Association of aquaporin-1 with tumor migration, invasion and vasculogenic mimicry in glioblastoma multiforme

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Received July 25, 2017; Accepted November 11, 2017

DOI: 10.3892/mmr.2017.8265

Abstract. The present study aimed to assess the expression and functional role of aquaporin-1 (AQP1) in glioblastoma multiforme (GBM) migration, invasion and vasculogenic mimicry (VM). In the primary human gliomas and human glioma-derived cell lines tested, it was observed that the expression of AQP1 was upregulated. In addition, it was demonstrated that silencing of AQP1 expression resulted in decreased migration and invasion, in addition to vasculogenic mimicry *in vitro*. It was additionally observed that silencing of AQP1 expression resulted in *in vivo* inhibition of tumor growth, a decrease in the expression of invasion-associated protein, and suppression of VM formation. Based on these data, it was concluded that AQP1 may serve a role in GBM migration, invasion and VM formation, and that it may serve as a novel diagnostic/prognostic biomarker and a potential therapeutic target.

Introduction

Glioma is the most common malignant tumor of the central nervous system in adults. According to the WHO classification of tumors of the nervous system, glioma may be further divided into four grades (grades I-IV) with increasing malignancy (1). Grade IV, additionally termed glioblastoma multiforme (GBM), is the most malignant type of brain tumor. Despite the improved survival associated with modern surgical, chemotherapy and radiotherapy treatments, the prognosis of patients with glioma remains poor due its rapid and invasive growth, its genetic heterogeneity, and a lack of understanding of its underlying molecular mechanisms (2,3).

Vasculogenic mimicry (VM) refers to non-endothelial tumor cell-lined microvascular channels in aggressive, malignant and genetically dysregulated tumors (4,5). A previous report indicated that VM had been implicated in invasion, metastasis and cancer progression (6). However, there is limited data regarding the correlation with VM and abnormally-expressed genes in human gliomas. Aquaporins (AQPs) are a family of water-selective transmembrane transport channels that allow rapid movement of H_2O across normally hydrophobic cell membranes (7). In previous studies, AQP1 and AQP4 have received the most attention due to their contributions to brain edema (8,9). In brain tumors, studies have demonstrated that AQP1 expression is increased with the grade of malignancy, and was associated with tumor blood vessels (10,11). However, the role of AQP1 remains speculative in glioma.

In the present study, the AQP1 expression was measured in clinical glioma tissue samples and GBM cell lines. Subsequently, short hairpin (shRNA)-mediated AQP1 silencing was used to assess the potential effects of AQP1 on migration, invasion and VM formation using two types of GBM cell lines *in vitro* and *in vivo*. The present study aimed to assess the expression and functional role of aquaporin-1 (AQP1) in human GBM migration, invasion and VM formation.

Materials and methods

Clinical samples and cell lines. All the clinical glioma tissues samples were obtained from Guangzhou Overseas Chinese Hospital (Guangzhou, China) and, according to criteria from the World Health Organization (WHO), classified into 4 grades with increasing malignancy: Grade I pilocytic astrocytoma; grade II astrocytoma; grade III anaplastic astrocytoma; and grade IV GBM, the most malignant brain tumor. For each grade, 10 cases were used in the present study. All samples were freshly frozen in liquid nitrogen and stored at -80˚C until RNA extraction. The present study was approved by the Institutional Review Boards of the Guangzhou Overseas Chinese Hospital, and all participants provided written informed consent. The GBM cell lines A172 and U251, and the normal glial cell line HEB were obtained from the Shanghai Cell Collection (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) at 37˚C in a 5% CO_2 humidified atmosphere.

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Key words: aquaporin-1, migration, invasion, vasculogenic mimicry, glioblastoma multiforme

Cell transfection. The shRNA-AQP1 was synthesized and cloned into the pSUPER-retro-puromycin plasmid (Shangahi GenePharma Co., Ltd., Shanghai, China). The shRNA-AQP1 sequence was: 5'-GATCACACACAACTTCAGCAACTC GAGTTGCTGAAGTTGTGTGTGATC-3, and the negative control sequence was: 5'-CACCGTTCTCCGAACGTGTCA CGTCGAAACGTGACACGTTCGGAGAA-3'. The plasmids above were combined with PIK vector, and lentiviral vectors were constructed. Following 10 h transfection (multiplicity of infection=20) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA), according to the manufacturer's protocol, the transfected A172 and U251 cells were selected with puromycin, and the stably transfected cell lines were prepared by monoclonal screening. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were used to assess the transfection efficiency as detailed below.

