Original Article

AZT sensitizes hepatocellular carcinoma cells to As_2O_3 by up-regulating the arsenic transporter aquaglyceroporin 9

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Background: Even though the mechanism is unknown, previous studies have demonstrated that the traditional chemotherapy agents, 3'-azido-3'-deoxythymidine (AZT) and arsenic trioxide (As_2O_3), can synergetically inhibit the growth, migration and the invasion of hepatocellular carcinoma cells. This study aimed to investigate the role which aquaglyceroporin 9 (AQP9), an arsenic channel protein which is widely distributed in the liver tissues, plays in the process of As_2O_3 combined with AZT in inhibiting growth, migration and the invasion of hepatocellular carcinoma cells.

Methods: HepG2 and MHCC97H cells were treated using As_2O_3 (2 μM) combined with a wide range of different concentrations of AZT (0, 10, 20 μM) for 48 h, and the proliferation inhibition rates were detected using a MTT assay. AQP9 mRNA/protein expression was determined by RT-PCR and western blotting assays, respectively. Next, HepG2 cells were electro-transfected with AQP9 siRNA to disturb the expression of AQP9, which was verified by RT-PCR and western blotting. The effect of the 2 μM As_2O_3 combined with 20 μM AZT (the optimum synergy inhibition concentration investigated previous) on the migration and invasion of HepG2 cells was then measured by wound healing assay and transwell migration assay and invasion assays, respectively, before and after an AQP9-siRNA transfection.

Results: The proliferation inhibition rate from the combination of As_2O_3 and AZT on the hepatocellular carcinoma cells was significantly higher than the rate from As_2O_3 alone ($P<0.05$), and it had a greater dose-dependent effect when it was compared to AZT. A low dose of As_2O_3 (2 μM) combined with AZT (20 μM) also significantly inhibited the migration and invasion of liver cell carcinoma. The expression levels of AQP9 in the combination of As_2O_3 (2 μM) and AZT (20 μM) group were significantly higher than those from the As_2O_3 alone group and the blank control group ($P<0.05$). After silencing AQP9, the effect of the combination between As_2O_3 and AZT for the migration and invasion of liver cell carcinoma was significantly reduced ($P<0.05$).

Conclusions: AZT improves the efficiency of As_2O_3 on inhibiting the proliferation, migration and invasion of hepatocellular carcinoma cells by up-regulating the expression of AQP9, which has important implications in reducing the poisonous side effect of As_2O_3 for cancer therapies.

Keywords: HepG2; 3'-azido-3'-deoxythymidine (AZT); arsenic trioxide (As_2O_3); aquaglyceroporin 9 (AQP9); proliferation; migration; invasion

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Introduction

Arsenic trioxide (As_2O_3) is a highly efficacious drug (1) and has been shown to be successful as a first-line treatment option in induction therapy for the patients with acute promyelocytic leukemia (APL) and as a maintenance therapy (2,3). Furthermore, As_2O_3 has also been reported to have anti-proliferative and proapoptotic activities in some solid tumors, including lung adenocarcinoma, hepatocellular carcinoma and cervical cancer (4-6). As a drug that has chemo-preventative qualities, As_2O_3 has been demonstrated to induce apoptosis through the generation of a reactive oxygen species and a superoxide compound (7,8). This leads to cell arrest through the processes of demethylation and alteration in the expression level of the cell cycle-related genes leading to cell cycle arrest (9), induction DNA damage, and the disruption of the mitochondrial transmembrane potential with the release of cytochrome c and caspase activation (10). However, As_2O_3 is a double-edged sword during cancer treatment. Its cardiotoxicity, neurotoxicity and other side effects (11-14) are a significant obstacle for further application. Side effects such as cardiotoxicity and neurotoxicity present a major obstacle for its use in a clinical environment.

3'-azido-3'-deoxythymidine (AZT) is a thymidine analogue, which has been shown to have antitumor effects against adult T-cell leukemia/lymphoma and pancreatic cancers. As Namba reported, AZT could resensitize the gemcitabine-resistant pancreatic cancer cells for gemcitabine via the inhibition of the Akt-GSK3β-Snail pathway (15). Accordingly, our previous results showed that As_2O_3 combined with AZT has significantly important synergistic inhibitory effects on the proliferation, migration and the invasion abilities of the HepG2 cells (16,17). However, the mechanism is still unknown.

