Basic Study

Auphen and dibutyryl cAMP suppress growth of hepatocellular carcinoma by regulating expression of aquaporins 3 and 9 in vivo

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Aim: To investigate whether the regulation of aquaporin 3 (AQP3) and AQP9 induced by Auphen and dibutyryl cAMP (dbcAMP) inhibits hepatic tumorigenesis.

Methods: Expression of AQP3 and AQP9 was detected by Western blot, immunohistochemistry (IHC), and RT-PCR in HCC samples and paired non-cancerous liver tissue samples from 30 hepatocellular carcinoma (HCC) patients. A xenograft tumor model was used in vivo. Nine nude mice were divided into control, Auphen-treated, and dbcAMP-treated groups (n = 3 for each group). AQP3 and AQP9 protein expression after induction of xenograft tumors was detected by IHC and mRNA by RT-PCR analysis. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and histological evaluation were used to detect apoptosis of tumor cells, and the concentration of serum α-fetoprotein (AFP) was measured using RT-PCR and an ELISA kit.

Results: The volumes and weights of tumors decreased significantly in the Auphen- and dbcAMP-
INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant cancer worldwide; however, the mechanism of hepatocarcinogenesis is unknown, and a reliable prognosis is still lacking\(^{[1,2]}\). Thus, novel treatment regimens that allow for the prevention and retardation of HCC still need to be identified. Aquaporins (AQPs) consist of 13 small, hydrophobic, integral, transmembrane, water channel proteins, which have an important role in the control of water movement, fluid transport, and cell migration\(^{[3]}\). AQPs are closely associated with cancer biological functions and have been identified in > 20 human cancer cell types\(^{[4]}\). AQP3 is overexpressed in HCC, which is related to tumor grade, stage, metastasis and prognosis, and may be helpful in diagnosis of HCC when combined with serum α-feto-protein (AFP)\(^{[5]}\). AQP9 expression is reduced in HCC and mainly located in non-tumorigenic liver tissue\(^{[6]}\). Furthermore, AQP9 has been found to be involved in cell proliferation in many cell types such as those in the skin, colon, and cornea. Serna et al\(^{[7]}\) found that AQP3 was positively associated with cell proliferative activity. Several \textit{in vivo} and \textit{in vitro} experiments have shown that AQP3 can promote cell proliferation and migration\(^{[8-10]}\). Some researchers suggest that AQP9 could be a novel target for drug therapy in liver cancer patients because its transport activities do not extend to charged neutral molecules, such as purine, pyrimidine, and urea, including permeability to 5-fluorouracil. Besides, Jablonski et al\(^{[11]}\) found that decreased AQP9 expression in HCC can increase resistance of HCC cells to apoptotic stimulation, and AQP9 expression decreases with the degree of tumor cell differentiation. Thus, the targeted regulation of AQP3 and AQP9 may provide significant therapeutic benefits to HCC patients.

Recently, agents modulating the expression of AQPs have been reported, which contain heavy metals\(^{[12-20]}\), quaternary ammonium salts\(^{[21-23]}\), or mineral salts\(^{[24]}\). Although these agents are valuable in characterizing the effect of AQP regulation in cells, they are not suitable for clinical application because of their toxic side effects and poor selectivity. These modulators have various therapeutic traits, such as anticancer, antirheumatic, and antibiotic properties. Au(III) compounds and isoelectronic and isostructural Pt(II) compounds can be used as anti-tumor drugs\(^{[25-27]}\). An Au(III) complex has been shown to have effective antiproliferative \textit{in vitro} against various cancer cells with high cytotoxic potency and selectivity. It is possible that these properties arise from their possible inhibition of histone deacetylase\(^{[28]}\). Martins et al\(^{[29]}\) reported that an Au(III) complex was a selective and potent inhibitor of AQP3. In addition, Auphen showed antiproliferative traits in tumor cells \textit{in vitro}\(^{[30-32]}\). Yamamoto et al\(^{[33]}\)
found a protein kinase A (PKA) activator (dibutyryl cAMP; dbcAMP) and a PKA inhibitor (cycloheximide) can increase and reduce the expression of AQP9, respectively, thereby demonstrating that PKA-based approaches can increase AQP9 expression. There are currently no reports describing the effects of Auphen on AQP3 and dbcAMP on AQP9 in HCC in vivo. Therefore, we used nude mice subcutaneously xenografted with human HCC SMMC-7721 cells to study their effects.