RT-qPCR assay. Total RNA was extracted from the tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) was used, according to the manufacturer's protocol. qPCR was performed to detect the expression levels of AQP1 mRNA using the LightCycler 480 detection system (Roche Diagnostics, Indianapolis, IN, USA). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 3 min, followed by 45 cycles of 95˚C (15 sec) and 60˚C (30 sec). The primer sequences of AQP1 were: Forward, 5'-TCATCTACGACTTCATCCTGGC-3' and reverse, 5'-GGAAGCTCCTGGAGTTGATGT-3'. β-actin mRNA levels were used for normalization: Forward 5'-GTC CACACCCGCCACCAGTTC-3' and reverse 5'-TCCCACCAT CACACCCTGGTG-3'. The qPCR results were analyzed and expressed as relative mRNA levels of the Cq value, which was then converted to a fold change (12).

Western blot analysis. The tissue samples and cells were lysed in a radio-immunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40] containing a protease inhibitor cocktail (Roche Diagnostics), and the protein concentration was measured using a micro bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 50 μg per lane of the total cell lysates was resolved on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, Inc.). The PVDF membranes were blocked with 5% non-fat dry milk for 1 h at room temperature, and followed by immunoblot detection and visualization with enhanced chemiluminescence western blot detection reagents (Pierce; Thermo Fisher Scientific, Inc.). Immunoblotting was performed with AQP1 antibodies (Abcam, Cambridge, UK; cat. no. ab15080; 1:1,000) at 37˚C for 2 h, followed by incubation with the horseradish-peroxidase-conjugated immunoglobulin G secondary antibodies (Abcam; cat. no. ab97023; 1:5,000) for 1 h at room temperature. GAPDH (Abcam; cat. no. ab8245; 1:1,000) levels were used for the control and normalization. The protein bands were scanned and quantified using ChemiDoc MP imaging analysis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as the relative grey value.

Transwell migration and invasion assay. The transfected A172 and U251 cells were trypsinized and resuspended to a density of $5x10^5$ cells/ml in serum-free medium. A total of 200 μl cell suspension was added to the upper chamber of each well in 24-well polycarbonate Transwell membrane inserts (BD, 353097, USA) coated with 40 μl extracellular matrix (ECM) Matrigel (invasion assay; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or without Matrigel (migration assay), and 600 μl Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum was added in the lower chamber. Following incubation for 24-48 h at 37˚C, cells on the upper membrane surface were removed by careful wiping with a cotton swab, and the Transwells were fixed in 4% paraformaldehyde solution for 30 min at room temperature and stained with 0.2% crystal violet solution for 30 min at room temperature. Migrated and invaded cells adhering to the underside of the Transwell were counted using an inverted microscope (magnification, x400).

VM assay. A total of 50 μl ECM Matrigel (Sigma-Aldrich; Merck KGaA) was dropped onto 18-mm glass coverslips in 6-well plates and incubated at 37˚C for 1 h. Subsequently, the transfected A172 and U251 cells ($2x10^5$ cells/well) were seeded onto the coated coverslips. Following incubation for 24-48 h, the cells were fixed in 4% paraformaldehyde-PBS for 10 min at room temperature, oxidized in a 0.5% periodic acid solution for 5 min and rinsed with PBS. The coverslips were dried at room temperature and the VM images were captured at x400 magnification (CX71, Olympus Corporation, Tokyo, Japan).

In vivo xenograft experiments. A total of 6 female BALB/C nude mice (weight, 20-22 g) at the age of 4 weeks were obtained from the Laboratory Animal Centre of Jinan University (Guangzhou, China). The animals were maintained on a 12 h light/dark cycle under room temperature (24±1˚C) and humidity of 50±10%, and free fed with standard forage and clean water. They were randomly divided into two groups (three mice per group). The silenced A172 cell and negative control cell suspensions ($5x10^6$ cells/ml) in 200 μl serum-free medium were subcutaneously injected into the flanks of nude mice. Tumor growth was measured twice per week for 4-5 weeks. Following 5 weeks, tumor samples were carefully isolated, weighed and analyzed by hematoxylin-eosin (HE) staining. The experimental protocol was approved by the Laboratory Animal Ethics Committee of Jinan University (Guangzhou, China).