Since As_2O_3 shows its cytotoxic activity only when entering cells, the arsenic carrier appeared to be an important player for modulating arsenic sensitivity. Previous studies have shown that aquaglyceroporin 9 (AQP9), which is mostly expressed in tumor cells and human leukocytes, was supposed to be related to the absorption of As_2O_3. This indicated that it has a critical role in determining the sensitivity of the tumor cells towards the As_2O_3 induced cytotoxicity (18-20). In acute myeloid leukemia cells, azacytidine facilitates the uptake of As_2O_3 by up-regulating the arsenic transporter AQP9 (21). Moreover, all-trans retinoic acid (ATRA) up-regulated AQP9, contributes to a significantly increased As_2O_3-induced cytotoxicity on combination with As_2O_3 (20). Chen and his colleagues found that AQP9 played a key role in the proliferation, apoptosis and metastasis of androgen-independent prostate cancer cells (22), which shows the same effects with our previous work in the HepG2 cells. Thus, we proposed that one of the mechanisms of synergism between AZT and As_2O_3 might be through a modulation of AQP9 expression.

Methods

Chemicals and reagents

AZT and 3-(4,5-dimethyl thiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Chemical Co. (St Louis, Michigan, USA). As_2O_3 was obtained from the Shui Kou Shan mining bureau of Hengyang industrial company, China. Other cell culture supplies were purchased from HyClone (Logan, UT, USA). The antibody to AQP9 and β-actin was purchased from ImmunoWay Biotechnology Co. (Newark, DE, USA). Peroxidase-Conjugated AffiniPure Goat anti-Rabbit IgG (H+L) was purchased from ZSGB-BIO Co. (Beijing, China). Real-time reverse transcription polymerase chain reaction (RT-PCR) related reagents were purchased from Promega (Madison, WI, USA). siRNA-AQP9 was purchased from Ambion (Austin, TX, USA). Transwell boyden chamber was purchased from Corning (Corning, NY, USA).

Cell culture

The human hepatocellular carcinoma cell lines HepG2 and MHCC97H cells were purchased from JRDUN Biotechnology Co. (Shanghai, China). The hepatocellular carcinoma cell lines HepG2 and MHCC97H were cultured in DMEM with a high glucose content containing 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin/streptomycin at 37 ℃, and were incubated in a 5% CO_2 humidified atmosphere. Cells were harvested in a logarithmic phase for experiments.

Cell proliferation assay

The Cellular viability of the HepG2 and MHCC97H cells was assessed by the reduction process of MTT to formazan as described earlier (23). Cells were seeded into 96-well microtiter plates at a density of 1×10^4 cells/well with DMEM. After a period of 24 h, the culture medium in each well was replaced with a fresh medium and treated with a

Table 1 Primers for PCR

Primer name	Forward sequence	Reverse sequence
AQP9	CTTTGGACGGATGAAATGGTT	GAGTCAGGCTCTGGATGGTG
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

2 μM As_2O_3 solution combined with different concentrations of AZT (0, 10, 20 μM) for an additional 48 h. Then, 10 μL of a MTT solution (5 mg/mL in PBS) was added to each well. After a 4 h incubation at 37 ℃, the formazan salt crystals were dissolved in 100 μL of lysis solution (10% SDS in 0.01 M HCl). The absorbance (OD) values were measured spectrophotometrically at 490 nm using a microplate reader (Bio-RAD, Benchmark Plus, USA). Each experiment was performed in triplicate. The inhibition rate (IR) was calculated as follows:

IR (%)=1 – (Mean A_{490} of experiment – A_{490} of blank/A_{490} of control – A_{490} of blank).

Quantification of gene expression

Total RNA was extracted using TRIzol Reagent, according to the manufacturer's protocol, and dissolved into RNase-free water. Then, 1 μg of RNA was reversely transcribed to cDNA. Afterward, AQP9 and β-actin mRNA were quantified by q-PCR using the SYBR Green Quantitative PCR kit following the manufacturer's instructions. The primers for PCR are exhibited in *Table 1*. Reaction was carried out in a total volume of 20 μL for 40 cycles of 2 min at 95 ℃, 15 s at 95 ℃, 60 ℃ at 30 s and 30 s at 72 ℃. The amplification reaction of AQP9 with respect to β-actin was analyzed with the comparative CT (ΔΔCT) method. Experiments were performed in triplicates.

