

Effects of Acetazolamide Combined with or without NaHCO₃ on Suppressing Neoplasm Growth, Metastasis and Aquaporin-1 (AQP1) Protein Expression

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Abstract: This study was made to explore the effects of acetazolamide on tumor growth, metastasis and the possible mechanisms. The mice bearing with Lewis lung carcinomas were taken as the animal model. The effects of acetazolamide were compared with the combination treatment of NaHCO₃ on tumor growth, metastasis and carbonic anhydrase activity in lung and tumor tissues using imidazole-Tris technique. And also the possible role of AQP1 in tumor tissues was investigated by Western blot and immuno-histochemical analysis. Results showed that acetazolamide alone could sharply reduce the number of lung metastasis and primary tumor growth, and appeared in a dose-dependent manner. Acetazolamide significantly inhibited carbonic anhydrase activity in tumor tissue. After the addition of NaHCO₃, the suppression of acetazolamide on tumor growth, number of metastasis and carbonic anhydrase activity in primary tumor tissue could not be altered significantly, but the inhibitory rate of metastasis in lung and carbonic anhydrase activity in lung tissue appeared to show a reversal trend in the dose dependency from the acetazolamide treatment alone. The exactly mechanisms need to be clarified in future. Western blot and immunohistochemical analysis demonstrated that AQP1 expression in the tumor tissue was higher than both tissue of normal and treated with acetazolamide treatment alone. Combination with NaHCO₃ could not synergistically inhibit the expression of AQP1 with acetazolamide. The results suggested that the mechanism of acetazolamide on anti-tumor

especially on its anti-metastasis actions might partly involve either inhibiting the carbonic anhydrase activity or reducing AQP1 water channel protein expression, whatever if treated with or without NaHCO_3 .

Key words: acetazolamide, aquaporin, carbonic anhydrase, tumor, metastasis

Abbreviations: AQP1-aquaporin-1; CA-Carbonic anhydrase

1. Introduction

Tumor metastasis is the major characteristic of carcinoma and the direct cause of clinical death. Carbonic anhydrases (CA) are a family of zinc-binding metalloproteinases that catalyze reversible hydration of carbon dioxide, produce H^+ and HCO_3^- , and induce pH decrease [1]. Some CA isozymes (CA IV, IX, XII) were prominently found to be expressed only in tumor cells [2]. The cell surface CA might play an important role in controlling the level of protons and bicarbonate in the immediate vicinity of tumor cells by sensing pH and tipping the proton balance across the cell membrane. It has been shown that acidic pH enhances invasive behavior of tumor cells [3, 4]. Acetazolamide is a kind of sulfanilamide served as a carbonic anhydrase inhibitor.

Teicher et al. [5] reported that CA inhibitors, as part of a chemotherapy regimen enhances the chemotherapeutic drug effects and help the delay in tumor growth. Several new type CA inhibitors have acted as the effective compounds on suppressing tumor cell growth *in vitro*, such as on leukaemia, non-small cell lung cancer, melanoma, ovarian, renal, prostate, and breast cancer cell lines [6]. Although several mechanisms of action of CA inhibitors exist, it is believed that they could lead to the suppression in the acidification of tumor cell extra-environment after directly inhibiting tumor related CA isozymes. That seems to be contrary to the theory described above. Therefore, we postulated that there must be other pathways on suppressing tumor metastasis by CA inhibitors.

Aquaporins, is a large family of membrane proteins that function as highly selective water channels [7]. Recently published articles have drawn attention to their role in physiology[21]and several human diseases involving rapid water transport and they have been identified as potential targets for therapeutic intervention. AQP1 as the first characterized water channel protein[8], was identified in erythrocyte membranes, renal proximal tubule, choroids plexus, eye, lung, vascular endothelium, hepatobiliary epithelium [9], and some tumor cells themselves [10]. Most tumors have shown to exhibit high vascular permeability and high interstitial fluid pressure [11,12], but the transport pathways for water within tumors remain unknown.