Invasion-associated protein measurement and HE staining. The isolated tumor tissues were divided into two parts: One for the measurement of the invasion-associated proteins αvβ3 integrin (Abcam, Cambridge, UK; cat. no. ab78289; dilution, 1:500), 72 kDa type IV collagenase (MMP-2; Abcam; cat. no. ab37150; dilution, 1:500) and matrix metalloproteinase-9 (MMP-9; Abcam; cat. no. ab38898; dilution, 1;1,000) using western blot analysis as aforementioned; and the other for HE staining.

The tissues were fixed in a 4% paraformaldehyde-PBS solution for 30 min at room temperature, and sliced into 3-5 μm sections following paraffin-embedding. Slides were stained as follows: 70% ethyl alcohol for 10 sec; diethylpyrocarbonate-treated water for 5 sec; hematoxylin with RNAase

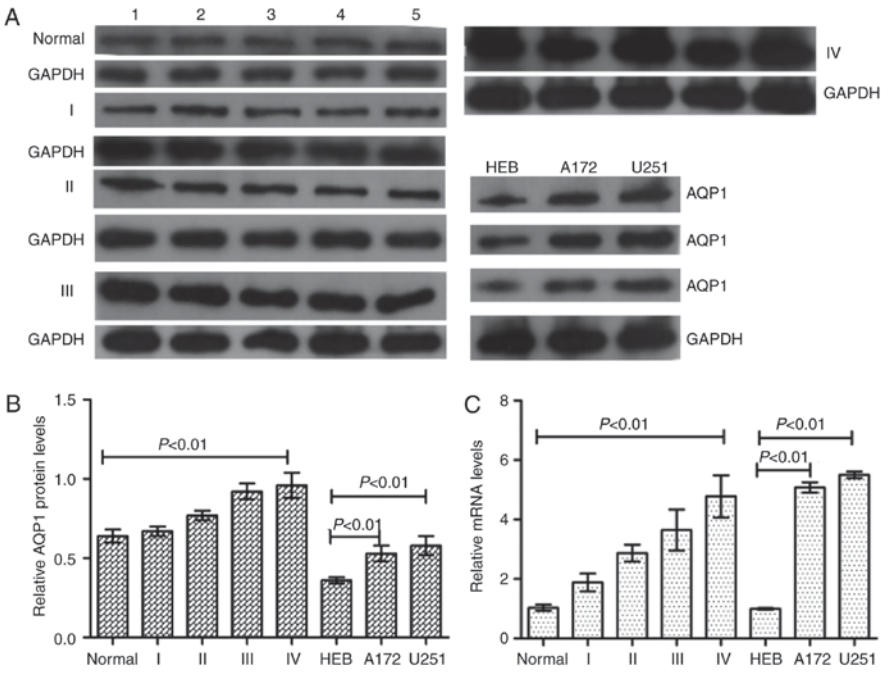

Figure 1. Expression of AQP1 protein and mRNA in primary glioma tissues samples, and HEB, A172 and U251 cells. (A) Western blot analysis of AQP1 protein in glioma tissues of different grades and glial cell lines. (B) Gray scale analysis of relative AQP1 expression levels in the western blot images. (C) The relative AQP1 mRNA levels, as analyzed by reverse transcription-quantitative polymerase chain reaction. The relative expression of AQP1 is presented as the ratio of the expression level of GAPDH. Values are expressed as the mean ± standard deviation. n=3. AQP1, aquaporin-1.

inhibitor for 20 sec; 70% ethyl alcohol for 30 sec; eosin Y in 100% ethyl alcohol for 20 sec followed by dehydration with a series of alcohols for 30 sec each; and xylene for 2 min.