In the present study, we assessed the anti-oncogenic effects of Auphen and dbcAMP in vivo and investigated whether their underlying mechanisms regulate AQP3 and AQP9 expression. We also analyzed the correlation between AQP3 and AQP9 expression and clinicopathologic features of HCC, which demonstrated that both AQP3 and AQP9 play an important role in HCC tumor development and clinical prognoses. Taken together, our results significantly contribute to the evaluation of the anti-oncogenic effects of Auphen and dbcAMP in vivo.

MATERIALS AND METHODS

Drugs
Auphen was synthesized according to a previously described method[31] and prepared at a concentration of 1 mmol/L by adding dimethylsulfoxide (DMSO) and normal saline (NS). dbcAMP was purchased from Calbiochem (San Diego, CA, United States) and prepared at a concentration of 3 mmol/L using NS. The purity of the complex was > 98% based on elemental analysis.

Patients and specimens
All patients were recruited between 2002 and 2012 at the Liver Cancer Institute and Zhongshan Hospital (Fudan University, Shanghai, China). In accordance with the protocol approved by the Zhongshan Hospital Research Ethics Committee, all patients participating in this study provided informed consent. HCC specimens and paired normal liver tissues from 30 patients, and their clinicopathologic information were obtained from the Liver Cancer Institute and Zhongshan Hospital (Table 1). The collected HCC tissues had no selection bias. All patients had: (1) a pathological diagnosis of HCC; (2) tumor stages diagnosed based on the 2002 TNM staging system of the Union for International Cancer Control; and (3) tumor differentiation determined using the Edmondson grading system. All patients were not being treated with any anti-tumor medications before collecting the biopsy samples.

Immunohistochemistry
Endogenous peroxidase was blocked with 3% H2O2. Sections were treated with a primary antibody against AQP3 (1:50; Abcam, Cambridge, MA, United States) or AQP9 (1:50; Abcam), and were washed three times in phosphate-buffered saline. Then, sections were incubated with a biotinylated goat anti-rabbit IgG (1:250 dilution; Abcam). The reaction was visualized using a diaminobenzidine (DAB) substrate chromogen solution (Gene Technology, Shanghai, China). The sections were counterstained with Mayer’s hematoxylin, and the slides were examined under an optical microscope.

Total RNA extraction and real-time PCR
Total RNA was prepared from tumor tissues using RNAiso Plus (TaKaRa, Tokyo, Japan), and reverse transcribed using PrimeScript RT Master Mix (TaKaRa) with the GeneAmp-PCR system 7500 (Applied Biosystems, Foster City, CA, United States). The cDNA obtained was amplified using SYBR Premix Ex Taq (TaKaRa) on the Master cycler ep realplex4 PCR system (Eppendorf, Hamburg, Germany) with the primers listed in Table 2. The cycling parameters were: 1 min at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The mRNA expression levels were computed after normalization against β-actin mRNA levels using the 2−ΔΔCt method. Each assay was performed in triplicate.

Xenograft tumor model in nude mice
SMMC-7721 cells (5 × 10⁶ cells) were injected subcutaneously into each flank of nine male BALB/c nude mice (Shanghai Slac Laboratory Animal Co.

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**Table 1 Clinicopathological data of the hepatocellular carcinoma cohort**

| Clinicopathologic parameters | Frequency | %  |
|-----------------------------|-----------|----|
| All cases                   | 30        |    |
| Gender                      |           |    |
| Male                        | 23        | 76.7|
| Female                      | 7         | 23.3|
| Age (yr)                    |           |    |
| < 50                        | 5         | 16.7|
| > 50                        | 25        | 83.3|
| Tumor size (cm)             |           |    |
| < 5                         | 8         | 26.7|
| > 5                         | 22        | 73.3|
| Serum HBsAg                 |           |    |
| Positive                    | 24        | 80.0|
| Negative                    | 6         | 20.0|
| Serum AFP (ng/mL)           |           |    |
| < 25                        | 4         | 13.3|
| > 25                        | 26        | 86.7|
| Cirrhosis                   |           |    |
| Presence                    | 27        | 90.0|
| Absence                     | 3         | 10.0|
| UICC stage                  |           |    |
| I + II                      | 13        | 43.3|
| III + IV                    | 17        | 56.7|
| Metastasis/Recurrence       |           |    |
| Yes                         | 18        | 60.0|
| No                          | 12        | 40.0|
| Edmondson grade             |           |    |
| Low (1 / II)                | 10        | 33.3|
| High (III / IV)             | 20        | 66.7|
Table 2 Primer sequences used for the amplification of different genes by quantitative PCR