In reviewing the previous published studies on AQP1 and carbonic anhydrase, especially carbonic anhydrase II and carbonic anhydrase IV, we found that they shared many common biological characteristics. For instance, they are all widely distributed in fluid transporting tissues including the kidney, lung, brain, eye, erythrocytes, pancreas and some glands and having the function of facilitating reabsorption and secretion of water [13]. AQP1 has the tendency to increase the response to estrin [14] and developmentally increase after birth [15]. In addition, AQP1 is capable of transporting CO_2 [16],

CO₂ is a substrate of carbonic anhydrase in catalyzing the formation of HCO₃⁻, which suggests a close relationship in biological characteristics between carbonic anhydrase and aquaporins.

As a carbonic anhydrase inhibitor, acetazolamide mainly used for edematous diseases such as glaucoma, mountain sickness, congestive heart failure, drug-induced edema, and to correct metabolic alkalosis. Parkkila et al. [17] have shown that acetazolamide alone can inhibit the invasive potential of cancer cells *in vitro*, but the mechanism of action has not been clarified. Our laboratory have indicated that acetazolamide inhibited gene expression of AQP1 in rat kidney and testis, where we have established a AQP1 expression system by using *Xenopus* oocytes to study the effect of acetazolamide on water transport function of AQP1, and confirmed that acetazolamide was a direct inhibitor of AQP1 [18-21]. Hence we have hypothesized that the reductive action of acetazolamide on AQP1 gene expression and water transportation might be contributed to their effects on cancer growth and metastasis.

In this study, we characterized and evaluated the expression of AQP1 in tumor tissues and its relation with carbonic anhydrase activity, and further explored the possible mechanisms of acetazolamide on tumor metastasis *in vivo*. It is well known that the most serious side effect of CA inhibitor is metabolic acidosis after a long period of administration. In order to correct the acidification of the extracellular milieu, animals were treated with acetazolamide that was combined with NaHCO₃ for comparison.

2. Methods

2.1 Lewis-lung-carcinoma *in vivo* model

Female C57BL/6 mice weighing 18-20 g were purchased from the Experimental Animal Center of Peking University (Gradell , Certificate №11-00-0004). Lewis lung carcinoma cells were provided by the Chinese Medical Science Institute and were maintained in C57BL/6 mice by subcutaneous injections in the axillary region of 0.2 ml of homogenized tumor tissues [tumor tissue (g): 0.9% Sodium chloride (ml) = 1:3] that were prepared from donors who were similarly inoculated for experimental tumor transplantation purposes.

2.2 Drug treatment and tissue collection

Acetazolamide was purchased from Sigma and given at a volume of 0.1 ml/mice. NaHCO₃ 30 mg/kg/day ig was applied. The animals were divided into nine groups of 10 mice each, control (C): normal animals; model (M): with tumor implantation; acetazolamide treatment (AH, AM, AL): corresponding to 80, 40, 20 mg/kg/day ig; acetazolamide combined with NaHCO₃ (ABH, ABM, ABL): corresponding to acetazolamide 80, 40, 20 mg/kg/day and NaHCO₃ 30 mg/kg/day ig (the dosage of NaHCO₃ used here was according to the dose clinically used in human and converted to mice dosage in order to neutralize the metabolic acidosis).

Treatment was initiated the first day after tumor transplant for a period of 20 days. On day 21, animals of every group were killed to calculate the tumor weight ratio in comparison to the body and lung weights (g/g). Also the numbers of lung metastases were counted under microscopy as well.

Primary tumor and lungs were then surgically resected and the tissue specimens were snap-frozen in liquid nitrogen for further analysis.

2.3 Carbonic anhydrase activity assay

The tissue specimens of lung and tumor were sonicated in a lysis buffer containing 0.3 M sucrose. The activity of CA in tissue was analyzed according to an established procedure [22]. One enzyme unit (EU) of CA activity was defined as the amount of homogenate necessary to halve the time of the control. CA activity was generally calculated from the formula:

$$\text{CA (EU/mg prot)} = [\log(B/S)]/[(\text{prot})\log 2]$$

Where B and S are the times measured for paired boiled inactivated enzyme and active sample, respectively. (prot) is milligrams of protein used for the measurement.