Statistical analyses. The data were analyzed by one-way analysis of variance and data from the multiple groups were also analyzed using analysis of variance with repeated measures. The Student's t-test to determine statistical significance using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). In addition, the least significant difference post-hoc test was employed where equal variances were assumed, while Dunnett's T3 test used when equal variances were not assumed. The results are expressed as the mean ± standard deviation with at least three times. Two-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

AQP1 is upregulated in glioma tissues and cell lines, and is associated with malignancy grade. The expression of AQP1 in normal brain tissues, glioma tumor tissues and glioma cell lines was analyzed by western blotting and RT-qPCR analysis. Western blotting and RT-qPCR analysis demonstrated that AQP1 was expressed at higher levels in glioma tumor tissues and cell lines compared with normal brain tissues and HEB cells, and was positively associated with glioma malignancy (glioma grades; Fig. 1), meaning that a higher malignant grade of glioma was associated with a higher AQP1 expression level.

Silencing of AQP1 inhibits GBM migration and invasion. To examine the role of AQP1 in glioma cells, AQP1 was stably silenced using shRNA-AQP1 in A172 and U251 cells. As presented in Fig. 2, RT-qPCR analysis and western blotting

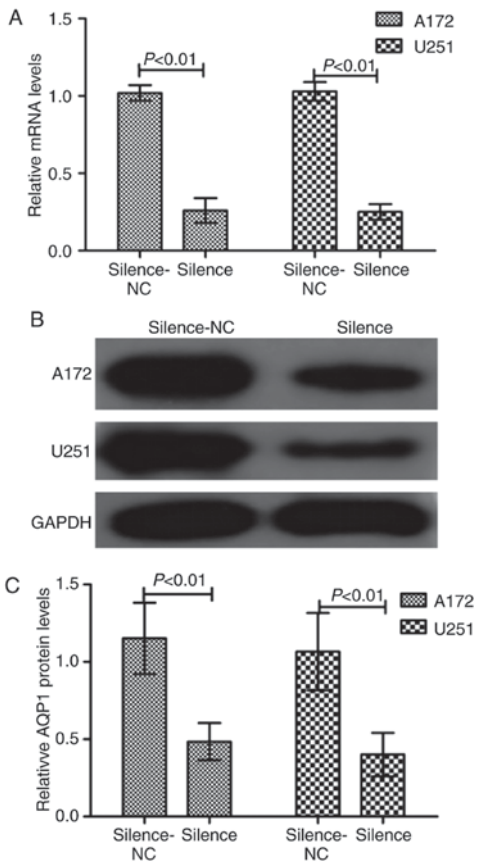

Figure 2. Expression of AQP1 in glioblastoma multiforme cells following transfection. (A) Expression level of AQP1 mRNA in each group. (B) The western blotting images of AQP1 protein in each group. (C) Gray scale analyses of the relative AQP1 expression levels in the western blotting. The relative expression of AQP1 is presented as the ratio of the expression level of GAPDH. Values are expressed as the mean ± standard deviation. n=3. AQP1, aquaporin-1; NC, negative control.