Western blotting analysis

Cells were collected and lysed in a RIPA lysis buffer (Solarbio, Beijing, China) containing phenylmethylsulfonyl fluoride (MP Biomedicals, Santa Ana, CA, USA). The concentration was determined using the BCA protein assay kit (Solarbio, Beijing, China). 50 μg of the total protein was separated by 10% SDS-PAGE solution and blotted at the target protein and the β-actin antibody on a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with a 5% skimmed milk powder buffer for 90 min, in Tris-Buffered Saline Tween, and then was probed with an anti-AQP9 in a 1:300 diluted solution or an anti-β-actin antibody in a 1:5,000 diluted solution in the TBST. After being rinsed 3 times with a PBS, the membrane was re-probed with a HRP-linked anti-rabbit IgG in a 1:10,000 diluted TBST. Results were intuitively observed by the Immobilon Western chemiluminescent HRP Substrate (Millipore Corporation, Billerica, USA), according to the manufacturer's protocol. Experiments were performed in triplicates.

RNA interference

HepG2 cells were electro-transfected with AQP9 siRNA according to the recommendations. After being digested by pancreatic enzyme, the cells were collected, counted and adjusted for density to 1~2×10^6/mL, 200 rpm 10 min. The supernatant was then removed and the cells were resuspended in a 100 μL nucleofector solution. The experiential cells were divided into the mock group (the blank control, only added nucleofector solution), the AQP9 siRNA group (transfected with AQP9 siRNA) and the NC group (transfected with NC-siRNA which were marked by GFP). After transfecting, 500 μL of DMEM (preheated) containing 10% heat-inactivated fetal calf serum was added to the cup, then removed. The samples for the 6-well microtiter plates were cultured in a 5% CO_2 humidified atmosphere at 37 ℃. The transfection efficiency of AQP9 siRNA was detected by q-PCR and western blotting. The primers of AQP9-siRNA are as following: sense 5'-UGACCUCAACUACAUGGUUTT-3' and antisense 5'-AACCAUGUAGUUGAGGUCATT-3'.

Wound healing assay

HepG2 cells were divided into a blank control group (HepG2 cells treated with the same volume of DMEM), HepG2 cells treated with the drug (2 μM As_2O_3 and 20 μM AZT) and the siRNA-AQP9 HepG2 cells treated with the drug (2 μM As_2O_3 and 20 μM AZT) groups. After a 12 h incubation time in 6-well plates, an artificial wound of

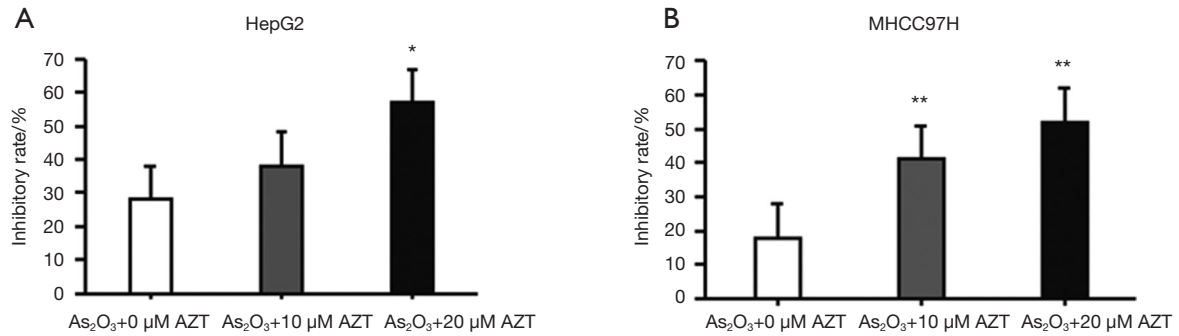

Figure 1 Effect of As_2O_3 combined with AZT on cell viability inhibition. As_2O_3+0 μM AZT: treated with 2 μM As_2O_3; As_2O_3+10 μM AZT: treated with 2 μM As_2O_3 and 10 μM AZT; As_2O_3+20 μM AZT: treated with 2 μM As_2O_3 and 20μM AZT. Compared with As_2O_3 alone, *P<0.05, **P<0.01.

the scratched cells was made using a 200 μL pipette tip. Then, it was rinsed with a PBS and the drugs from the corresponding group were added and cultured for 0, 24 and 48 h. The photos were captured using the microscope at a magnification of ×40. The migration distance of the wound was measured using the Image Pro Plus software. The experiment was repeated at least 3 times, and the data was normalized to the average of the control.

Transwell migration and invasion assays

At the 48 h post-transfection time, the above 3 groups of cells (5×10^4 cells per well) were moved into DMEM without FCS and the added drugs were then plated into the upper chambers of the transwell plates, and the medium supplemented with a 20% FCS was plated into the lower chambers. The membrane was fixed in a methanol solution, and then stained using hematoxylin after 24 h of incubation. Stained cells were visualized and counted under a light microscope (200×). The analysis of the cell invasion was consistent with that of cell migration. The cell invasion was detected using the Transwell assay with the Matrigel Invasion Chambers. Experiments were performed in triplicates.

