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ORIGINAL RESEARCH

The Mechanism of miR-29 in Bladder Cancer Released by Exosomes into Brain Microglia to Promote M2 Polarization and Angiogenesis in Brain Metastasis of Bladder Cancer

Haopeng Tan, MD; Yongzhao Li, MD; Jianhua Li, BD; Kun Wang, BD; Yongqiang Xiao, MD

ABSTRACT

Objective • To explore the role of miR-29 in bladder cancer, released by exosomes into brain microglia to influence its polarization and promote angiogenesis. This, in turn, would help design therapeutic strategies for brain metastasis caused by bladder cancer.

Methods • The relative expression of miR-29 in normal bladder and bladder cancer cells was compared by qPCR technology, and the difference of specific binding between PI3K and has-miR-29a in the NC group and mimic group was verified by luciferase activity. Bladder cancer cells T24 were transfected with miR-29 NC, mimic, or neferine and divided into miR-29-NC group, miR-29-mimic group, miR-29-NC-neferine group, and miR-29-mimic-neferine group. Then they were co-cultured with microglia BV2 in a 1% hypoxia environment. The protein expressions of p-PI3K, p-AKT, p-AMPK, p-PGC-1α, p-PPARγ, CD206, and HIF1α in glial cells BV2 were detected by Western blot. The effect of each group on angiogenesis was observed by the tube formation experiment. A glioma mouse model was established, and the number of blood vessels and tumor proliferation were observed by pathological section H&E staining, to assess the effect of miR-29 on angiogenesis.

Results • qPCR and dual-luciferase reporter assay showed that miR-29 was highly expressed in bladder cancer compared with normal bladder cells. The binding of miR-29 to PI3K led to the degradation of PI3K mRNA. Protein expression analysis showed that miR-29 inhibited PI3K and p-AKT in bladder cancer cells, and promoted the expression of p-AMPK, p-PGC-1α, p-PPARγ, CD206, and HIF1α. *In vivo* experiments demonstrated that miR-29 could promote the cell volume of bladder cancer cells and increase the number of blood vessels in bladder cancer cells, while neferine could inhibit the above effects.

Conclusion • miR-29 can regulate PI3K/AMPK/PGC-1α/PPAR-γ signaling in microglial cells, promote their polarization to M2, and ultimately promote neovascularization in bladder cancer. (*Altern Ther Health Med.* 2024;30(12):318-323).

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INTRODUCTION

Bladder cancer, as the most common malignant tumor of the urinary system, is the 9th most common malignant tumor in the world.[1] It is one of the ten most common types of cancer in the world, with approximately 550 000 new cases each year.[2]

The incidence of bladder cancer is expected to rise continuously over the next decade.[3] In China, the mortality rate of bladder cancer is the highest among urological tumors, and despite the improvement of surgical treatment, chemotherapy, and immunotherapy in recent years, the 5-year recurrence and metastasis rates are still very high.[4] This warrants further research and an in-depth understanding of the various mechanisms and pathways involved in bladder cancer.

miR-29a is a family of microRNAs (miRNAs) that are closely related to cell proliferation and have numerous functions, including but not limited to the regulation of multiple target genes and play a key role in cell proliferation, differentiation, growth, and apoptosis.[5] Microglia are a key component of the central nervous system (CNS); they are the resident macrophages of the CNS. These monocytes are distributed throughout the brain, and they function as key immune effector cells in the CNS.[6] Microglia associated with

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glial cells are the most multifunctional cells in gliomas, accounting for approximately 30-50% of the total cell number.[7-10] LGALS3BP in microglia plays an important role in angiogenesis, and the phosphorylation levels of PI3K and AKT are increased during hypoxia through the activation of the PI3K/AKT signaling pathway.[11] AMPK (AMP-activated protein kinase) and AKT also directly or indirectly regulate reciprocal phosphorylation.[12] In addition, several studies have shown that AMPK is involved in neuro-regenerative processes. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a downstream target of AMPK that regulates mitochondrial biogenesis and reactive oxygen species synthesis.[13]

