

Aquaporin 5 expression increases mucin production in lung adenocarcinoma

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Abstract. Our recent studies have demonstrated that AQP5 is involved in the metastatic potential of lung cancer. Here, our aim was to explore the effects of AQP5 expression on mucin production in lung adenocarcinoma. We tested MUC5AC and MUC5B mucin production induced by AQP5 expression in lung adenocarcinoma metastasis. Lung adenocarcinoma cells with different levels of AQP5 expression were used in this study. In another set of experiments, deletion of AQP5 was studied using AQP5 (-/-) mice. Significantly increased expression of MUC5AC and MUC5B mucin was found in AQP5 high-expressing tumor cells, which suggested that mucin production induced by AQP5 may contribute to the enhanced metastatic potential in lung adenocarcinoma cells. Our results also showed that AQP5 expression increases MUC5AC and MUC5B mucin production and that this may be partly through the EGFR signaling pathway. In brief, our results provide evidence that mucin production induced by AQP5 expression may play important roles in enhanced metastasis potential in lung adenocarcinoma.

Introduction

As membrane protein, aquaporin (AQP) mainly facilitates osmotic fluid transport (1-4). Recent studies have discovered that AQP has unexpected functions other than just facilitating osmotic water transport. For example, AQP-promoting cell migration was found in colon, pancreatic and other cancer cells. By using resected NSCLC tissue samples (IHC), it was shown that AQP5 expression was highly increased in human non-small cell lung cancer (5), which suggests the potential

role of AQP in promoting cancer metastasis. Moreover, our recent study (6) demonstrated that AQP5 is involved in the metastasis potential of lung cancer, and also suggested that mucin expression induced by AQP may play roles in the progression of lung cancer metastasis.

Interestingly, studies have demonstrated that AQP5 was strongly expressed predominantly in lung adenocarcinomas and poorly expressed in non-mucinous bronchioloalveolar carcinoma (BAC) (5). It has been suggested that mucin phenotype is an independent prognostic factor (7-13) in cancer progression. Studies also showed MUC5AC high-expression is associated with the early post-operative metastasis potential in mucin-secreting non-small cell lung cancer (14-16), and MUC5B mucin is upregulated in breast cancer (17). Expression of mucin genes is tissue-specific and that the expression is altered during the pathogenesis of several diseases, which suggests human mucin expression is tightly regulated and may play important roles during carcinogenesis. Our previous studies also showed that deletion of AQP5 was associated with downregulation of MUC5AC expression in airway epithelial cells (18,19). It is thus possible that the above observed enhanced metastasis potential associated with AQP5 in lung cancer may be partly due to the up-regulation of mucin.

Studies also suggested that AQP may interact with some downstream signaling pathways. Kang showed AQP5 protein expression can influence colon cancer development through its interaction with Ras/ERK pathway. Our recent study also demonstrated different expression of AQP5 leads to the changed activation of EGFR signaling pathway in SPC-A1 cell line (6).

Considering EGFR, one core signaling pathway known for its potential role in tumor cell metastasis (20-23) and regulation of mucin production (15,24). It is thus important to examine the roles of mucin in AQP5-promoting metastasis potential of lung adenocarcinoma, and the underlying mechanism. This study, using lung adenocarcinoma cell lines with different expression of AQP5 demonstrated that mucin may be a unique biomarker which is associated with malignancy in lung adenocarcinoma metastasis induced by AQP5.

Materials and methods

Cell culture and transfection. Human pulmonary adenocarcinoma cell lines (LTEP-A2 and PC-9) grown in RPMI medium

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(Hyclone, USA) supplemented with 10% FBS (Hyclone) were cultured in 5% CO₂ incubator at 37°C, and divided into two groups: mock group (transfected with pEGFP-N1 plasmid and showed no significant change of AQP5 expression) as control and AQP5 group (transfected with pEGFP-N1/AQP5 plasmid showed AQP5 high-expression).

