig. 5d). KEGG pathway enrichment further showed that C6-ceramide was associated with “cell cycle” and “DNA replication” (Fig. 5e). These findings were partly consistent with our experimental results.

**Discussion**
Lung cancer is a major public health concern, and BM is an important cause of death in patients with lung cancer. Among all brain tumors, BM is 10-times more common than primary brain tumors [43], and NSCLC is the most common source of BM [44]. Despite tremendous advances in the treatment of NSCLC, the prognosis of patients with BM is still poor [7]; thus, it is particularly important to explore the pathogenesis of BM in NSCLC in order to improve patient survival. In this study, we explored the biological functions and molecular mechanisms of C6-ceramide in NSCLC cells and found that C6-ceramide could not only promote NSCLC cell apoptosis, proliferation and migration but also inhibit the ability of cells to penetrate the BBB model via downregulation of the PI3K/AKT/mTOR signaling pathway.

Ceramide is the core component of the sphingolipid metabolism pathway and is mainly produced by the de novo synthesis pathway in the endoplasmic reticulum and the salvage synthesis pathway in the lysosome[19, 45]. Ceramide was involved in cell apoptosis, inflammation, and the cell cycle and acts as a bioactive lipid in cellular signaling pathways, such as heat shock response [46, 47]. Studies have shown that ceramide participates in the pathogenesis of various diseases, particularly malignant tumors. A previous study confirmed that exogenous ceramide C16 can target xIAP and cIAP1 at the cellular level, inhibit the growth of resistant colorectal and breast cancer cells, and promote cell apoptosis [48]. Additionally, Braicu et al. [49] found that the levels of ceramide in the serum of patients with ovarian cancer were significantly lower than those in healthy individuals, suggesting that ceramide in the blood may be a biomarker for patients with ovarian cancer. In this study, we demonstrated that NSCLC cells treated with C6-ceramide showed typical apoptotic morphology and that C6-ceramide increased the percentage of apoptotic NSCLC cells. These results were consistent with the findings of Kurinna et al. [50], demonstrating that C6-ceramide could induce apoptosis in A549 cells. We also found that C6-ceramide inhibited cell proliferation and migration and could pass through the in vitro BBB model.

The PI3K signaling pathway plays important roles in regulating cell growth and proliferation in a variety of cancers. Cortes et al. showed that PIK3R2 is highly expressed in colon and breast cancers and activates the PI3K signaling pathway to promote tumor progression [51]. Moreover, the PI3K signaling pathway has been reported to regulate the tumor microenvironment in NSCLC [52]. Studies have confirmed that the expression of AKT in melanoma-derived BM is significantly higher than that in extracranial metastases, and bioinformatics cluster analysis showed that the PI3K/AKT signaling pathway is tightly clustered, suggesting that this pathway is activated in BM [53]. In our study, C6-ceramide increased the expression of apoptosis-related proteins and blocked NSCLC cells from penetrating the BBB model through dephosphorylation of components of the PI3K/AKT/mTOR signaling pathway. Treatment of NSCLC cells with LY294002, a PI3K inhibitor, further reduced the ability of cells to penetrate the BBB. These findings further confirmed that C6-ceramide suppressed BM via the PI3K/AKT/mTOR signaling pathway. Moreover, RNA-Seq showed that C6-ceramide was associated with “cell cycle” and “DNA replication”. These findings were consistent with our experimental results.

The BBB is a highly selective semipermeable barrier that serves as a window between the peripheral blood circulation and the central nervous system [54]. The destruction of the BBB is the main cause of tumor metastases [55], and constructing a reasonable BBB model in vitro is the basis for studying the
pathogenesis of BM. Currently, in vitro BBB models are typically constructed using two devices, i.e., transwell chambers and microfluidic chips [56]. In this study, a traditional transwell chamber was used to build a double-layered BBB model using HUVECs and HAs. We also evaluated the permeability of HRP, a reagent with a molecular weight of 40 kDa, and expression levels of tight junction-related proteins to test the effectiveness and availability of the BBB model. We found that coculture of cells for 72 h minimized HRP permeability and maximized the expression of tight junction-related proteins, consistent with a study of BM in breast cancer by Mustafa et al. [57]. Additionally, these results suggested that the in vitro BBB model constructed in this study could better simulate the physiological effects of the human BBB.

To the best of our knowledge, this is the first study to comprehensive explore the biological functions and molecular mechanisms of C6-ceramide in NSCLC cells. Moreover, we also successfully constructed an in vitro BBB model and tested its effectiveness and availability. However, there were some limitations to our study. First, although we found that C6-ceramide promoted NSCLC cell apoptosis through the PI3K/AKT/mTOR signaling pathway, we did not explore the involvement of other mechanisms. In addition, the results of RNA-Seq for PC-9 cells treated with C6-ceramide partly confirmed the findings of molecular experiments. However, further cellular studies of the mechanism and additional RNA-seq analyses may be required to further confirm our findings.