Statistical analysis

The SPSS 19.0 software was used to perform comparative analysis of cell viability and the AQP9 expression by way of a one-way analysis of the variance. It was followed by a LSD-t-test, and all dates were presented as mean ± standard deviation (SD). The criterion of statistical difference was taken as P<0.05.

Results

Effect of As_2O_3 combined with different concentrations of AZT on the viability of HepG2 and MHCC97H cells

The inhibition effect of As_2O_3 combined with the different concentrations of AZT in human liver cancer HepG2 and MHCC97H cells were detected by MTT assay. As_2O_3 (2 μM) combined with AZT (20 μM) significantly inhibited the growth of HepG2 and MHCC97H cells at the 48 h mark (*Figure 1*) (P<0.05 or P<0.01). The results showed that As_2O_3 combined with AZT inhibited cell growth, and the optimum synergy inhibition concentration was 2 μM As_2O_3 combined with 20 μM AZT, which indicated a dose-dependent inhibition of cell growth.

As_2O_3 combined with AZT up-regulated the expression of AQP9 mRNA/protein in HepG2 cells

To explain the modulating effect of As_2O_3 and AZT in the HepG2 cells, a real time-PCR was performed to detect the expression of the AQP9 mRNA. As shown in *Figure 2*, As_2O_3 (2 μM) and AZT (20 μM) efficiently promoted the expression of AQP9 mRNA (P<0.05). A western blotting result showed that the combination of treatments also significantly increased the protein levels of the AQP9 group (*Figure 3*). These results indicated that As_2O_3 combined with AZT could up-regulate the expressions of AQP9 mRNA/protein.

AQP9 siRNA inhibited the expression of AQP9

Since the NC-siRNA marked by GFP gene (express green fluorescent protein) is transfected into the cell, the

GFP protein can be expressed in the cell and emit green fluorescence. Therefore, GFP was marked to observe the transfection efficiency. As shown in *Figure 4*, the green fluorescent cells were significantly increased at 10 h, so the transfection efficiency could be detected at 24 h. qPCR result showed that the transfection efficiency was nearly 70% (*Figure 4C*), which could meet the experimental requirements. The western blotting results showed that the protein level of AQP9 was consistent with the mRNA level (*Figure 4D*).

The effects of AQP9 on transversal migration of HepG2 cell

The results of the wound healing assay (*Figure 5*) showed that As_2O_3 combined with AZT could significantly inhibit the scratch healing ability of the HepG2 cell after a 24-h period. However, the effect was decreased after transfecting with AQP9 siRNA. Scratches in the blank control group basically healed 48 h after scratching. The scratch width of the AQP9-siRNA group was slightly narrower than that of the 24-h group, but there was no significant change in As_2O_3 when combined with the AZT group. These results indicated that As_2O_3 combined with AZT can significantly inhibit transversal migration of HepG2 cells, which may be controlled by AQP9.

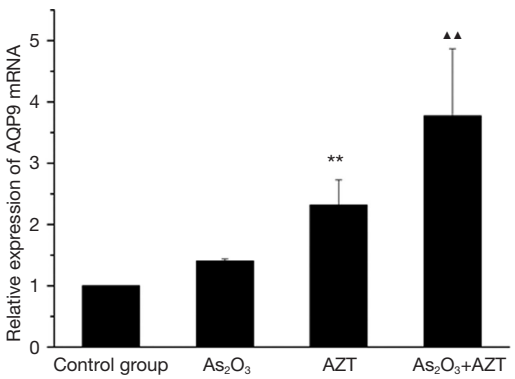

Figure 2 The expression levels of AQP9 mRNA in human liver cancer HepG2 cells. Compared with control group, As_2O_3, and AZT, respectively, ▲▲P<0.01; compared with control group, **P<0.01.

The effects of AQP9 on longitudinal migration and invasion of HepG2 cells

As *Figure 6* illustrates, the number of transmembrane cells in the drug-treated group was significantly less than that in blank control group (P<0.01). However, the effect was reduced after transfecting with AQP9 siRNA. These results showed that As_2O_3 combined with AZT could significantly inhibit the longitudinal migration and the invasion of the HepG2 cells, which may be related to AQP9.