Mammalian cell expression vector, pEGFP-N1, was purchased from GenScrip (NJ, USA). Human AQP5 plasmid was constructed and clones were confirmed by restriction analysis and DNA sequencing of both strands (Bioasia, Shanghai, China). Cells were transfected with pEGFP-N1 plasmid (the mock group) or the recombinant pEGFP-N1/AQP5 plasmid (the AQP5 group). Stable AQP5-transfected LTP-A2 and PC-9 cells were selected by G418 following previous protocols (25). Stable AQP5-transfected tumor cell lines with and without stable AQP5 high-expression were obtained and used in the study. Pictures were taken by phase contrast microscopy (Leica, Germany).

Mice. Experiments were done in weight and sex-matched male nude mice (BALB/CA-nu/nu) (6-week-old), which were obtained from the Shanghai Experimental Animal Center (Chinese Academy of Sciences, China). AQP5 (+/+) and AQP5 (-/-) mice with a C57BL/6 genetic background (gift from Prof. A.S. Verkman, University of California, San Francisco) were used in this study. All mice were bred and housed in pathogen-free barrier. Animal protocols were approved by the ethics committee on animal experiments of the University of Fudan Animal Care Committee, Shanghai, China.

Histology and immunofluorescence. Cells or lung tissue were fixed in 4% paraformaldehyde and embedded in paraffin or OCT for paraffin or frozen slices, respectively. Paraffin-embedded slices were stained with hematoxylin and eosin. Cell or tissue sections of adult mice immunofluorescence staining was dealt with as previously (25). Anti-AQP5 primary antibody (1:100, Chemicon, USA) and rabbit anti-mouse IgG antibody conjugated to Cy3 (1:100, Molecular Probe, USA) were used for IF staining.

Immunohistochemistry. All of the specimens were formalin-fixed and paraffin-embedded. Serial sections (3–5 μm thick) were cut. The sections were processed for immunohistochemical analysis as previously (25). Images of immunohistochemistry staining were photographed under microscope (Leica). Immunohistochemistry stain was performed following manufacturer's protocols (Maixin-Bio, China), and the following primary antibodies were used: rabbit monoclonal anti-AQP5 (Chemicon), mouse monoclonal MUC5AC and MUC5B (Cell Signaling, USA). Antibodies and secondary antibodies were used according to manufacturer's instructions. Immunohistochemistry scoring results were obtained based on staining intensity and distribution of immunopositive cells, which was expressed as mean optical density (MOD), semi-quantitative analysis was done by image plus software.

Real-time quantitative PCR. Total RNA was isolated and cDNA was reverse transcribed according to manufacturer's instruction. Primer sequences included: β-actin, sense: 5'CCTGTA CGCCAACACAGTGC3', antisense: 5'ATACTCCTGCT

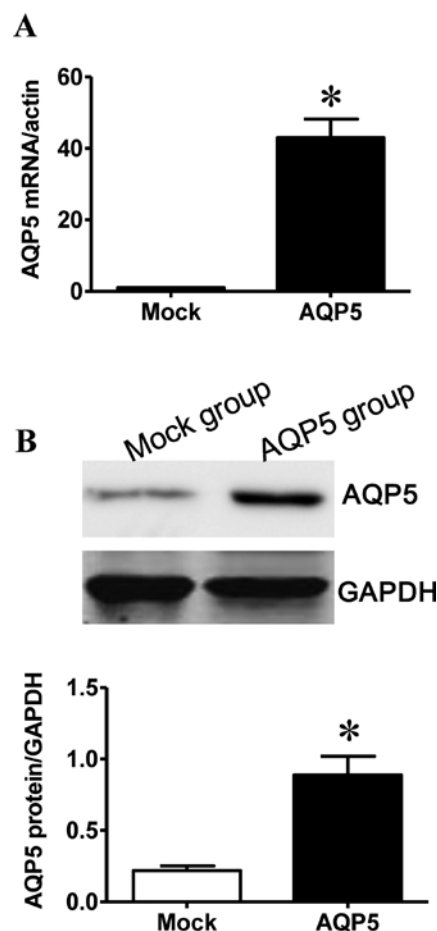


Figure 1. Plasmid transfection and selection of stable cell lines. Cell lines stably transfected with mock or AQP5 plasmids, and detection of AQP5 protein expression by immunofluorescence staining, quantitative real-time RT-PCR and Western blot analyses. Quantitative real-time RT-PCR (A) and Western blot analyses (B) for the examination of AQP5 mRNA and protein expression, showed higher AQP5 expression in the AQP5 group, compared with the mock group (n=4–6, *P<0.01).