Microglia are involved in angiogenesis through the expression of a range of angiogenic factors.[14] Hypoxia activates hypoxia factor-1a (HIF-1α) signaling, which activates a series of cellular processes, including metabolic changes and angiogenesis, to maintain cell viability.[15-19] CD206 protein is an important immune molecule involved in the regulation of immune responses and anti-inflammatory processes. Microglia are characterized by the production of anti-inflammatory cytokines and growth factors such as IL-10, TGF-10, CD-206, and ARG1(Recombinant Human Arginase-1).[20] CD206 and HIF-1α can promote neovascularization under hypoxia. Increased expression of HIF-1α leads to an increase in microglia and induces their polarization and differentiation from M2 to M1 type, which triggers the release of pro-inflammatory mediators.[21] The peroxisome proliferator-activated receptor gamma PPAR γ is a group of lipoprotein-activated transcription factors. In our study, reduced PI3K-AKT phosphorylation and AMPK phosphorylation activity in microglia increased M2 polarization of p-PGC-1α and p-PPARγ, which, together with HIF-1α, promoted angiogenesis, thereby accelerating the onset and progression of brain metastases from bladder cancer. Therefore, studies targeting microglia may provide an experimental basis for the treatment of brain metastases.

The complexity of the role of tiny molecules in human cells and the current lack of understanding of the regulatory mechanisms of miR-29 in bladder cancer presents many obstacles to its therapeutic intervention. Therefore, further studies exploring the mechanism of miR-29 within bladder cancer and ultimately deciphering the role played by miR-29 in regulating microglia are crucial, providing a theoretical basis for the treatment of brain metastasis from bladder cancer.

MATERIALS AND METHODS

Cell co-culture

The murine BV-2 microglia cell line, developed by Dr. V. Bocchini (Blasi et al., 1990), was kindly provided by Dr. Grace Y Sun from the University of Missouri, Columbia, MO. This cell line is not only highly purified but also exhibits morphological, phenotypic, and functional characteristics similar to primary cultured microglia, making it relatively easy to culture. Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The BV2 cells were grown in DMEM medium containing 10% fetal bovine serum, 0.1 mM non-essential amino acids, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HUVEC cells were grown in a complete medium with growth supplements (Cell Applications Inc, San Diego, CA). All cells were grown at 37 °C in 5% CO_2. Recombinant Galectin-3 (R&D Systems, Minneapolis, MN) was added to media as needed.

Quantitative reverse transcription (qPCR)

The cells in each group were digested and collected with 0.25% EDTA trypsin, and total RNA was extracted by adding TRIzol, and its purity and concentration were detected by UV spectrophotometer. Subsequently, total RNA was reverse transcribed into cDNA on a 20 μL reverse transcription system: 4 μL of 5× RT buffer, 0.5 μL of RNase inhibitor, 0.5 μL of oligodeoxynucleotide (dT), 0.5 μL of ReverTra Ace, and DEPC-treated water, in a final volume of 20 μL. Q-PCR was carried out in a fluorescence Q-PCR instrument, including an initial denaturation at 98 °C for 2 min, followed by 40 denaturation cycles at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 30 s. The cDNAs obtained were subjected to Q-PCR in the following system (10 μL): 1 μL of cDNA, 0.5 μL of upstream primer, 0.5 μL of downstream primer, 5 μL of 2× TransStart SYBR Green qPCR supermix and 3 μL ddH_2O.Each experiment was performed four times and each sample was repeated three times. Relative expression was observed by Q-PCR to observe the relative expression of miR-29 in normal bladder cells (SV-HUC) and bladder cancer cells (T24), and to determine the binding site of miR-29 and PI3K.

Primers upstream of miR-29	CCG TCA CGT GCC ACT GCA CGT GCG
Downstream primer of miR-29	GGG TCA CAC GTG TGG TGC ACG TGC
Upstream primer of PI3K	GCA CTT GGT AAT CGG AGG ATA G
Downstream primer of PI3K	CAC CAA TCC CAA GGA CAT AAG A

Dual luciferase reporter assay

A putative hsa-miR-29a 3' binding site was identified in the 3' UTR of the PI3K 3' genome according to predictions based on the target database. Wild-type (WT) and mutant (MUT) sequences were amplified and cloned by polymerase chain reaction (PCR). The PI3K 3' UTR WT and PI3K 3'UTR MUT vectors were established respectively by Xunyu Biological Co. Ltd. Subsequently, the constructed plasmids were mixed with hsa-miR-29a 3' (NC/mimic) and co-transfected into T24 cells within 48 hours. Subsequently, the cells in each group were digested with 0.25% EDTA trypsin, collect and add 100 μL of solute salt, followed by incubation on ice for 5 minutes. Subsequently, completely dissolve the cells and transfer 20 cell lysates to a black microplate. Mix the lysates with the luciferase reaction solution to assess luciferase activity. Simultaneously, employ the vibration method to mix the microplate with renal lipase reaction solution for assessing renal lipase activity. Each experiment was performed in triplicate.

