Original Article
Use of aquaporins 1 and 5 levels as a diagnostic marker in mild-to-moderate adult-onset asthma

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Abstract: Characteristic features of asthma include airway inflammation and hyperactivity, mucus hypersecretion, mucosal edema, and airway remodeling. These features could be due to pathological water transport across pulmonary epithelia and aquaporins (AQPs) have recently been isolated as key proteins in fluid transportation in the human respiratory tract. We aimed to evaluate the role of aquaporins in the pathogenesis of asthma and their possible use a diagnostic marker of the disease. A total of 110 hospitalized and outpatients with mild to moderate adult-onset asthma were invited to participate in this study and 34 submitted an induced sputum sample adequate for analysis. The amount of AQP1, AQP5 and MUC5AC were measured with ELISA assay. The amount of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17 in both serum and sputum were measured with Cytometry Bead Array (CBA kit).

Our results suggest that sputum AQP5, AQP1 and MUC5AC are all in a good correlation (r=0.498 between AQP5 and AQP1, r=0.529 and r=0.661 between MUC5AC and AQP5 or AQP1, respectively, all P<0.05). The AUC value for AQP1 and AQP5 to diagnose asthma were 0.729 and 0.745, respectively.

In conclusion, water homeostasis plays an important role in maintaining adequate fluid transportation within the lung and is involved in the pathogenesis of asthma. Our results suggest that AQPs may influence pulmonary physiology that their dysfunction can contribute to pulmonary pathogenesis, such as asthma. Moreover, their quantification could serve as biomarkers for the diagnosis of asthma.

Keywords: Asthma, aquaporin 1, aquaporin 5, disease models, humans

Introduction
Asthma is the most common chronic respiratory disease and is associated with increased morbidity and mortality. Airway inflammation and hyperactivity, mucus hypersecretion, mucosal edema, and airway remodeling are characteristic features of the disease [1, 2]. Pulmonary epithelial water transport is a vital component of lung physiology; fluid movement between the airspace and cellular and/or interstitial and vascular compartments contribute to an adequate airway fluid balance [3, 4]. Under pathological conditions, dysregulation of transepithelial water transport appears to play an important role in the development of pulmonary edema [3, 4]. However, the detailed mechanisms and specific contribution of water transportation in the development of pulmonary edema is still unclear.

Aquaporins (AQPs) constitute a family of proteins highly expressed in the membrane of tissue cells involved in water transportation (e.g., blood vessels, urinary, respiratory and digestive tract) [4-9]. They are water-permeable channels located within the cell membrane, or pore proteins, that play an important role in selective water transfer through the cell wall. To date, 13 different AQPs have been identified and cloned in mammals. Among those, AQP1 and AQP5 constitute the principal routes for osmotically driven water transport in the human respiratory tract. While AQP1 is expressed in microvascular endothelial cells (MECs) and surrounding connective tissue cells, AQP5 is present in the apical membrane of airway epithelial cells [6, 8]. AQP1 may help regulate the permeability of plasma to the vascular walls [10], while AQP5 may be involved in the formation of mucus within the respiratory tract [6, 11, 12]. Loss of func-
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In this study, we measured AQP5, cytokines and cell counts in blood and induced sputum samples from mild to moderate adult-onset asthma patients. The correlation between AQP5 expression level and its possible use as a diagnostic marker for asthma was also explored.

Materials and methods

Subjects

Both hospitalized and outpatients with mild to moderate adult-onset asthma, according to the Chinese Medical Association of Respiratory Disease Branch Asthma Study Group diagnostic criteria, were recruited in the department of respiratory medicine of the Chinese Traditional Medicine Hospital of Xinjiang Medical University between March, 2012 and July, 2013. Patients with severe asthma or acute exacerbation of asthma, those with a history of cardiac or other respiratory diseases, those who reported a respiratory tract infection less than two weeks before sputum collection or received glucocorticosteroids less than a month before collection, current or past smokers (0.5 package/day) were excluded from the study, as well as pregnant women and those who refused to participate in research. Furthermore, eleven healthy subjects without any respiratory diseases were recruited from the surrounding community of hospital as control between March and December 2012. This study was approved by the Chinese Traditional Medicine Hospital of Xinjiang Medical University’s ethics committee. All patients were informed of the study’s experimental nature and gave written informed consent.