2.4 Western Blot analysis

Homogenized tissue specimens were solubilized in a sample buffer and heated to 60°C for 15 min [23]. The total protein concentration was measured by Lowry's method, using bovine serum albumin as a standard. Each sample containing 50 µg of protein was loaded on a 12% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. The SDS-PAGE gels were stained with Coomassie brilliant blue to confirm equivalence of the samples. The nitrocellulose membranes were blocked in blotting buffer containing 5% nonfat dry milk in Tris-buffered Saline (TBS, 50 mM pH 7.4), followed by incubation with anti-AQP1 antibody (rabbit anti-human IgG, a gift from Dr. Yang BX, University of California, San Francisco) diluted 1:1000 in blotting buffer at 4°C overnight. The membranes were washed three times for 5 min with TBS containing 0.05% Tween-20 (TBST) and incubated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG which was diluted in 1:5000 in TBS buffer. After washing three times for 5 min with TBST buffer, staining was developed with BCIP/NBT. Stained bands were scanned and the pixel intensity was quantified using Gel Doc 2000 Image system (Bio-Rad, USA) (n = 6-9 for each experiment).

2.5 Immunohistochemistry

On day 21st of treatment, mice were killed. Tissue specimens were then surgically resected into small blocks and transferred immediately into cold fixative solution (4% paraformaldehyde in PBS 0.1 M, pH 7.4). The tissue blocks were then rinsed in PBS, embedded in paraffin, and sectioned at 5 µm. The paraffin sections were deparaffinized with xylene and rehydrated in a gradient series of ethanol. Slides were then submerged in 3% hydrogen peroxide to quench any endogenous peroxidase activity, washed with distilled water, and heated in citrate buffer (10 mM, pH 6.0) in a microwave oven for 15 min, then cooled and washed with PBS. Non-immune serum for blocking was applied to eliminate nonspecific staining. Sections were incubated overnight at 4°C with rabbit anti-human AQP1 antibody. The sections were washed with PBS and incubated with the secondary antibody of biotinylated goat anti-rabbit IgG for 40 min, rewashed with PBS and incubated with peroxidase-conjugated streptavidin for 40 min. The peroxidation activity was visualized by incubating the sections with a peroxidase

substrate solution (DAB kit) after sufficient washing. The sections were counterstained with hematoxylin and mounted. Non-immune rabbit serum was used as a negative control.

2.6 Statistical analysis

Data were expressed as a mean \pm SD. The comparison of differences in metastasis number and tumor weight indexes between treatment group and model group were evaluated with Mann-Whitney *U*-test. The comparisons of AQP1 expression level in the lungs and CA activity in tissues between treatment group and model group were done by one way analysis of variance (ANOVA). All statistical analyses were performed using SPSS version 10.0, $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Effect of acetazolamide with or without NaHCO₃ on primary tumor growth and formation of spontaneous lung metastasis

The murine Lewis lung carcinoma is known to produce spontaneous lung metastasis. To determine whether combination treatment with acetazolamide and NaHCO₃ could reduce the number of lung metastasis synergistically, we compared the effect of two medications. Data that was collected from the treatment with acetazolamide alone were previously reported in Life Sciences [24]. We found that acetazolamide treatment alone could sharply reduce the number of lung metastasis and primary tumor growth. The tumor weight index were 19.9 ± 2.8 , 20.2 ± 2.8 , 19.5 ± 3.4 ($p < 0.05$) and the inhibition rate of metastasis were 62.0, 72.5, 77.7 % ($p < 0.05$) corresponding to the doses of 20, 40, 80 mg/kg/day, appeared in a dose-depend manner.