| Gene   | Forward primer sequence                    | Reverse primer sequence                    |
|--------|-------------------------------------------|-------------------------------------------|
| AQP3   | 5'-CACACGGCGCATCTTTGCTA-3'                | 5'-TGCCCCACACACACACAGATA-3'               |
| AQP9   | 5'-CTTAAACATCTACAAGCCACTT-3'              | 5'-TCTCAGCCAGCTACTGATCTTC-3'              |
| AFP    | 5'-ACCTGTTGTGGCCGTCATGC-3'                | 5'-GCCGCCGTACACCTGACGGT-3'                |
| β-actin| 5'-TCACCACACTGTGCCCATCT-3'                | 5'-CACGCCAAGCCTCATIGCGCAC-3'              |

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Ltd., Shanghai, China) with an age of 4 wk and a weight of 18-20 g. Tumor diameters were measured in three dimensions with a Vernier caliper to diagnose tumorigenesis at 21 d. A nodal diameter up to 0.5 cm was defined as a tumor. The frequency of tumor production was 100% at 21 d after inoculation. The protocol for the treatment of animals was approved by the Ethics Committee of Zhongshan Hospital. When the tumor diameters measured at least 1-2 cm, the nude mice with tumors were divided into three groups with no selection bias (n = 3); group 1 was treated with a placebo; group 2 with Auphen at a dose of 1 mmol/L; and group 3 with dbcAMP at a dose of 3 mmol/L. Tumor volumes were measured with calipers using the formula: (width)² x length/2. Mice were followed for 2 wk. All mice were euthanized after 4 wk, and the tumors were removed and used for further analysis. The inhibition rate of tumor progression was calculated using the following formula: inhibition rate (%) = (NS treated tumor weight (g) - drug treated tumor weight (g)/NS treated tumor weight (g)) x 100%. Part of the tumor tissues were stored at -80 °C. The remaining tissues were fixed in 10% formalin, embedded in paraffin, and used for real-time PCR.

**ELISA for measurement of AFP in serum of nude mice**

Blood collected from the tail vein under aseptic conditions was centrifuged at 2600 rpm at 10 °C for 10 min. The level of AFP was detected using an ELISA kit from HUMAN (GmbH, Wiesbaden, Germany). The ELISA method was based on the affinity of biotin to streptavidin immobilized on the surface of a microtiter well. A complex was formed by mixing the enzyme-antibody conjugate with the serum. The zymolyte was added following incubation and washing to develop a color. The strength of the color was directly proportional to the concentration of AFP in serum. An ELISA reader was used at 450 nm to detect the optical density of the reaction.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay**

After fixation with 10% formalin for 4 h, the tumor tissues were embedded in paraffin. Transferase-mediated dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (KGI Biotechnology, Nanjing, China). After being deparaffinized, the samples were mixed with 3% H2O2 for 10 min at room temperature, then incubated in a wet box with fluorescein dUTP for 1 h at 37 °C. After treatment with horseradish peroxidase, the tissues were stained with DAB and counterstained with methyl green. The matched groups had the same treatment except for treatment with the fluorescein dUTP. A light microscope was used to visualize the nuclei of the tissue, which showed a brown color as a positive result.

**Histological evaluation**

Tissues were immobilized in formalin, embedded in paraffin, and cut into 4 μm sections. The sections were then stained with hematoxylin and eosin (HE) as previously described[34].

**Statistical analysis**

Fisher’s exact test for nonparametric variables and Student’s t-test (two-tailed) for parametric variables were used. Changes in animal survival were estimated by the Kaplan-Meier method and analyzed using Cox regression analysis and univariate analysis. Data are presented as the mean ± SD. Statistical analysis of data was performed with one-way analysis of variance among three groups using SPSS, version 19.0 software (SPSS, Armonk, NY, United States). A P-value < 0.05 was regarded as statistically significant.