TGCTGATCC3'; AQP5, sense: 5'CTGTCCATTGGCCTGT CTGTC3', antisense: 5'GGCTCATACGTGCCTTTGATG3'; MUC5AC, sense: 5'GAGGGCAACAACGTCATCTCC3', antisense: 5'TCTTGGTTCAGCCACCTTCACA3'; MUC5B: 5'CTGTACGGGAACCCTAAGGAAA3', 5'ACCGCAAG ACAGTCGCATTTA3'. Real-time PCR conditions for amplification of target gene by standard procedure, and analyzed by Rotor-Gene 3000 sequence detection system.

Western blot. Total protein was extracted from cells using RIPA kit (Pierce, USA). Protein was electrophoresed on a polyacrylamide gel and transferred to Hybond-C nitrocellulose membranes. The membranes were incubated with anti-AQP5 (Chemicon), EGFR or phospho-EGFR (Cell Signaling) with 1:1000 dilution at 37°C for 2 h, then with peroxidase-conjugated goat anti-rabbit IgG at room temperature for 1 h. GAPDH was used as the internal control. Protein was visualized using enhanced chemiluminescence (ECL) methods. Membranes were washed three times and then exposed to X-ray film. Analyses of the results were performed as described (26).

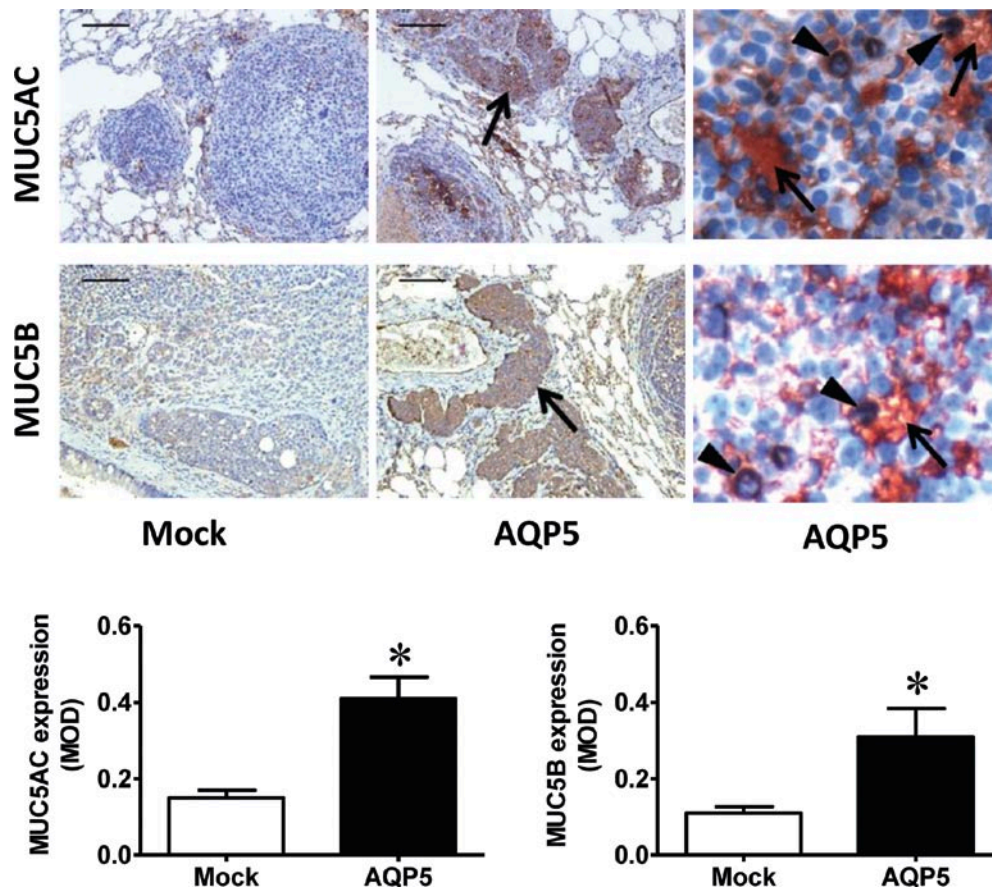


Figure 2. MUC5AC and MUC5B production induced by AQP5 expression. Staining or co-staining for MUC5AC, MUC5B and AQP5 protein expression by immunohistochemistry staining. Results showed significantly increased MUC5AC and MUC5B expression in AQP5 high-expressing group. Co-staining for AQP5 and MUC5AC expression by immunohistochemistry showed significantly increased MUC5AC and MUC5B expression in tumor lesions of AQP5 high-expressing group, arrows indicate MUC5AC or MUC5B expression (red), arrowheads indicate AQP5 expression (black). (n=6, *P<0.01).

Tumor metastasis in vivo. LTP-A2 cells (1×10^6) were injected i.v. through tail vein into nude mice. Mice were then monitored for potential lung tumor colonization and metastases by *in vivo* fluorescence imaging (27). Metastatic lesions were identified by fluorescence signals (Night OWL LB 981 *in vivo* imaging system, Berthold, Germany), and analyzed by WinLight32 software. Mice were sacrificed 4 weeks later, and lungs were harvested for hematoxylin/eosin staining and AQP5 immunofluorescence staining. Metastatic lesions were identified by histology and fluorescence analyses: paraffin-embedded sections were stained with hematoxylin and eosin. Lung frozen sections were observed directly or for immunofluorescence detection by standard procedures. The number of tumor colonies in lungs was counted.