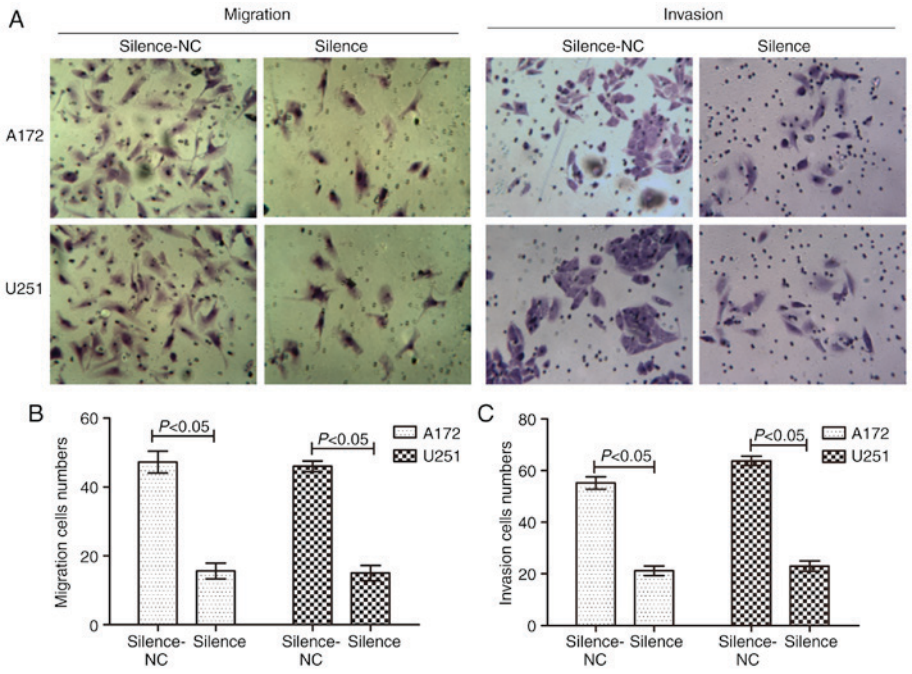

Figure 3. Silencing of aquaporin-1 expression inhibits glioblastoma multiforme cell migration and invasion *in vitro*. (A) Graphical representation of Transwell migration and invasion assays in each group (magnification, x400). (B) The statistical results of migrated cell numbers. (C) The statistical results of invaded cell numbers. The data are presented as the mean ± standard deviation. n=3. NC, negative control.

demonstrated that the transfection was successful in reducing the expression levels of AQP1. Following transfection, a Transwell assay demonstrated that the migratory and invasive capacity were markedly reduced in the silenced group, demonstrating that the number of migrated and invaded cells decreased (Fig. 3). There was a statistical difference between the NC and transfected groups (P<0.05). These results suggested that silencing of AQP1 reduced the abilities of migration and invasion in GBM cells.

Silencing of AQP1 inhibits the development of VM in vitro. VM is associated with tumor blood supply and metastasis. The number of vessels (nodes) and the remodeling of the microcirculation are used as histological markers of tumor progression. In *in vitro* experiments, the vessel numbers of the network channels, which reflect VM development, were calculated and analyzed. It was observed that A172 and U251 cells in the silence-NC group, which express high levels of AQP1, formed classical VM networks on Matrigel. Following silencing of AQP1 via transfection, it was observed that the classical VM networks became less obvious, and the number of vessels decreased significantly in the silence group, compared with the silence-NC group (Fig. 4). These results suggested that AQP1 may regulate the development of VM in GBM cells *in vitro*.

Silencing of AQP1 inhibits tumorigenesis and induces invasion-associated protein expression. In order to study the effect of AQP1 on tumorigenesis and tumor infiltration *in vivo*, the transfected A172 and U251 cells were planted into the nude mouse xenograft model. Across the 35 days, there was a marked decrease in tumor weight in the silence group compared with the silence-NC group (Fig. 5A and B). In addition, western blot analysis of invasion-associated proteins demonstrated that in the case of decreased AQP1 expression, the expression of αvβ3

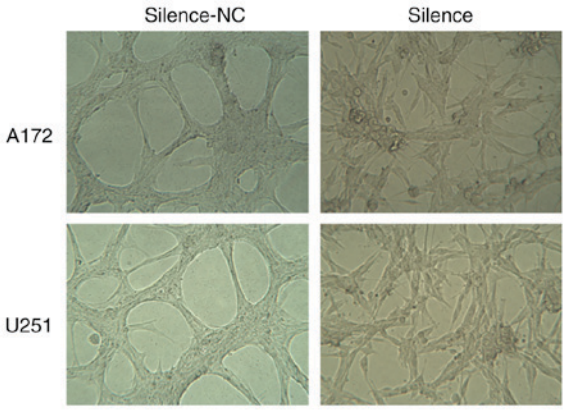

Figure 4. AQP1 induces the development of vasculogenic mimicry (VM) *in vitro*. Following silencing of AQP1, network formation was assessed using an inverted microscope (magnification, x200). AQP1, aquaporin-1; NC, negative control.

integrin, MMP-2 and MMP-9 in the silence group decreased (Fig. 5C and D). There were statistical differences between the silence-NC group and the silence group (P<0.05). These results suggested that silencing of AQP1 reduced the abilities of tumorigenesis and tumor infiltration *in vivo*.