After adding the NaHCO₃, the suppression of acetazolamide on tumor growth and metastasis was almost the same as a single therapy, but the inhibition rate of lung metastasis appeared to show a reversal in the dose dependency from the acetazolamide treatment alone, which suggested the NaHCO₃ might deprave the action of acetazolamide on metastasis especially in combination with higher dose of acetazolamide (Table1). The exact mechanisms need to be clarified.

3.2 Effect of acetazolamide with or without NaHCO₃ on carbonic anhydrase activity

Carbonic anhydrase activity was measured by imidazole-Tris technique. The results that are displayed that acetazolamide treatment significantly inhibited CA activity in primary tumor tissue and lungs. Increased doses of acetazolamide showed a dose-dependent inhibitory effect. After adding NaHCO₃, the result showed a trend to reverse the dose dependency of acetazolamide on CA activity in the lung. Whereas in primary tumor tissue, the NaHCO₃ did not affect the action of acetazolamide dramatically, and appeared a dose dependent suppression and the tendency was same as that acetazolamide treated alone (Table 2).

Table 1. Effect of acetazolamide treatment with NaHCO₃ on experimental tumor growth and metastasis in mice (mean \pm SD). Acetazolamide and NaHCO₃ i.g. were used for 21 days. C: normal, M: tumor model, ABL: low dose of acetazolamide at 20 mg/kg and NaHCO₃ 30 mg/kg, ABM: acetazolamide at medium dose of 40 mg/kg and NaHCO₃ 30 mg/kg, ABH: high dose of acetazolamide at 80 mg/kg with NaHCO₃ 30 mg/kg. ^aTumor weight index means the ratio of tumor weight to body weight (g/g) \times 100. ^bInhibition rate of lung metastases = Model-Treatment/Model-Control(lung wet weight) \times 100%. *p < 0.001, ** p < 0.01, *** p < 0.05 vs Model.

Group	Number	Tumor weight index ^a	Number of Metastasis	Lungs wet weight(mg)	Inhibition rate of lung metastases % ^b
C	9			170.8 \pm 17.1*	
M	7	25.3 \pm 7.3	18.6 \pm 3.9*	250.0 \pm 30.2	0
ABL	8	18.6 \pm 3.7***	11.0 \pm 2.6*	192.5 \pm 17.4*	72.8
ABM	9	18.0 \pm 5.2***	7.2 \pm 1.9*	199.7 \pm 23.6**	64.6
ABH	6	19.8 \pm 6.5	7.2 \pm 2.3*	201.8 \pm 38.9***	61.0

Table 2. Effect of Acetazolamide or combination with NaHCO₃ on total CA activity (mean \pm SD). C: normal, M: tumor model, B: NaHCO₃, AL: acetazolamide 20 mg/kg, AM: acetazolamide 40 mg/kg, AH: acetazolamide 80 mg/kg, ABL: acetazolamide 20 mg/kg and NaHCO₃ 30 mg/kg, ABM: acetazolamide 40 mg/kg and NaHCO₃ 30 mg/kg, ABH: acetazolamide 80 mg/kg and NaHCO₃ 30 mg/kg. *p < 0.001, **p < 0.01, ***p < 0.05 vs Model.

	Number	CA activity in lung (EU/mg prot)	CA activity in primary tumor (EU/mg prot)
C	9	1.98 \pm 0.39	
M	7	2.09 \pm 0.42	0.99 \pm 0.20
B	9	1.68 \pm 0.25***	0.78 \pm 0.20***
AL	8	1.16 \pm 0.33*	0.79 \pm 0.23
AM	7	0.95 \pm 0.27*	0.65 \pm 0.19**
AH	8	0.71 \pm 0.25*	0.57 \pm 0.10*
ABL	8	0.78 \pm 0.24*	0.89 \pm 0.08
ABM	9	1.31 \pm 0.26*	0.77 \pm 0.14***
ABH	6	1.98 \pm 0.38	0.65 \pm 0.16**

3.3 AQP1 protein expression

Western blot demonstrated that the AQP1 expression in the lungs bearing tumor was higher than that of normal mice tissue. Acetazolamide clearly inhibited the protein expression of AQP1 in the treated group in comparison to the tumor transplanted model group. The dose of 20 mg/kg is the most effective group. Adding NaHCO_3 , there was a trend of decrease in the suppression action of acetazolamide (Figure 1) but not dramatically, which was identical with the changes of CA activity in the lung and the rate of metastasis in lungs.