Discussion

To date, reports have shown much progress in the antineoplastic combined chemotherapy protocols of As_2O_3 with other drugs. As a telomerase inhibitor, AZT inhibits the infinite replication potential in the z tumor cell proliferation by inhibiting the telomerase activity (24). Also, research has shown that the combined application of the

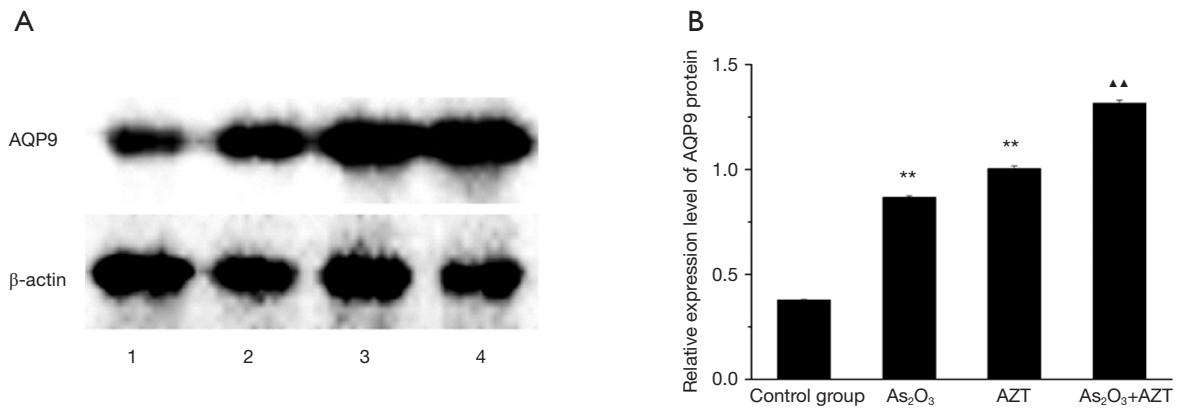

Figure 3 The expression levels of AQP9 protein in human liver cancer HepG2 cells. 1: Control group without treatment with any drugs; 2: As_2O_3 group treated with 2 μM As_2O_3; 3: AZT group treated with 20 μM AZT; 4: As_2O_3+AZT group treated with 2 μM As_2O_3 and 20 μM AZT. Compared with control group, **P<0.01; compared with control group, As_2O_3, and AZT, respectively, ▲▲P<0.01.