**RESULTS**

**Expression of AQP3 and AQP9 differs in HCC**

We investigated AQP3 and AQP9 levels in HCC tumor samples and their coupled non-neoplastic counterparts. Representative mRNA images show that AQP3 (Figure 1A) was overexpressed whereas AQP9 (Figure 1B) was expressed at a low level in HCC tissues compared with their non-neoplastic counterparts, although the levels of AQP3 and AQP9 were similar in both tumors and noncancerous tissues (Figure 1A, samples 2 and 20; Figure 1B, samples 22 and 26). The results were confirmed by positive staining and protein expression analyses of AQP3 (Figure 2) and AQP9 (Figure 3). As shown by immunohistochemical analyses, HCC samples showed intense staining of AQP3 in the membrane and cytoplasm, and weak membrane staining of AQP9, while the noncancerous tissues presented mainly weak expression of AQP3 and strong expression of AQP9, indicating that AQP3 was

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overexpressed and AQP9 was reduced in HCC tissues.

Correlation of AQP3 and AQP9 expression with clinicopathologic traits and prognosis
To explore the relevance between AQP3 and AQP9 expression and clinicopathologic traits and prognosis, we analyzed the expression data in Table 3, and found that AQP3 and AQP9 expression correlated with liver neoplasm stage \( (P = 0.029 \text{ and } P = 0.003, \text{ respectively}) \), metastasis \( (P = 0.026 \text{ and } P = 0.031, \text{ respectively}) \), and tumor differentiation \( (P = 0.016 \text{ and } P = 0.047, \text{ respectively}) \). Other clinical characteristics, including age, gender, tumor size, hepatic sclerosis, serum hepatitis B surface antigen (HBsAg), and serum AFP, were not correlated with the expression of AQP3 or AQP9 (Table 3).

Correlation of AQP3 and AQP9 expression with overall survival
We explored whether AQP3 and AQP9 expression correlated with the clinical progression and prognosis of HCC, by examining patients’ overall survival rates. The overall survival of the patients with low AQP3/high AQP9 was greater than that of the patients with high AQP3/low AQP9 \( (P = 0.045, P = 0.020) \) (Figure 4A and B), which was independent of whether they received HCC-tailored treatment or not. The results suggest that AQP3/AQP9 could be used for assessing the clinical prognosis of HCC. The parameters used to assess the influence on overall survival involved AQP3 and AQP9 expression, age, gender, tumor size, liver cirrhosis, serum HBsAg, serum AFP, tumor stage, metastasis, and tumor differentiation. The data according to the Cox proportional hazards test showed that AQP3 and AQP9 expression, tumor stage, metastasis, and tumor differentiation were independent prognostic parameters of survival (Table 4). Hence, the results show that AQP3 or AQP9 expression correlated with a poor prognosis in HCC patients.

Effect of Auphen and dbcAMP on expression of AQP3 and AQP9 in tumor-bearing nude mice
To obtain a better understanding of the regulation of AQP3 and AQP9 in HCC, we determined the effect of Auphen and dbcAMP on the expression of AQP3 and AQP9 \textit{in vivo}. Expression of AQP3 in Auphen-treated tumors was lower than that in the control mice. Expression of AQP9 in dbcAMP-treated tumors was higher than that in the control mice. Decreased levels of AQP3 and increased levels of AQP9 were confirmed at both the mRNA level (Figure 5A and B), and the protein level by Western blot (Figure 5C and D), and

![Figure 1](https://example.com/figure1.png)

**Figure 1** mRNA levels of AQP3 and AQP9 in hepatocellular carcinoma and normal liver tissues. A: mRNA levels of AQP3 in 30 paired HCC tissues and normal liver tissues measured by RT-PCR; B: mRNA levels of AQP9 in 30 paired HCC tissues and normal liver tissues measured by RT-PCR. HCC: Hepatocellular carcinoma; N: Normal liver tissues; T: HCC tissues.
Effect of regulation of AQP3 and AQP9 on hepatic tumor growth

To gain further insights into the roles of AQP3 and AQP9 in growth of HCC, we characterized the roles of Auphen and dbcAMP in vivo. The growth rates and volumes of Auphen- and dbcAMP-treated tumors were found to be lower than those in the controls. There was a marked decrease in tumor size in the Auphen- and dbcAMP-treated mice when compared with the control mice ($P < 0.01$; Figure 6), and the weights of tumors in the three groups were $0.06 \pm 0.01$, $0.07 \pm 0.01$, and $0.19 \pm 0.03$ g, respectively. There were significant differences between the Auphen- and dbcAMP-treated mice and the control mice ($P < 0.01$). The results suggest that low expression of AQP3 and high expression of AQP9 suppressed tumor growth of HCC in vivo. Our study clearly indicates that Auphen and dbcAMP reduced the serum AFP density in mice.

an immunohistochemical assay (Figure 5E).