Statistical analysis. Statistics were conducted by SPSS 10.0 software. Values are represented as the mean \pm SD. Statistical analyses were performed by t-test and One-way analysis of variance (ANOVA). All P-values were two-sided, and differences at P<0.05 were considered statistically significant.

Results

Plasmid transfection. LTP-A2 and PC-9 cell lines stably transfected with mock or AQP5 plasmids were selected. Fig. 1 shows AQP5 mRNA and AQP5 protein expression by using

quantitative real-time RT-PCR and Western blot analyses. Of note, significantly increased AQP5 mRNA and protein expression were found in AQP5 group, compared with mock cells.

Increased MUC5AC, MUC5B mucin production in AQP5 group. In this study, we tested roles of mucin including MUC5AC, MUC5B mucin induced by AQP5 in lung adenocarcinoma metastasis. Lung adenocarcinoma cells with different expression of AQP5 were used in this study. Staining or co-staining for MUC5AC, MUC5B and AQP5 protein expression by immunohistochemistry. Results demonstrated significantly increased MUC5AC or MUC5B expression in tumor lesions of AQP5 group, compared with the mock group. Arrows indicate MUC5AC or MUC5B expression (brown), arrowheads indicate AQP5 expression (black) (Fig. 2). The results suggested increased MUC5AC, MUC5B mucin production is associated with AQP5 expression.

Increased metastasis potential of AQP5 high-expressing cells in vivo. As mentioned above, nude mice were used to study tumor cell metastasis *in vivo*. After LTP-A2 cells were injected through tail veins, lung metastases were monitored 4 weeks later. Histology study showed a number of well-demarcated tumor metastasis nodules in lungs and AQP5 high-expressing tumor cells injection produced many more metastatic nodules in lungs of AQP5 group, compared to the mock group.