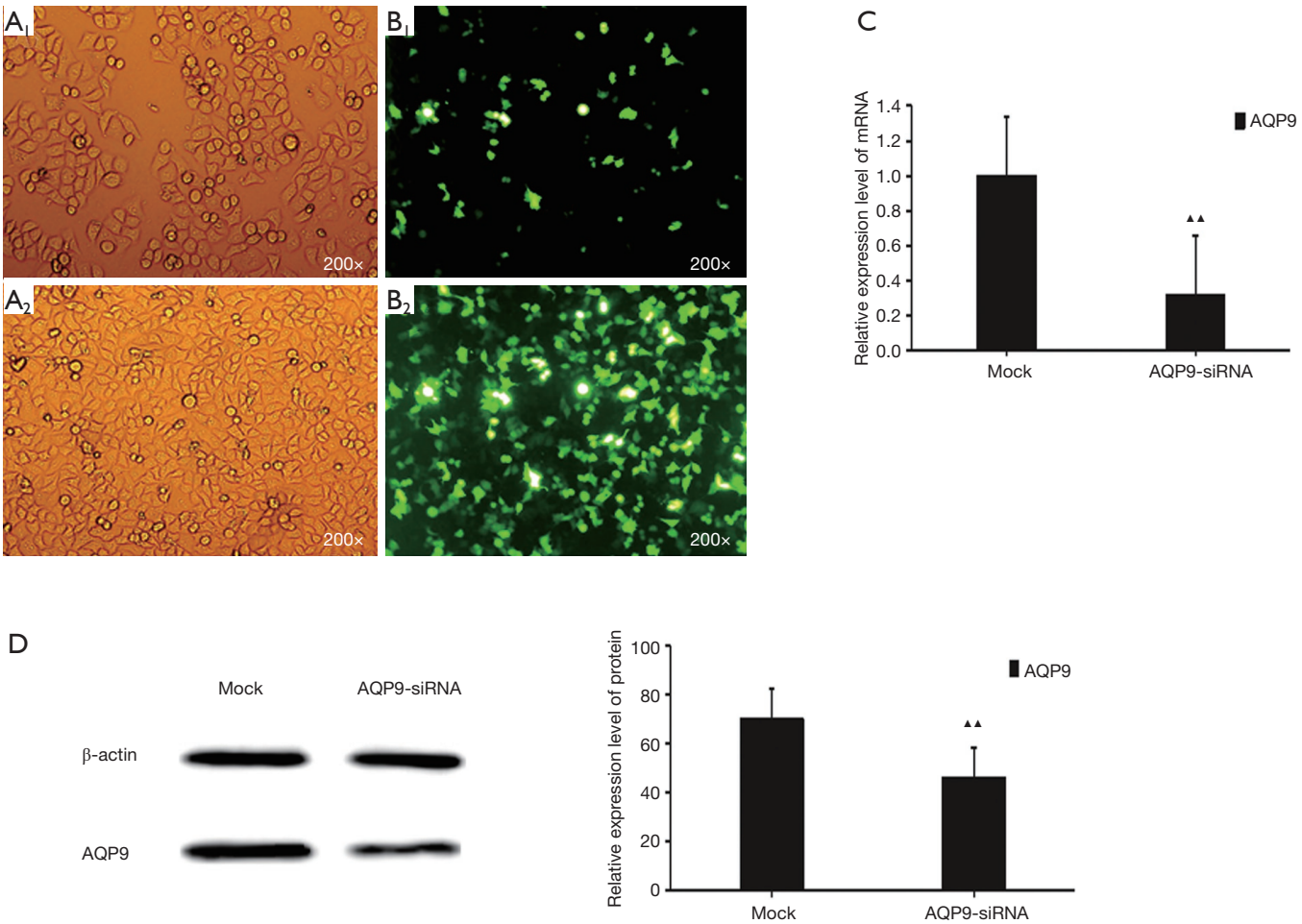

Figure 4 The HepG2 cells transfected with AQP9. Cells observed under light microscope at 10 h (A_1) and 24 h (A_2), respectively (×200). Cells observed under fluorescence microscope at 10 h (B_1) and 24 h (B_2), respectively (×200). The cells transfected showed green fluorescence. (C,D) Transfection effects were tested by q-PCR and Western blotting, respectively. Compared with Mock, ▲▲P<0.01.

AZT with emodin can co-inhibit the cell proliferation of leukemia KG-1a, which demonstrates that by constraining the activity of telomerase, Pt-AZT suppresses Bcl-2 expression and inhibits the growth of hepatocellular carcinoma in mice (25,26). Furthermore, our previous research found that the combination of As_2O_3 and AZT has a remarkable therapeutic effect on the liver carcinoma cell, HepG2 (8). However, its mechanism of action has not been published in the literature.

AQP9 is widely distributed in liver tissues, which plays a significant role in the physiopathology proceedings of the liver. Li and his colleagues found that AQP9 was down-regulated in the liver carcinoma cells when compared with normal liver tissues. Meanwhile, over-expression of AQP9 inhibited the proliferation of the SMMC-7721 liver carcinoma cells (27). Other research has indicated that AQP9 promoted the intake of some micro molecule neutral solutes such as monocarboxylic acid, 5FU, platinum, and As_2O_3 (28). Tang and his partner reported that the over-expressed AQP9 increased the effects of As_2O_3 on inhibiting proliferation, migration, and the invasion of liver carcinoma cells (29). Here, we found the excitation of caspase-3 was also enhanced, which is consistent with our previous study on cell biological behavior of As_2O_3 combined with AZT against liver cell carcinoma HepG2. However, Gao and his colleagues reported that melanoma cells had resisted chemotherapy and radiation when there was an over-expressed AQP9 (30). These conflicting results were probably due to the effect of AQP9 being dependent on different types of cells.