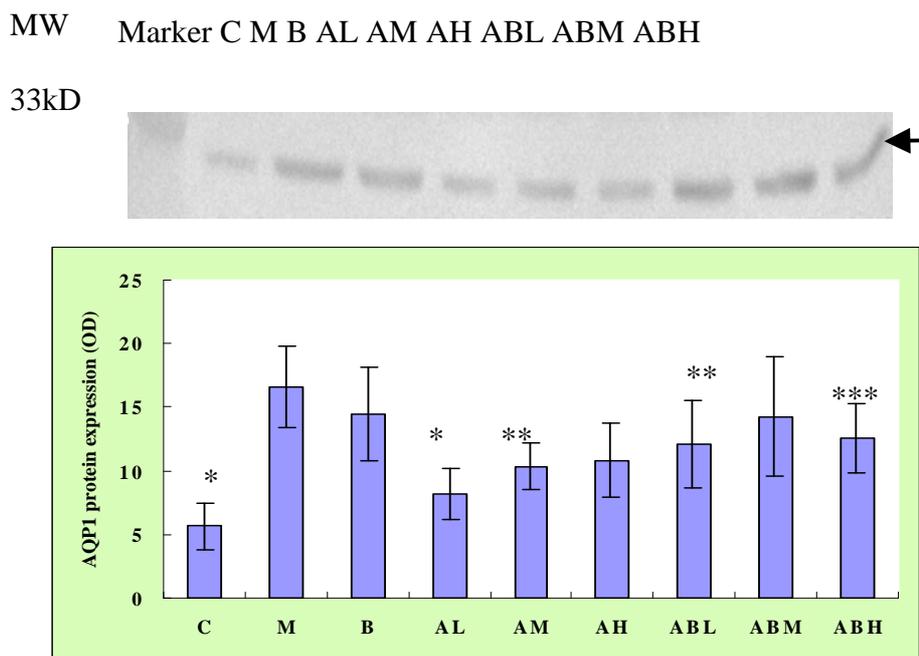


Figure 1. The AQP1 protein level in the lung specimen of each group. Arrow points to the 28 kD AQP1 protein. C: normal, M: tumor model, B: NaHCO_3 , AL: acetazolamide 20 mg/kg, AM: acetazolamide 40 mg/kg, AH: acetazolamide 80 mg/kg, ABL: acetazolamide 20 mg/kg and NaHCO_3 30 mg/kg, ABM: acetazolamide 40 mg/kg and NaHCO_3 30 mg/kg, ABH: acetazolamide 80 mg/kg and NaHCO_3 30 mg/kg. $n = 6-9$, mean \pm SD, * $p < 0.001$, ** $p < 0.01$ *** $p < 0.05$ vs Model.

3.4 Localization and expression of AQP1 in tumor tissues

Immunohistochemical localization of AQP1 in mice tumor and lung was labeled in capillaries, postcapillary venules and bronchiolar basal membrane (Figure 2, positive staining was showed in dark color). Immunostaining showed strongly staining for AQP1 in mice bearing Lewis lung carcinoma (Figure 3. A), but presented a diminished staining in treated by acetazolamide at the medium dose (40mg/kg, Figure 3. B) which was consistent with the result of Western blotting. Combination with NaHCO_3 (30 mg/kg) could not dramatically inhibit the expression of AQP1 (Figure 3.D) , in comparison with mice bearing Lewis lung carcinoma (Figure 3. C).