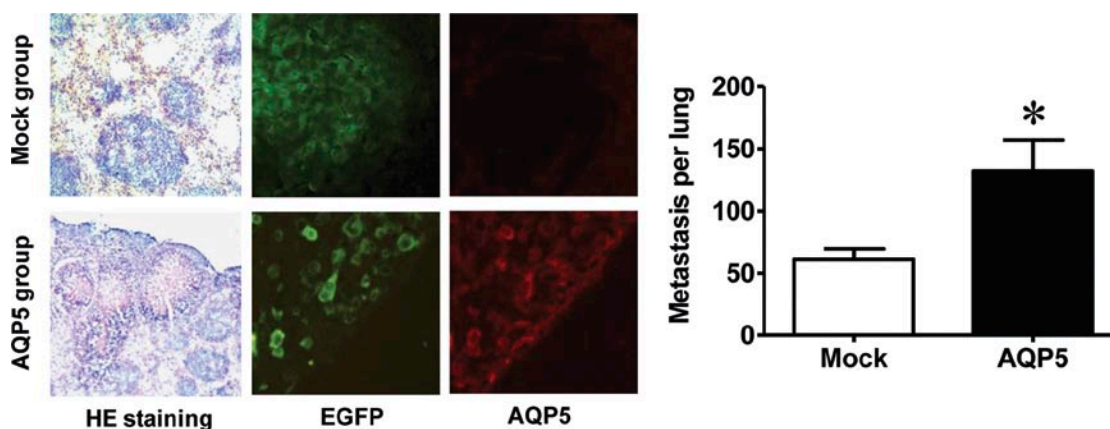


Figure 3. Enhanced metastasis potential of AQP5 high-expressing lung adenocarcinoma cells *in vivo*. Optical imaging and fluorescence image measurements confirmed tumor metastasis in all mice. The results showed lung histology after tail vein injection of the mock or AQP5 high-expressing tumor cells, with more regions of tumor lesions generated in lungs of the AQP5 group, compared with the mock group, which suggested more tumor metastasis nodules. Data summary showed tumor colonies of metastasis (as representative optical image of lung slides) (n=6, *P<0.01). Fluorescence images showed AQP5 protein expression (red) and EGFP (green) expression by immunofluorescence staining.

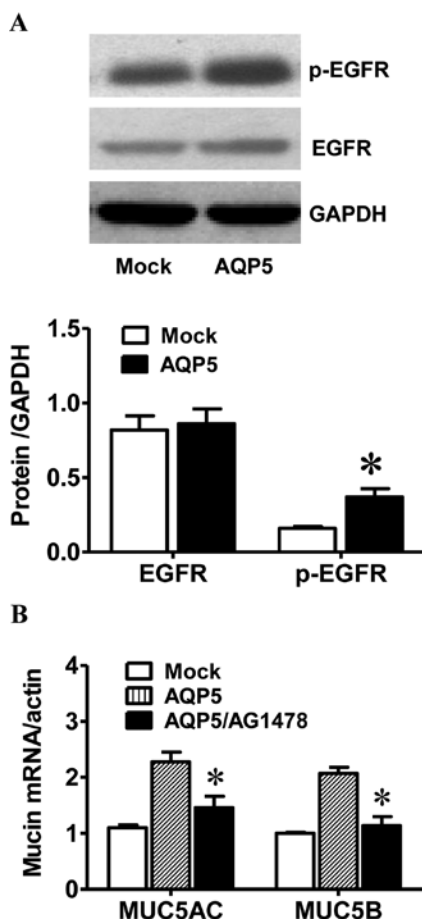


Figure 4. Interaction of AQP5 with EGFR/mucin pathway. By Western blot analysis, the results showed AQP5 high-expressing tumor cells demonstrated significantly enhanced activation of EGFR pathway, compared with the mock group (n=4-6, *P<0.05) (A). Moreover, quantitative real-time RT-PCR analysis showed significantly increased MUC5AC and MUC5B mRNA expression in AQP5 high-expressing tumor cells, compared with mock group. Pretreated with a special inhibitor of EGFR signaling pathway (AG1478), real-time RT-PCR showed significantly down-regulation of MUC5AC and MUC5B mRNA expression in AQP5 high-expressing tumor cells (n=6, *P<0.01) (B). The data suggest that interaction of AQP5 and EGFR/mucin pathway may play a role in the enhanced metastasis potential in lung adenocarcinoma.

Immunofluorescence staining in tumor lesions showed significantly higher AQP5 expression after injection of AQP5 high-expressing cells, compared to mock cells (Fig. 3). These data suggested that AQP5 expression increases metastasis potential of lung adenocarcinoma cells *in vivo*.