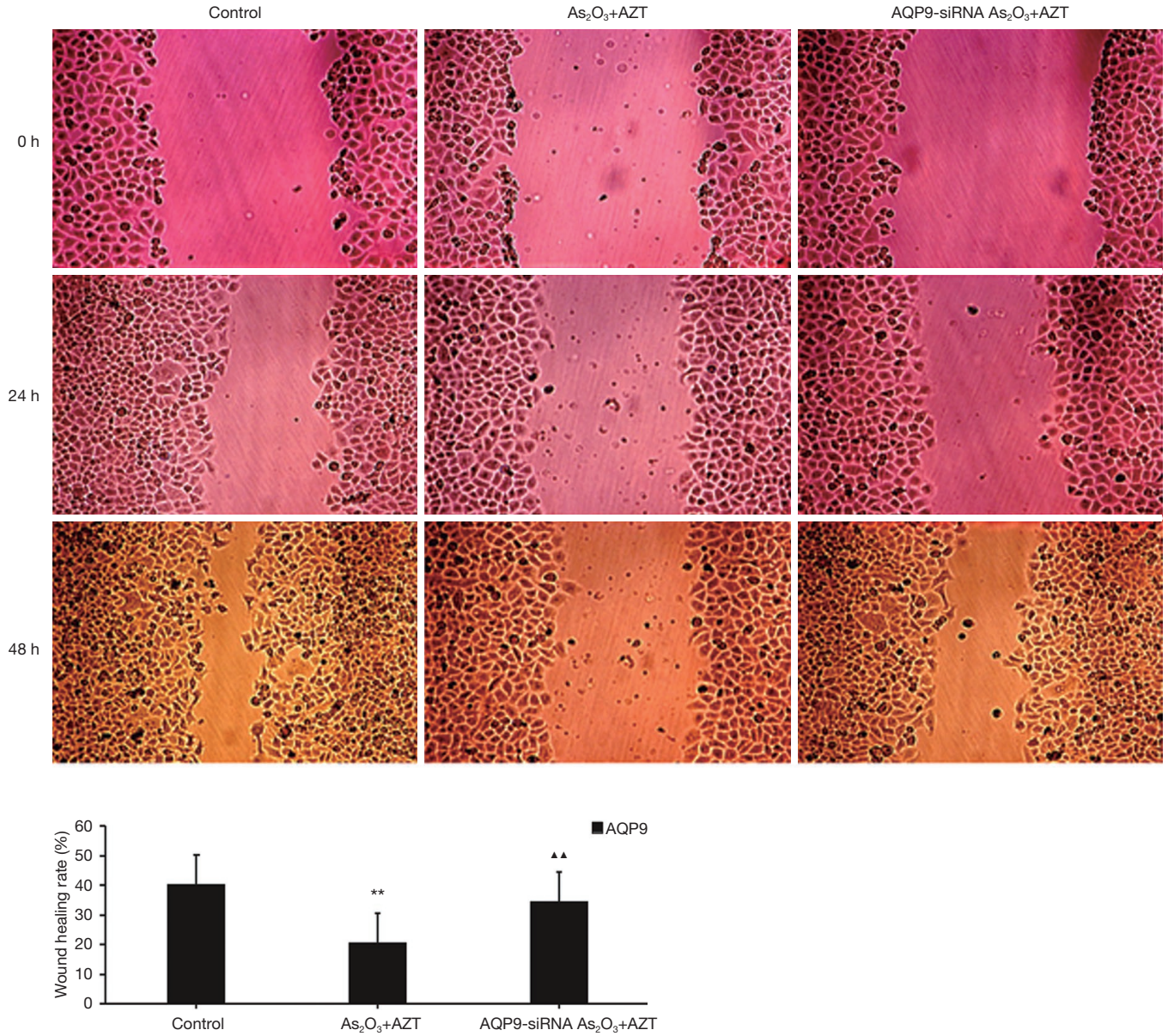

Figure 5 The effects of AQP9 on transversal migration of HepG2 cell (×200). Human liver cancer HepG2 cells; As_2O_3+AZT: HepG2 cells treated by combination of 2 μM As_2O_3 and 20 μM AZT; AQP9-siRNA As_2O_3+AZT: HepG2 cells treated by combination of 2 μM As_2O_3 and 20 μM AZT after transfected with AQP9 siRNA. Compared with control, **P<0.01; compared with combination of 2 μM As_2O_3 and 20 μM AZT, ▲▲P<0.01.

In the present studies, we found that the proliferation inhibition rate of the combination of As_2O_3 and AZT on the hepatocellular carcinoma cells was significantly higher than As_2O_3 alone, and it had a dose-dependent effect for AZT. In addition, a low dose of As_2O_3 combined with AZT also significantly inhibited the migration and invasion of liver cell carcinoma. Furthermore, as shown by the results of qPCR, the expression levels of AQP9 in combination of As_2O_3 (2 μM) and AZT (20 μM) group were higher than those of As_2O_3 alone groups, as well as the blank control groups, which was also verified by the western blot test results.

To further investigate whether AQP9 plays a key role in the process of the combination of As_2O_3 and AZT against