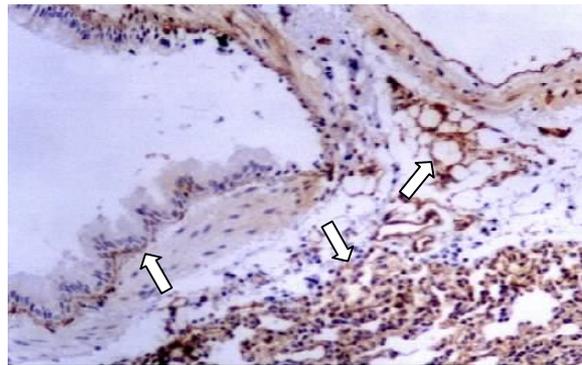


Figure 2. Localization of AQP1 in mice lung. AQP1 positive staining were found in capillaries, postcapillary venules, bronchiolar basal membrane and alveolar epithelial cells as arrows pointed(M group $\times 100$).

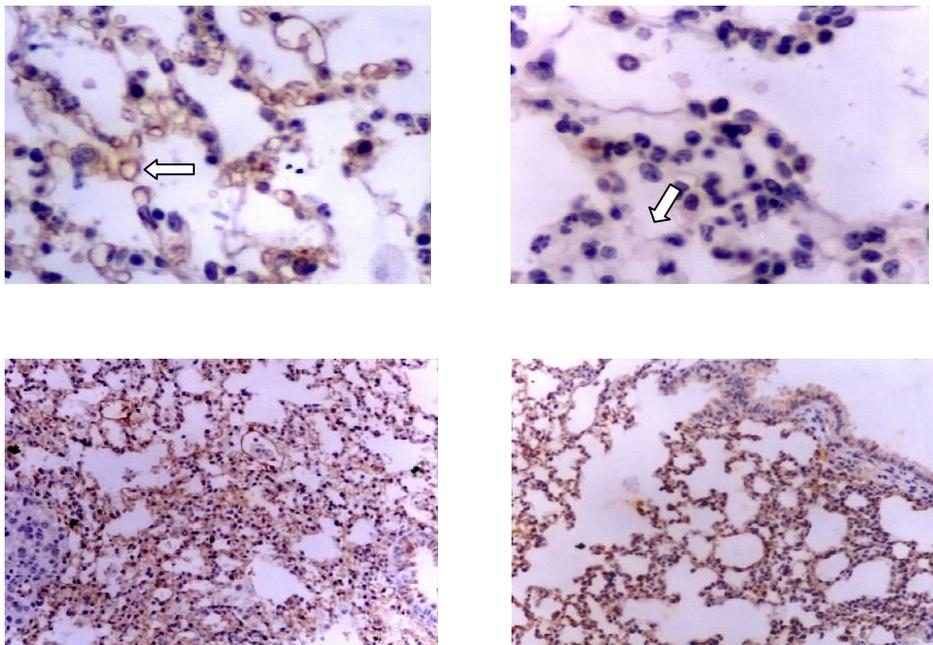


Figure 3. Localization and expression of AQP1 protein in the lungs after treatment with or without NaHCO_3 . The arrow indicate the expression of AQP1 in alveolar epithelial cells. A: M group, showed strongly positive for AQP1 in mice bearing Lewis lung carcinoma ($\times 400$); B: treated with acetazolamide showed a thin staining of AQP1 at the dose of 40 mg/kg ($\times 400$), C: M group ($\times 100$), D: treated with acetazolamide (40 mg/kg) and NaHCO_3 (30 mg/kg) which could not inhibit the expression of AQP1 dramatically in comparison with mice bearing Lewis lung carcinoma ($\times 100$).