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Protein Expression Analysis

T24 cells were transfected with miR-29 NC OR miR-29 mimic and stimulated with or without Neferine (specific AKT inhibitor) for BV2 cells (cellular stimulation dose), and the above two types of cells were co-cultured in a 1% hypoxic environment, so they were classified into 4 groups: miR-29 NC group; miR-29 mimic group; miR-29 NC + neferine group; miR-29 mimic + neferine group. At the end of the culture, BV2 cells were lysed for protein, and the protein expression of p-PI3K, p-AKT, p-AMPK, p-PGC-1α, p-PPARγ, CD206, and HIF1α was observed using the Western Blot technique to analyze the protein expression in bladder cancer since protein expression effects of miR-29 and PI3K/AKT pathway within brain microglia.

Assessment of tumorigenesis and intra-tumor neovascularization in nude mice

Twenty healthy SPF-grade BALB/c-nu mice, weighing 20 ± 4 g, half female and half male, were used, purchased by Henan Skibbes Biotechnology Company Limited (License No.: SCXK (Yu): 2020-0005). The above four groups of T24 and BV2 co-cultured cells were planted in the back of nude mice to establish an animal model of tumorigenesis in nude mice. All mice were kept in a standard laboratory environment (25 ± 2°C, 60 ± 5% humidity, and 12/12 h light/dark cycle) with free access to food and water, cultured for 4 weeks, and the tumor volume on the backs of nude mice was measured using vernier calipers for each group. After completion of the test, mice were anesthetized using 3% chloral hydrate and executed by cervical dislocation. The brain glioma cells to be observed were extracted, and the value-added of the brain glioma cells and angiogenesis were observed by hematoxylin and eosin (H&E) staining to analyze the effect of bladder cancer on brain gliomas, as well as the effect on angiogenesis.

RESULTS

Q-PCR detection of miR-29 expression in normal bladder cells and bladder cancer

The relative miR-29 expression in normal bladder cells and bladder cancer cells was observed by qPCR, and the relative expression of miR-29 in normal bladder cells was observed to be higher in the walls of bladder cancer cells. The effect of miR-29a on PI3K transcription by miR-29a expression in the miR-29a/PI3K axis was also observed in T24 cells using a dual luciferase reporter assay, and the effect of miR-29a on PI3K transcription was determined in T24 cells. Luciferase reporter assay in T24 cells, and the putative wild-type PI3K 3' UTR WT-binding sequence, as well as the mutant sequence, were determined. There was a difference in PI3K 3' UTR WT and hsa-miR-29a 3' binding, while the PI3K 3 ' UTR MUT and hsa-miR-29a 3' binding did not differ. The results suggest that the binding of miR-29 to PI3K promotes the degradation of PI3K mRNA, and the experimental results are shown in Figures 1A and B. The binding of miR-29 to hsa-miR-29a 3'UTR WT and hsa-miR-29a 3'UTR MUT was not observed.

Figure 1. (A) Statistical Analysis of Observed miR-29 Relative Expression Data in SV-HUC cells and T24 cells. (B) Statistical Analysis of miR-29 Relative Expression Data to PI3KWT and PI3KMUT Binding Proteins in SV-HUC Cells and T24 Cells.