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**Figure 2  Levels of AQP3 in hepatocellular carcinoma of different differentiation.** A: Analysis of AQP3 protein expression by immunohistochemistry. a: AQP3 expression decreased in normal liver tissues; b: AQP3 expression was weak in well-differentiated HCC samples; c: AQP3 expression decreased in normal liver tissues; d: AQP3 expression was moderate in moderately differentiated HCC samples; e: AQP3 expression decreased in normal liver tissues; f: AQP3 expression was high in poorly differentiated HCC samples. B: The protein levels of AQP3 in HCC of different degrees of differentiation corresponded to their immunohistochemistry results. Tumor 1, well-differentiated HCC sample; tumor 2, moderately differentiated HCC sample; and tumor 3, poorly differentiated HCC sample. HCC: Hepatocellular carcinoma.
compared with the control mice ($P < 0.01$; Figure 7A and B). The TUNEL assay revealed more apoptotic changes in tumor tissues in the Auphen and dbcAMP groups (Figure 7C). Light microscopy was employed to detect the results of HE staining (Figure 7D). The Auphen and dbcAMP groups showed more apoptotic cells, which were revealed as karyopyknosis, and had a cytoplasmic red color.

**DISCUSSION**

HCC is the fifth most fatal malignant tumor worldwide, with no effective therapy. Thus, it is particularly important to identify novel targets for more effective treatments for this disorder. AQPs are known to be related to carcinogenesis and cancer malignancy\(^{[35]}\). Hu et al\(^{[36]}\) showed that AQPs had strong correlations with
tumor proliferation and metastasis. Also, it is suggested that AQPs are of great diagnostic and prognostic value in tumor tissues\cite{37,38}. Modulation of AQP expression has a wide range of clinical applicability, as suggested by the results from mice with AQP gene deletions and has a wide range of clinical applicability, as suggested by the results from mice with AQP gene deletions.

Table 3  Correlation of AQP3 and AQP9 expression levels (low and high) to clinico-pathological data

| Factors                        | AQP3 OR (95%CI) | P value | AQP9 OR (95%CI) | P value |
|--------------------------------|----------------|---------|----------------|---------|
|                                | Low | High       |                |         |
| Gender                         |     |            |                |         |
| Male                           | 4   | 19         | 0.526 (0.074-3.748) | 0.603 |        |
| Female                         | 2   | 5          |                | 9       |        |
| Age (yr)                       |     |            |                |         |
| < 50                           | 1   | 4          | 1.000 (0.091-11.03) | 1.000 |        |
| > 50                           | 5   | 20         |                | 22      |        |
| Tumor size (cm)                |     |            |                |         |
| < 5                            | 3   | 5          | 3.800 (0.580-24.90) | 0.300 |        |
| > 5                            | 3   | 19         |                | 19      |        |
| Serum HBsAg                    |     |            |                |         |
| Positive                       | 5   | 19         | 1.316 (0.124-13.98) | 1.000 |        |
| Negative                       | 1   | 5          |                | 4       |        |
| Serum AFP (ng/mL)              |     |            |                |         |
| < 25                           | 3   | 2          | 11.00 (1.271-95.23) | 0.041 |        |
| > 25                           | 3   | 22         |                | 21      |        |
| Cirrhosis                      |     |            |                |         |
| Presence                       | 6   | 21         | 2.116 (0.096-46.56) | 1.000 |        |
| Absence                        | 0   | 3          |                | 14      |        |
| UICC stage                     |     |            |                |         |
| I + II                         | 4   | 4          | 10.00 (1.341-74.55) | 0.029 |        |
| III + IV                       | 2   | 20         |                | 23      |        |
| Metastasis/recurrence          |     |            |                |         |
| Yes                            | 1   | 17         | 0.082 (0.008-0.839) | 0.026 |        |
| No                             | 5   | 7          |                | 8       |        |
| Edmondson grade                |     |            |                |         |
| Low (1 / II)                   | 3   | 3          | 14.00 (1.741-112.6) | 0.016 |        |
| High (III / IV)                | 3   | 21         |                | 18      |        |