4. Discussion

It is reported that some new sulfonamide derivatives possessing potent carbonic anhydrase (CA) inhibitory properties are effective on repressing tumor cell growth and metastasis *in vitro* [6]. In the present study and in our previous study, we have demonstrated that acetazolamide could dramatically reduce the rate of lung metastasis and primary tumor growth, which appeared in a dose-

dependent manner. Its inhibition rate of lung metastases was 77.7% (80 mg/kg/day i.g. for 21days). In addition, we observed that acetazolamide could significantly inhibit CA activity in tumor tissue and lung tissue. After adding NaHCO_3 , the suppression of acetazolamide on tumor growth, metastasis and CA activity could not be increased. Therefore, the suppressive effects of acetazolamide on some tumor-associated CA (I, II, IV, IV, IX, XII) isozymes activity may be one of the mechanisms in promoting its inhibitory effect on tumor metastasis.

As we know, when acetazolamide was introduced as a chemotherapeutic agent, a notable side effect was detected which is metabolic acidosis. Furthermore extracellular pH in solid tumors is more acidic than that in adjacent normal tissue [25]. Acidic extracellular microenvironment is beneficial to tumor growth and metastasis [26]. Hence, the neutralizing the metabolic acidosis can assist the anti-tumor effect of acetazolamide. However our results did not show that adding NaHCO_3 could synergize the effect of acetazolamide. This fact provides a compelling argument for a more thorough evaluation of metabolic acidosis as an underlying interfering factor or mechanism of action to CA inhibitors on its anti-tumor activity and leading the emergence of some other hypothesized mechanisms of acetazolamide.

In this study, the results of Western blotting analysis indicated that AQP1 protein expression in the lung of mice bearing tumor was higher than that in normal mice tissue. Acetazolamide dramatically inhibited the expression of AQP1 and addition of NaHCO_3 could not synergize the effect of acetazolamide. Immunohistochemical localization of AQP1 in mice lung was labeled in capillaries, postcapillary venules and bronchiolar basal membrane. Immunostaining showed a strongly positive for AQP1 in mice bearing Lewis lung carcinoma, but negative result in those animals treated with acetazolamide. This study suggests the possibility that tumors growth might have higher water permeability since that a general function of AQP1 is to increase membrane water permeability. We proposed that appropriate management of fluid in the vascular, interstitial, and cells would be in parallel to anti-tumor growth. Furthermore, the expression of AQP1 in tumors might reflect a role of this water channel in pathological processes including the development of effusions or edema due to changes in hydrostatic or tumor oncotic pressures. Ivanov et al. [27] reported that the high vascular permeability and high interstitial fluid pressure of most tumors might result from activation, as a consequence of the acidic tumor microenvironment, of trans-membrane aquaporins that are widely distributed in tumors. Therefore, the most straightforward explanation for our finding is that acetazolamide suppresses tumor metastases, at least partially by inhibiting the expression of AQP1. Our finding is consistent with the views of that transcript levels of AQP1 might serve as a new molecular prognostic marker in patients with renal cell carcinoma following nephrectomy [28], and might be a therapeutic target for human diseases in the coming future. The exact role of AQP1 in tumor growth and metastasis is being studied through gene transfection *in vitro* by our laboratory.

On the basis of our data *in vivo*, the possible mechanisms remain to be explored in detail. We have carried out a comprehensive proteomic analysis by investigating the protein targets of acetazolamide on tumor growth and tumor metastases, which will help us to understand the molecular insight for its antagonistic effects on carcinogenesis and metastasis[24]. Angiogenesis, the formation of new blood vessels, is required for sustaining tumor growth in the face of an accumulating cell mass and providing access to the systemic circulation so that tumor microemboli can be transported to distant sites and

established metastatic foci. In a previous study, our laboratory has demonstrated acetazolamide can repress the ability of the endothelial cell to participate in the angiogenic process, and the mechanism of acetazolamide on tumor metastases could be involved in reducing AQP1 water channel protein expression[29].

In conclusion, acetazolamide has a strong anti-tumor and anti-metastasis effect on mice bearing Lewis-lung-carcinoma. The mechanism on its anti-tumor effects especially on the anti-metastasis actions might partly involve either inhibiting the carbonic anhydrase activity or reducing AQP1 water channel protein expression, whatever if treated with or without NaHCO₃.

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