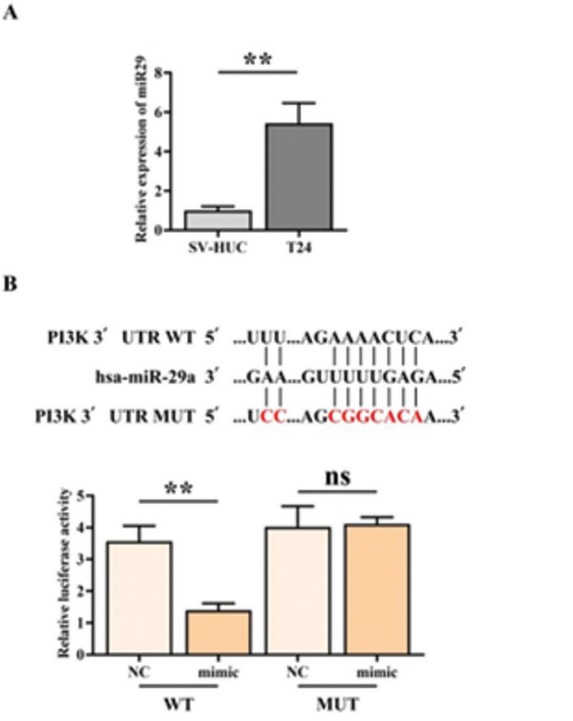
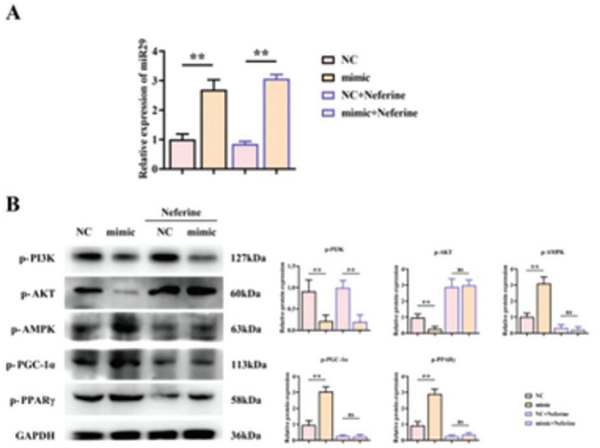


Figure 2. (A) Statistical Analysis of Relative Expression Data of NC Group, Mimic Group, NC-Neferine Group, and Mimic-Neferine Group. (B) Statistical Analysis of Protein Expression Results and Data of NC Group, Mimic Group, NC-Neferine Group, and Mimic-Neferine Group.



Effects of bladder cancer miR-29 on PI3K/AKT/p-AMPK in microglial cells

The relative expression of miR-29 in bladder cancer T24 cells transfected with the mimic group was found to be higher than that in the NC group by qPCR, and the co-culture of BV2 cells with or without neferine stimulation had no significant effect on the expression of miR-29 in T24 cells, as

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Figure 3. (A) Protein Expression Results and Statistical Analysis of Data in NC, Mimic, NC-Neferine, and Mimic-Neferine groups. (B) Observations and Statistical Analysis of Data in *In Vitro* Cell Culture Angiogenesis Experiments.

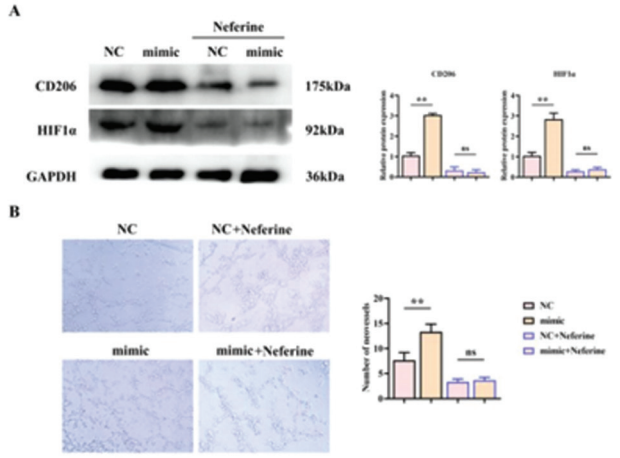
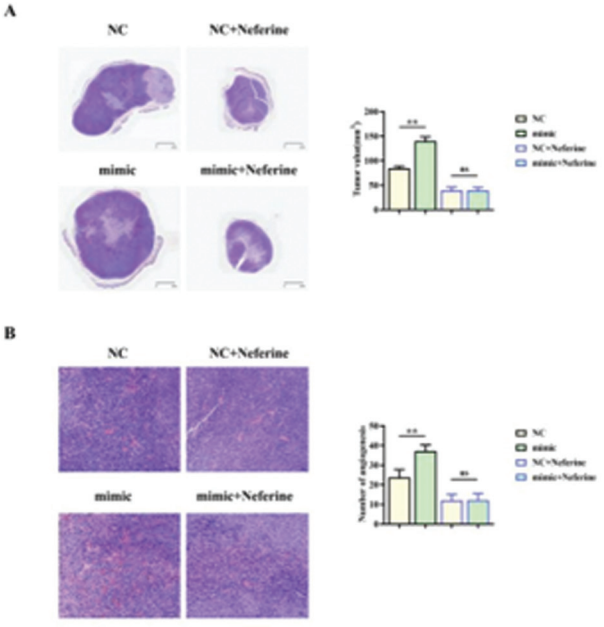