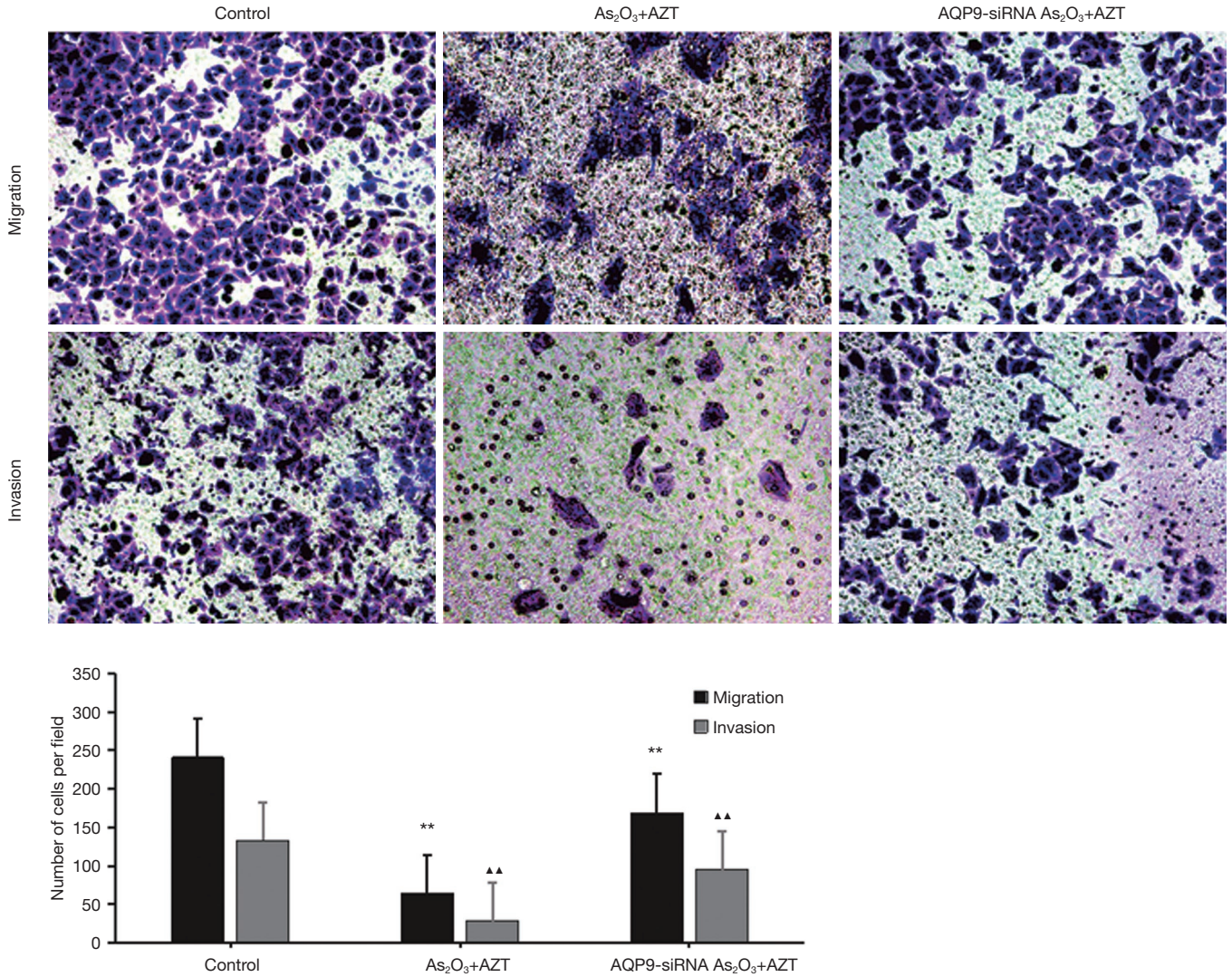

Figure 6 Transwell migration and invasion assays (×100). Control: human liver cancer HepG2 cells; As_2O_3+AZT: HepG2 cells treated by combination of 2 μM As_2O_3 and 20 μM AZT; AQP9-siRNA As_2O_3+AZT: hepG2 cells treated by combination of 2 μM As_2O_3 and 20 μM AZT after transfected with AQP9 siRNA. Compared with control **P<0.01; compared with combination of 2 μM As_2O_3 and 20 μM AZT, ▲▲P<0.01.

hepatocellular carcinoma cells and thus improves the treatment efficacy, the expression of AQP9 was silenced by electro-transfecting HepG2 cells with AQP9 siRNA. The results of the wound healing assay showed that the scratch width of the AQP9-siRNA group was dramatically narrower than that of As_2O_3 when combined with the AZT group. In addition, the number of transmembrane cells in the AQP9-siRNA group was significantly more than that of As_2O_3 combined with AZT group. These results suggest that AZT may increase the sensitivity of the hepatocellular carcinoma cells to As_2O_3 by up-regulating the expression of AQP9.

In conclusion, our study shows that As_2O_3 combined with AZT can co-inhibit the growth, migration and invasion of hepatocellular carcinoma cells by up-regulating the expression of AQP9. This has important implications in reducing the poisonous side effect of As_2O_3 in the cancer therapies.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.11.08). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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