Table 4  Cox regression analysis of patients with hepatocellular carcinoma

| Variables                      | Univariate | P value |
|--------------------------------|------------|---------|
|                                | HR (95%CI) |         |
| AQP3 expression (1 = low, 2 = high) | 4.948 | 1.037-23.605 | 0.045 |
| AQP9 expression (1 = low, 2 = high) | 10.835 | 1.488-78.877 | 0.019 |
| Gender (1 = Male, 2 = Female) | 2.681 | 0.852-8.436 | 0.092 |
| Age (1 < 50, 2 ≥ 50)           | 2.579 | 0.332-20.049 | 0.365 |
| Tumor size (1 < 5 cm, 2 ≥ 5)   | 1.261 | 0.334-4.759 | 0.732 |
| Serum HBsAg                    | 0.575 | 0.153-2.154 | 0.411 |
| (1 = Positive, 2 = Negative)   | 1.102 | 0.293-4.146 | 0.886 |
| Serum AFP (1 < 25 ng/mL, 2 ≥ 25 ng/mL) | 1.401 | 0.373-5.268 | 0.618 |
| Cirrhosis                      | 0.015 | 0.027-0.485 | 0.003 |
| UICC stage (1 = I + II, 2 = III + IV) | 0.228 | 0.065-0.804 | 0.021 |
| (1 = Yes, 2 = No)              | 0.560 | 0.175-1.791 | 0.328 |
| Edmondson grade                | 0.307 | 0.092-1.038 | 0.026 |

modulus\cite{40}. Nevertheless, these processes are computationally intensive, and water permeability modulus has no connection with inhibitory potency. Various chemotherapeutic drugs depend on high doses of toxic compounds to destroy cancer cells completely, and are frequently associated with serious side effects, tumor relapse, and progression to highly malignant states. Gold-based complexes have been reported to depress AQP3, with Auphen being the most efficient\cite{29,41}. Auphen has a greater ability to depress glycerol permeation via AQP3 than water permeation. Computational modeling has shown that gold-containing inhibitors have a mutual action to control cancer cell migration, with Cys40 located in the extracellular domain of AQP3\cite{40}. These advantageous properties of Auphen indicate its potential suitability for use in adoptive therapy against cancer. The PKA pathway is one of the main signal transduction pathways that regulate cell proliferation, mRNA expression, and enzyme activation, and dbcAMP increases the expression of AQP9 via the activation of PKA\cite{33}. Lee et al\cite{42} found that dbcAMP can inhibit cancer cell migration, thereby suggesting that cAMP-mediated PKA and CAM (EPAC)-mediated Ras related protein 1 (RAP1) could be therapeutic targets.

Our previous studies have revealed that Auphen inhibited AQP3 and dbcAMP increased AQP9 in HCC cells in a dose-dependent manner in vitro. However, whether regulating AQP3 and AQP9 can be used as potential targets for HCC treatment has not been confirmed.
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Figure 4  High AQP3 and low AQP9 expression predict worse survival in hepatocellular carcinoma patients. A and B: Survival of 30 hepatocellular carcinoma patients, including both untreated and HCC-treated patients using Kaplan-Maier analysis. High AQP3 and low AQP9 levels resulted in lower patient survival. HCC: Hepatocellular carcinoma.

Figure 5  Effects of Auphen and dibutyryl cAMP on AQP3 and AQP9 expression. A: mRNA levels of AQP3 in tumors from nude mice; B: mRNA levels of AQP9 in tumors from nude mice; C: Protein levels of AQP3 and AQP9 in tumors from nude mice; D: Immunohistochemical analysis of a subcutaneous tumor: a: Analysis of AQP3 expression in the control group; b: Analysis of AQP3 expression in the Auphen group; c: Analysis of AQP9 expression in the control group; d: Analysis of AQP9 expression in the dbcAMP group. (magnification × 200, bar = 50 μm). All data represent the mean ± SD (n = 3). *P < 0.01 vs the control; **P < 0.001 vs the control. dbcAMP: Dibutyryl cAMP.
Hence, in the present study, we characterized the functions of AQP3 and AQP9 in vivo.