Figure 4. (A) Observations on HE Staining of Gliomas in the NC, Mimic, NC-Neferine, and Mimic-Neferine Groups and Statistical Analysis of Data. (B) Observations on *In Vivo* Cell Culture Angiogenesis Experiments and Statistical Analysis of Data.



shown in Figure 2A. Protein expression of BV2 microglia was detected by Western Blot. The protein expression of p-PI3K and p-AKT was significantly decreased in the mimic group compared to the NC group, while the protein expression of p-AMPK, p-PGC-1α, p-PPARγ, CD206, and HIF1α was higher. After the neferine intervention was given to the BV2 cells, the expression level of p-AKT was significantly elevated, and the corresponding p-AMPK, p-PPARγ, and HIF1α expression levels were significantly increased in the BV2 cells, while miR-29 mimic group and miR-29 NC + neferine group p-AMPK, p-PGC-1α, p-PPARγ, CD206, and HIF1α protein expression decreased significantly (see Figure 2B). This result suggests that after the release of miR-29 overexpression in bladder cancer into microglia, it can bind to PI3K in BV2 cells and induce its deactivation by impeding downstream activation of the AKT pathway, but administration of the BV2 cell-specific AKT agonist neferine significantly inhibited the PI3K/AKT downstream p-AMPK, p-PGC-1α, and p-PPARγ signaling pathways, which have the function of influencing the polarization of BV2 microglia toward M2 and pro-angiogenesis.

Role of bladder cancer and microglia co-culture in influencing vascular neogenesis

The expression of CD206 and HIF-1α in BV2 cells was detected by Western blot, and the above two proteins can promote neovascularization under hypoxia. Thus, the present results are realistic. Compared to the NC group, the miR-29 mimic/overexpression group can significantly increase the expression of CD206 and HIF-1α in BV2 cells; whereas, the administration of neferine to BV2 cells to activate their AKT signaling significantly decreased the miR-29 overexpression-mediated expression of CD206 and HIF-1α, as shown in Figure 3A. The present results suggest that miR-29 in T24 cells can promote the function of M2 polarization and neovascularization of BV2 cells, which is mediated by the PI3K/AKT pathway. The results of tube-forming assay after stimulation of HUVEC cells with the above four groups of cell culture supernatants showed that the number of vascular tubes in the miR-29 mimic group of T24 cells was significantly higher than that in the miR-29 NC group, whereas after BV2 cells were given neferine to activate their AKT pathway, the number of neovascularizations decreased significantly, and there was no difference between the two groups, as shown in Figure 3B. Through the results of *in vitro* HUVEC angiogenesis experiments, it can be hypothesized that miR-29 can influence the angiogenesis of bladder cancer leading to brain metastasis by regulating the phenotype of brain microglia.

Tumorigenic experiments in nude mice to assess the role of miR-29 in bladder cancer and brain glial cells on tumors

Compared to the miR-29 NC group, the tumor volume of bladder cancer in the miR-29 mimic group was significantly increased; whereas, after the administration of neferine to brain microglioma cells, the tumor volume of both the miR-29 NC group and the miR-29 mimic group was significantly decreased; the results are shown in Figure 4A. Further observation of the H&E sections of the tumors revealed that the miR-29 mimic group could significantly increase the number of blood vessels in the tumor, while the number of neovascularization in the above two groups decreased significantly after neferine was given to the co-cultured brain microglia cells (see Figure 4B). The present results suggest that bladder cancer can affect the PI3K/AKT signaling pathway in the brain microglia through miR-29, which can promote the number of blood vessels in the brain metastases from bladder cancer, and then promote its proliferation.