AQP5 interacts with EGFR/mucin pathway. By Western blot analysis, our result showed AQP5 high-expressing cells demonstrated significantly enhanced expression of EGFR phosphorylation (p-EGFR), compared with the mock cells, although there was no difference of the total EGFR protein expression between the two groups. Quantitative real-time RT-PCR analysis showed significantly increased MUC5AC and MUC5B mRNA expression in AQP5 high-expressing cells, compared with mock cells *in vitro*. Considering increased MUC5AC or MUC5B mucin expression induced by AQP5 may partly be through EGFR signaling pathway, in this study, pretreated with a special inhibitor of EGFR signaling pathway (AG1478), real-time RT-PCR showed significant down-regulation of MUC5AC and MUC5B mRNA expression in AQP5 high-expressing cells (Fig. 4). These results suggested that enhanced mucin production induced by AQP5 expression might be partly through EGFR/mucin pathway.

Correlation of mucin production induced by AQP5 with tumor metastasis potential. Role of mucin in tumor metastasis potential has been extensively studied in recent years. The results demonstrated here show that a correlation exists between the metastatic potential and mucin production induced by AQP5 expression. A significant difference of mucin expression was found between groups with or without AQP5 high-expression. A positive relationship was noted between AQP5 and mucin expression, meanwhile, significant correlation was also noted between mucin expression and enhanced metastasis potential in AQP5 high-expression cancer cells. Therefore, increased expression of MUC5AC and MUC5B mucin may contribute to the enhanced metastasis potential in AQP5 high-expressing lung adenocarcinoma.

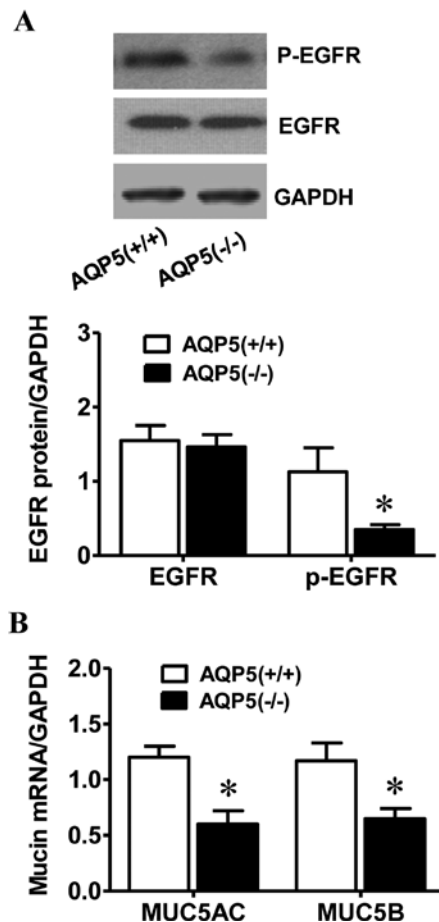


Figure 5. Deletion of AQP5 showed decreased activation of EGFR pathway and mucin production in mice. AQP5 high-expressing cells showed significantly activated EGFR pathway and up-regulation of MUC5AC and MUC5B mucin production, compared to mock cells. Deletion of AQP5 showed significantly decreased activation of EGFR pathway and MUC5AC, MUC5B mucin production in lung tissue, compared to wt mice (n=6, *P<0.01).

Deletion of AQP5 showed decreased activation of EGFR pathway and mucin production in mice. As mentioned above, AQP5 high-expressing cells showed significantly activated EGFR phosphorylation (p-EGFR) and up-regulation of MUC5AC and MUC5B mucin production, compared to mock cells. To test whether the observation was specific in AQP5 high-expressing cells, deletion of AQP5 by using AQP5 (-/-) mice served as control since AQP5 was abundantly expressed in lung tissue. In another set of experiments, deletion of AQP5 showed significantly decreased activation of EGFR pathway and MUC5AC and MUC5B mucin production in lung tissue, compared to wt mice (Fig. 5).