**Conclusion**

In conclusion, our study demonstrated that C6-ceramide not only promoted NSCLC cell apoptosis, proliferation, and migration but also inhibited the ability of cells to penetrate the BBB model via downregulation of the PI3K/AKT/mTOR signaling pathway (Fig. 6). Therefore, C6-ceramide may serve as a novel potential therapeutic target for patients with BM arising from NSCLC.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Medical Ethics Committee of Fujian Provincial Hospital.

**Consent for publication**

Not applicable

**Availability of data and material**

Not applicable

**Competing interests**

The authors declare that they have no conflicts of interest.
Funding

This study was supported by high-level hospital grants from Fujian Provincial Hospital, Fujian province, China (Grant number: 2018GSP008), Startup Fund for scientific research, Fujian Medical University (Grant number: 2017XQ2046 and 2018QH1130), and Health research talents training program of Fujian province (2019-ZQNB-1).

Authors' contributions

Yiquan Xu designed and conducted the experiments, and wrote the manuscript. Junfan Pan, Ying Lin and Yun Wu conducted the analysis of the clinical data. Yusheng Chen and Hongru Li contributed to the experimental design, the review and revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors gratefully acknowledge contributions from researchers from the Department of Virology of the Fujian Provincial Center for Disease Control and Prevention for their help.

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**Figures**
Figure 1

C6-ceramide results in promoted apoptosis in NSCLC cells. (a, b) C6-ceramide increased apoptosis rates in both H1299 and PC-9 cells. H1299 and PC-9 cells were treated with 50 μM C6-ceramide for 24 h, and then subjected to annexin V-FITC / propidium iodide (PI) staining, and analyzed by flow cytometry and fluorescence microscopy. (c) Morphological changes of H1299 and PC-9 cells after C6-ceramide treatment. The connections between cells disappeared and the cytoplasm of some cells was concentrated (red arrow), and some cells showed “bubbling” and debris in the cytoplasm (blue arrow). (d) The expressions of apoptosis-related proteins were determined by western blotting. Data were showed the average of three independent experiments with similar results. The data were presented as the mean ± SD, p values as determined by the t-test.
Figure 2

C6-ceramide suppresses NSCLC cells proliferation, migration, and BM in vitro. (a) CCK-8 assays were used to assessed the proliferative abilities of H1299 and PC-9 cells after treated by C6-ceramide. (b) Cell migratory capabilities were evaluated by wound healing assays. (c) Cells were stained with PI and analyzed by flow cytometry. C6-ceramide induced G1 phase arrest in NSCLC cells. (d) The effects of C6-ceramide on the ability of NSCLC cells to penetrate the BBB. H1299 and PC-9 cells were transfected with EGFP and inoculated into BBB model, the amounts of cells passing through BBB model was observed 24 hours later. Data were showed the average of three independent experiments with similar results. The data were presented as the mean ± SD, p values as determined by the t-test.
Figure 3

C6-ceramide inhibited BM via downregulating PI3K/AKT/mTOR signaling pathway in NSCLC cells. Western blotting detected the expression levels of p-AKT (Ser473) and p-mTOR (Ser2448) in H1299 and PC-9 cells treated with C6-ceramide for 24 h. The data were presented as the mean ± SD, p values as determined by the t-test.
LY294002 further enhanced the apoptotic effects of C6-ceramide in NSCLC cells. (a) Western blotting was performed to analyze the expression of apoptosis-related proteins. (b) The ability of NSCLC cells to cross the in vitro BBB model. H1299 and PC-9 cells treated with C6-ceramide and LY294002 showed a lower ability of crossing the BBB model than cells treated with C6-ceramide alone. These experiments were repeated twice, and the results were reproducible. The data were presented as the mean ± SD, p values as determined by the t-test.
Figure 5

Gene signatures and Pathways enrichment of C6-ceramide in NSCLC cells. (a) Venn chart identified 700 genes were expressed in PC-9 cells treated with C6-ceramide in comparison to matched control group. (b) Volcano plot revealing the differentially expressed genes in DESeq2 analysis with an adjusted p value less than 0.05 and a |log2FoldChange| more than 2. (c) The network relationship between upregulated
genes were analyzed base on STRING database. (d) KEGG pathway was performed to enrich the related pathways.

Figure 6

The schematic diagram shows the mechanism underlying C6-ceramide induces cells apoptosis and suppresses BM in NSCLC.
Supplementary Files

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- FigureS1.tif
- FigureS2.tif