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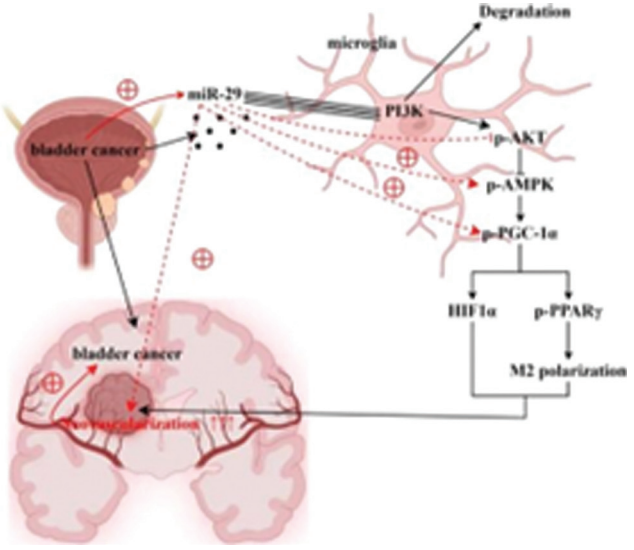
Figure 5. Schematic Representation of the Mechanism Through Which miR-29 in Bladder Cancer is Released into Brain Microglia Via Exosomes and then Promotes M2 Polarization and Ultimately Promotes Glioma Neovascularization by Regulating PI3K/AMPK/PGC-1α/PPAR-γ Signaling



DISCUSSION

Bladder cancer is a malignant tumor with extremely high morbidity and mortality, accounting for the highest rate of incidence of genitourinary tumors in China, while in Western countries, its rate of incidence is second only to prostate cancer. It was estimated that in 2021, there would be 83,730 new cases of bladder cancer and 17 200 related deaths in the United States.[22] Unfortunately, about 10% of all bladder cancer cases are diagnosed in the United States and 10-15% of bladder cancer patients already have metastases at the time of initial diagnosis. 15-30% of advanced bladder cancers eventually progress to advanced disease stage and are associated with poor prognosis.[23] After brain metastasis of bladder cancer, stereotactic radiosurgery and whole-brain radiotherapy can be an effective treatment for brain metastasis of certain malignant tumors.[24,25] The brain requires optimal oxygen and nutrients from the blood, and angiogenesis plays an important role in tumor growth. Anti-angiogenic methods that control angiogenesis at different stages can stop tumorigenesis and can be used as a complementary approach to cancer treatment.

In this experiment, we tried to investigate whether miR-29 has an effect on brain cells after brain metastasis from bladder cancer. The phosphoinositide 3-kinase (PI3K) cascade is a ubiquitous signaling pathway that promotes cell survival, proliferation, and metabolism, and it is the main downstream regulatory protein of miR-29. Therefore, the present study looked at miR-29 and PI3K signaling pathways as the entry point to investigate, and in addition, miRNAs mainly enter the tumor-adjacent cells through exosomes to play their regulatory roles[26-28] PI3K signaling plays an important role in metabolic control, immunity, angiogenesis, and cardiovascular homeostasis, and is one of the most common regulatory pathways in cancer.[28] Bladder cancer cells promote miR-29 expression, derived from the specific binding of miR-29 and PI3K. miR-29 specifically targets PI3K, contributing to PI3K degradation. The AGC kinase family members, AKT1, AKT2, and AKT3, are the downstream effectors of serine/threonine protein kinase and PI3K signaling pathways. Upon PI3K activation, cytoplasmic AKT1 is translocated to the membrane where it interacts with PIP3, leading to phosphorylation and activation of AKT1. AKT is critical for cell survival, growth, and metabolism.[29] The PI3K/AKT signaling pathway is a major and important signaling pathway that regulates M2 polarization in brain microglia.[30,31] We first analyzed the expression of PI3K and AKT and found that the phosphorylation levels of PI3K and AKT increased under hypoxia. Under glucose deficiency, AKT played an antagonistic role to AMPK in apoptosis. In addition, AMPK and AKT directly or indirectly regulated mutual phosphorylation. When glucose is abundant, AMPK activity remains limited, and AKT is relatively active, which promotes the growth, division, and metastasis of cancer cells. In addition, autocrine or paracrine signals of growth factors activate AKT in a positive feedback loop under the right environment.[12] AMPK is an important sensor of intracellular energy charge reduction, and its role is to increase catabolism and decrease synthesis. p-PGC-1α is an important regulator of the transcription of many energy homeostasis genes and is particularly involved in fuel oxidation and mitochondrial biology. Phosphorylation of AMPK increases the activation of the PGC-1α receptor to its promoter. AMPK activation leads to an increase in the expression of PGC-1α,[32] and AMPK requires p-PGC-1α activity to regulate the expression of several key players in mitochondrial and glucose metabolism.[33]