As shown by the immunohistochemical assays, 90.7% of the HCC samples showed strong membrane and cytoplasmic staining for AQP3 and weak membrane staining for AQP9, while the noncancerous tissues presented mainly weak expression of AQP3 and strong expression of AQP9, indicating that AQP3 and AQP9 could play important roles in the development of HCC. In addition, we have confirmed that AQP3 and AQP9 expression correlated with liver neoplasm stage, metastasis, and tumor differentiation. There was no significant correlation between AQP3 and AQP9 expression and age, gender, tumor size, serum HBsAg, or serum AFP. Our results indicated that AQP3 and AQP9 expression is related to tumor biological characteristics, such as rapid tumor development. These findings suggest that clinicopathologic features together with expression of AQP3 and AQP9 in tumor tissues could be valuable in prognosis assessment and design of individual treatment strategies for HCC.

To understand the functions of AQP3 and AQP9, we tested whether regulating AQP3 and AQP9 by Auphen and dbcAMP suppressed tumor growth in vivo. Doses of 1 mmol/L Auphen and 3 mmol/L dbcAMP suppressed tumor growth, with decreases of tumor sizes and weights. The Auphen-treated group had lower AQP3 expression and the dbcAMP-treated group had higher

Figure 6 Auphen and dbcAMP suppress hepatocellular carcinoma tumor growth. A: Tumor volumes of control (NS), Auphen-treated (Auphen), and dbcAMP-treated (dbcAMP) groups; B: Tumor volumes of control, Auphen-treated, and dbcAMP-treated groups; C: Tumor weights of control, Auphen-treated, and dbcAMP-treated groups; D: Tumor suppression rates of Auphen-treated, and dbcAMP-treated groups; E: Body weights of control, Auphen-treated, and dbcAMP-treated groups. All data represent the mean ± SD (n = 3). *P < 0.01 vs the control. dbcAMP: Dibutyryl cAMP.

Hence, in the present study, we characterized the functions of AQP3 and AQP9 in vivo.
AQP9 expression than the control group. Our study also is the first confirmation of the tumorigenic abilities of AQP3 and AQP9 in vivo. Inhibiting AQP3 and increasing AQP9 expression showed increased suppression of
tumor growth in nude mice. AFP has previously been reported to be a target for diagnosing and monitoring HCC. Collectively, our findings demonstrate that Auphen and dbcAMP decreased AFP expression and secretion in vivo. TUNEL assays revealed more apoptotic changes in tumor tissues in the Auphen and dbcAMP groups. We assume that this was the result of the anti-tumor activities of Auphen and dbcAMP. All these studies confirmed our findings that AQP3 and AQP9 exerted oncogenic effects in HCC. Our study also showed no differences in body weight, appetite, or behavior between Auphen- and dbcAMP-treated mice and the control mice, indicating the efficiency and nontoxicity of Auphen and dbcAMP. Combined with previous in vitro results showing inhibition of hepatoma cell proliferation, we conclude that Auphen and dbcAMP have anti-cancer effects both in vitro and in vivo. The results also showed that the Auphen-treated group had smaller tumors than the dbcAMP-treated group. It may be that AQP3 has a more important function than AQP9 in the development of HCC, although this still needs more research.

In summary, our in vivo and in vitro results provide a useful platform to study tumor growth and possible anti-tumor treatments. In the present study, the association of clinicopathologic and the expression results suggest that overexpression of AQP3 and low expression of AQP9 correlate with clinical prognosis of HCC. Because of the small sample size in our study, the relationship between AQP3 and AQP9 expression and metastasis still needs more studies in larger cohorts. Studies are ongoing to develop targeted and effective delivery systems for administering AQP3 and AQP9 in HCC patients. These studies should further emphasize the significance of AQP3 and AQP9 in HCC development. Our findings are consistent with previous results indicating that regulation of AQP3 and AQP9 levels in HCC cells leads to a decrease in cell proliferation. In general, the results we obtained from the regulation and control of function tests in vitro will be important in the implementation of future studies to explore tumor cell behavior, in vivo, to gain additional understanding into the function of AQP3 and AQP9 in hepatocarcinogenesis.

In conclusion, combining human clinical data and in vivo experiments, our study strongly suggests AQP3 and AQP9 as key players in HCC development. Further research should verify AQP3 and AQP9 as diagnostic biomarkers for HCC occurrence. Furthermore, an in-depth description of AQP3 and AQP9 regulation by Auphen and dbcAMP will provide a better understanding of the mechanisms of hepatocarcinogenesis, which could be used in the development of novel therapeutic drugs. Finally, our work further confirms the significance of AQP-driven hepatocarcinogenesis, emphasizing the importance of both basic and clinical knowledge of the roles of AQPs in HCC.

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