Discussion

Aquaporin (AQP) expression has been found in high-grade tumors of different tissues, and AQP expression in tumor cells was correlated with the metastatic potential. Studies showed AQP5 seemed to be an independent molecular marker associated with worse clinical outcomes of lung cancer. Chae *et al* found AQP5 over-expression was significantly associated

with worse disease-free survival in NSCLC (5). Our recent study also suggested AQP5 is associated with the enhanced metastasis potential (6). However, roles of AQP5 in lung adenocarcinoma metastasis need further exploration.

Mucins have been shown to affect the formation of tumor tissues and immune system in tumor, and it can facilitate tumor progression in tumor microenvironment (28-30), including angiogenesis and invasion (31). It was not excluded that AQP5 promoted cancer cell migration by other mechanisms. Studies of the relationship between AQP and mucin expression and its role on the metastasis potential of lung adenocarcinoma will certainly help in the understanding of the novel role of AQPs. However, interactions of AQP and mucin expression in lung adenocarcinoma cells remains poorly understood. Based on the above results, it was then suggested that such interactions of AQP and mucin expression can be altered to allow the tumor cells to migrate and induce metastasis. It is thus clear that any change in their expression will affect these functions and modify the behavior of cancer cells. Here we hypothesized that mucin may play a role in AQP5-induced cancer metastasis potential.

In our laboratory, AQPs and mucin expression has been extensively studied. We have found that AQP5 high-expression leads to increased MUC5AC mucin production in SPC-A1 cell line (6). Thus indicating the possibility that enhanced metastasis potential associated with AQP5 expression in tumors may be partly through up-regulation of mucin in some lung cancers, especially lung adenocarcinoma. In this study, MUC5AC and MUC5B mucin production and the metastasis potential were tested in lung adenocarcinoma cells with different AQP5 expression.

This study showed significantly enhanced MUC5AC and MUC5B mucin expression in AQP5 high-expressing cells. These data provide evidence that AQP5 interacting with mucin production may play important roles in lung adenocarcinoma metastasis. An *in vivo* study showed significantly enhanced lung metastases after intravenously delivering of AQP5 high-expressing cancer cells. Correlation analysis showed positive relationship between AQP5 and mucin expression, significant correlation was noted between mucin expression and enhanced metastasis potential in AQP5 high-expression cancer cells. Although various studies have suggested that AQPs may be associated with cancer cell metastasis (6,32), so far only few metastasis markers have been tested. These data provide novel mechanism that AQP5 is associated with increased metastasis potential in lung adenocarcinoma.

It is thus important to explore experimentally how AQP5 expression leads to enhanced metastasis potential. The underlying molecular mechanism mediated by AQP5 for promoting tumor metastasis in lung adenocarcinoma need clarification. Kang *et al* showed AQP5 expression can influence colon cancer development possibly through its interaction with the ERK pathway. Woo *et al* showed overexpression of AQP5 in NIH3T3 cells demonstrated a significant effect on Ras activity. Our recent study (6) suggested EGFR pathway play roles in human carcinogenesis. While, mucin expression in response to different stimulate is regulated by EGFR signaling pathway partially, we hypothesized EGFR/mucin pathways might play important roles in lung cancer potential associated with AQP5. Our study showed AQP5

high-expression leads to higher activity of EGFR pathway and increased mucin production, which suggested that AQP5 might interact with EGFR/mucin pathway directly or indirectly. Consistent with the above data, the effect was inhibited, when pretreated with a selective EGFR inhibitor (AG1478).

Here our results implicate the involvement of AQP5 in modulation of MUC5AC or MUC5B mucin expression in lung adenocarcinoma, which might be through EGFR signaling pathway in part, but why AQP5 expression could act on EGFR/mucin pathway needs further study. Taken together, these data provide novel mechanism for roles of AQP5 in lung cancer metastasis, and demonstrate the interaction between AQP5 and EGFR/mucin pathway in lung adenocarcinoma, suggesting MUC5AC or MUC5B mucin levels induced by AQP5 may provide information relevant to prognosis as well as treatment decisions.

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