Glial cells are a self-renewing population of tissue macrophages that play an important role in the development and stabilization of the CNS.[34] The microglia/macrophages are polarized into two phenotypes, namely, a typically activated pro-inflammatory (M1) phenotype and an alternatively activated anti-inflammatory (M2) phenotype.[35,36] The M2 state of microglia improves phagocytosis and produces cells with anti-inflammatory or tumor-supportive characteristics.[37,38] For example, the typical M2 state marker CD206, a C-type lectin that functions in endocytosis and phagocytosis, plays an important role in intra-immune homeostasis by removing unwanted mannoprotein.[39] CD206 is a C-type lectin that functions in endocytosis and phagocytosis and plays an important role in intra-immune homeostasis by removing unwanted mannoprotein. CD206 is a 175-kDa transmembrane protein encoded by the mannose receptor c-type 1 gene. It is predominantly expressed in macrophages, dendritic cells, and endothelial cells, where it functions as a receptor for glycolytic ligands, such as microbial antigens.[40] It is expressed in macrophages, dendritic cells, and endothelial cells. In neural tissues, CD206 expression has been observed in microglial cells[41,42] and astrocytes[43,44] CD206 promotes the activation of microglia, thereby increasing their growth and migration.[45] Microglia activation is dependent on glycolysis and mammalian targets of the rapamycin-hypoxia-inducible factor-1-α (HIF-1α)

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pathway. Activated microglia, in turn, secrete pro-inflammatory cytokines and adopt a phagocytic phenotype, whereas tolerant microglia can be induced by sustained exposure to pathogens.[46] Hypoxia-inducible factor-1 is an endogenous transcription factor that contributes to the cellular response to hypoxia. HF-1 is a heterodimer consisting of constitutively expressed HF-1 and HIF1α subunits. If oxygen is present, HIF-1α is degraded by oxygen-dependent hydroxylation with propanol. If oxygen concentration decreases, a stabilized HIF-1 complex is formed, which activates the transcription of genes encoding several proteins that are implicated in angiogenesis, glucose metabolism, and cell proliferation/survival. Significantly, HIF-1α induces the transcription of proteins associated with inflammation in various diseases, such as T-cell immunoglobulin and mucus vesicle 3, and vascular endothelial growth factor.[21] It follows that CD206 and HIF-1α promote angiogenesis in bladder cancer cells under hypoxic conditions. Angiogenesis is a key process in the growth and development of human cancer.[47] Microglia combined with macrophages not only target glioma cells but also affect angiogenesis and indirectly influence tumor growth. By means of M2 polarization, microglia in combination with macrophages can promote tumor proliferation, survival, and migration.[6]

CONCLUSION

Through the present study, we determined that bladder cancer can promote PI3K degradation in BV2 cells by promoting the expression of miR-29. The miR-29 enters the brain microglia, thus promoting the activation of the p-AKT/p-AMPK/p-PGC-1α pathway, which ultimately leads to the polarization of BV2 towards the pro-neovascular M2-type ultimately secreting pro-angiogenic factors such as HIF-1α, VEGF, and so on. Neovascularization factors assist in promoting the enhancement of neovascularization in brain metastatic tumors, so the present study provides a new therapeutic idea and a solid experimental basis for the prevention and treatment of brain metastasis of bladder cancer.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing interests.

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