Silencing of AQP1 inhibited the development of VM in vivo. *In vivo*, cancer tissues require an adequate blood supply for growth, and VM serves as an alternative pathway for maintaining this supply. In the *in vivo* experiment, VM was analyzed using the *in vivo* xenograft tumors via HE staining. HE staining demonstrated that A172 cells in the silence-NC group formed a large number of typical vascular structures (red arrows; Fig. 6). Following silencing of AQP1 via transfection, the number of vascular structures decreased significantly in the silence group,

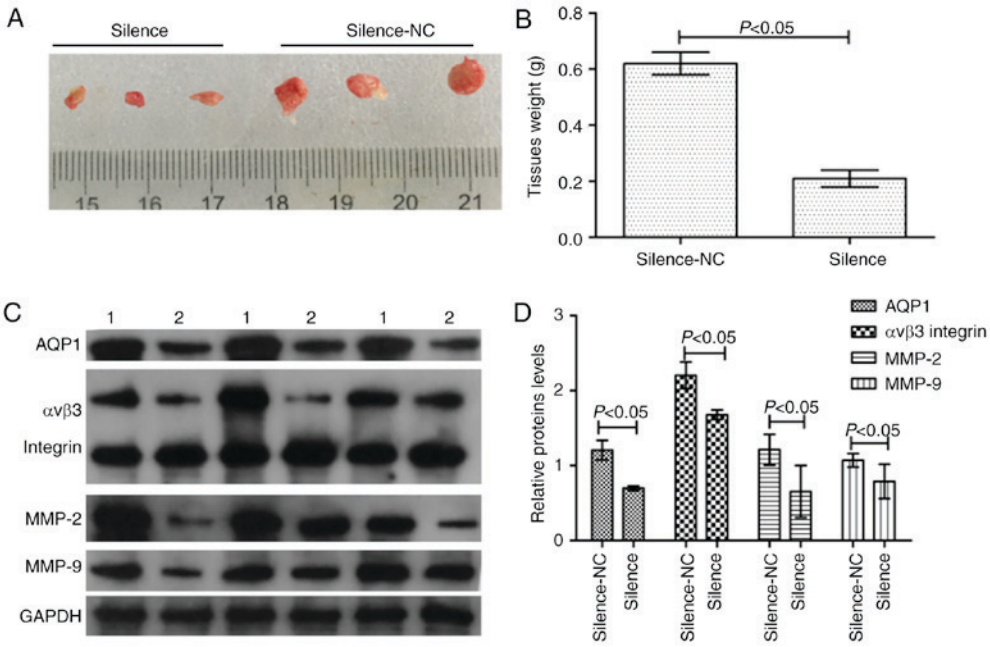

Figure 5. AQP1 regulates tumorigenesis *in vivo*. (A) Macroscopic appearance of xenotransplanted tumors. (B) Quantitative analysis of tumor weights. (C) The western blotting images of AQP1, αvβ3 integrin, MMP-2, MMP-9 protein. 1, Silence-NC group; 2, silence group. (D) Gray scale analysis of relative AQP1, αvβ3 integrin, MMP-2, MMP-9 expression levels in the western blotting. Values are presented as the mean ± standard deviation. n=3. AQP1, aquaporin-1; MMP-2, 72 kDa type IV collagenase; MMP-9, matrix metalloproteinase-9; NC, negative control.

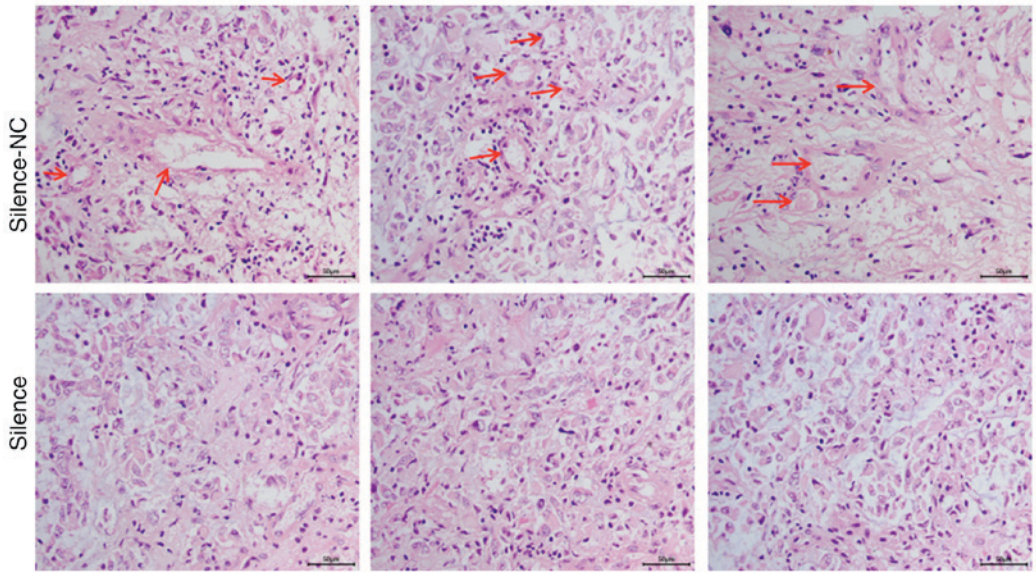

Figure 6. Aquaporin-1 induces the formation of vasculogenic mimicry *in vivo*. The red arrows indicate the vascular structures.

compared with the silence-NC group. These results suggested that AQP1 may regulate the development of VM *in vivo*.

Discussion

Using western blotting and RT-qPCR, the present study demonstrated that AQP1 expression levels were upregulated and positively associated with glioma grade, and that the highest expression levels were observed in GBM cells. Based on the characteristics of malignant glioma and this observation, it was hypothesized that AQP1 may be associated with GBM migration and invasion. In addition, a previous report revealed that AQP1 regulated cell volume via the rapid transmembrane transport of water, thereby promoting cell migration (13). In a study into the metastasis of lung cancer, the researchers reported that AQP1 led to extravasation and spread (14).

In order to verify the above hypothesis, shRNA-mediated AQP1 silencing was used to assess the potential effects of AQP1 on migration and invasion using two types of GBM cell line. The Transwell assay demonstrated that silencing of AQP1 was able to suppress GBM cell migration and invasion *in vitro*. In angiogenesis, tumor growth and metastasis, MMPs (including MMP-2 and MMP-9) and integrins (including αvβ3) degrade the ECM and release and/or activate growth factors, finally

resulting in cancer cell migration and invasion (15,16). Previous reports demonstrated that elevated αvβ3 integrin, MMP-2 and MMP-9 expression levels in tumor cells markedly increased the adhesion and migration of the tumor cells (17-20). In the present study, western blot analysis using *in vivo* xenograft tumor tissues demonstrated that invasion-associated protein (αvβ3 integrin, MMP-2 and MMP-9) expression decreased in the AQP1 silence group, indicating that silencing of AQP1 was able to suppress GBM migration and invasion *in vivo*. Additionally, AQP1 knockdown or inhibition may effectively inhibit cell proliferation, invasion and tumorigenesis in osteosarcoma and hepatocellular carcinoma (21,22). From the above data, it was inferred that AQP1 was indeed associated with GBM migration and invasion, and that silencing of AQP1 was able to suppress GBM migration and invasion, *in vitro* and *in vivo*.

Inhibiting angiogenesis is an important therapeutic approach in cancer (23,24). The proteins that regulate abnormal angiogenesis have attracted intense interest. AQP1, as a water channel membrane protein, was able to promote tumor angiogenesis by allowing faster endothelial cell migration in a mouse model of melanoma (25,26). In human glioma, it was demonstrated that elevated AQP1 in GBM cells led to a typical angiogenesis structure, and that AQP1 knockdown reduced VM structure formation *in vitro*. *In vivo*, VM channels are patterned networks with red blood cells readily detectable inside such channels, and are arranged in arcs, loops and networks (27). In the present *in vivo* xenograft experiment, HE staining exhibited typical VM channels in GBM cells and, following AQP1 silencing, the number of typical VM channels decreased. These results demonstrated that VM formation may be inhibited by regulating AQP1 expression in human GBM.

In conclusion, AQP1 was positively associated with glioma grade and promoted glioma cell migration, invasion and VM formation *in vitro* and *in vivo*. In the treatment of malignant glioma, the present study may provide a strategy and facilitate the development of AQP1-directed diagnostics and therapeutics against glioma. Further studies ought to be aimed at performing a systematic evaluation of AQP1 in gliomas of different grades, and correlating such findings with clinical survival parameters.

Acknowledgements

The present study was supported by the Scientific Research and Cultivation of Special Fund of the First Clinical Medical College of Jinan University (grant no. 2015109).

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