Sample collection and handling

A 3 ml fasting venous blood sample was collected in the morning, centrifuged under 4°C for 20 minutes at 3,000 rpm for 20 min and subsequently stored at -80°C until analysis of serum IL-2 (sIL-2), sIL-4, sIL-6, sIL-10, sTNF-α, sIFN-γ, and sIL-17 using a Cytometry Bead Array (CBA kit).

<table>
<thead>
<tr>
<th>Table 1. Physiological parameters</th>
<th>Asthma group N=34</th>
<th>Control group N=11</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (F/M)</td>
<td>28/6</td>
<td>8/3</td>
<td>0.795</td>
</tr>
<tr>
<td>Age</td>
<td>42.35±8.67</td>
<td>38.82±9.84</td>
<td>0.262</td>
</tr>
<tr>
<td>BMI</td>
<td>23.07±3.98</td>
<td>23.19±2.53</td>
<td>0.933</td>
</tr>
<tr>
<td>Course of disease</td>
<td>2.5 (0.3, 25)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ACT</td>
<td>19.03±2.74</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>6.56±14.98</td>
<td>0.50±0.28</td>
<td>0.024</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>48.79±24.35</td>
<td>27.45±11.39</td>
<td>0.000</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>42.03±21.91</td>
<td>67.64±9.27</td>
<td>0.000</td>
</tr>
<tr>
<td>Sputum AQP5</td>
<td>469.39±153.85</td>
<td>565.12±101.68</td>
<td>0.086</td>
</tr>
<tr>
<td>Sputum AQP1</td>
<td>0.89±0.21</td>
<td>0.79±0.39</td>
<td>0.372</td>
</tr>
<tr>
<td>Sputum MUC5AC</td>
<td>65.33±17.84</td>
<td>57.84±12.48</td>
<td>0.223</td>
</tr>
<tr>
<td>Baseline predrug FVC (% predicted)</td>
<td>97.68±15.99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Baseline predrug FEV 1 (% predicted)</td>
<td>81.61±10.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.83±0.48</td>
<td>1.80±0.51</td>
<td>0.881</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.67±0.41</td>
<td>0.46±0.30</td>
<td>0.221</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.47±1.35</td>
<td>2.02±0.87</td>
<td>0.340</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.68±0.50</td>
<td>0.64±0.60</td>
<td>0.845</td>
</tr>
<tr>
<td>TNF-α</td>
<td>6.64±22.50</td>
<td>1.73±1.13</td>
<td>0.523</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.22±0.69</td>
<td>1.64±0.60</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-17</td>
<td>19.75±3.91</td>
<td>17.01±6.09</td>
<td>0.213</td>
</tr>
<tr>
<td>sIL-2</td>
<td>1.35±0.69</td>
<td>1.03±1.13</td>
<td>0.573</td>
</tr>
<tr>
<td>sIL-4</td>
<td>0.15±0.16</td>
<td>0.18±0.31</td>
<td>0.830</td>
</tr>
<tr>
<td>sIL-6</td>
<td>105.27±113.54</td>
<td>59.65±46.82</td>
<td>0.528</td>
</tr>
<tr>
<td>sIL-10</td>
<td>0.31±0.31</td>
<td>0.04±0.08</td>
<td>0.183</td>
</tr>
<tr>
<td>sTNF-α</td>
<td>1.95±1.84</td>
<td>2.23±0.91</td>
<td>0.807</td>
</tr>
<tr>
<td>sIFN-γ</td>
<td>1.82±1.31</td>
<td>1.70±0.40</td>
<td>0.881</td>
</tr>
<tr>
<td>sIL-17</td>
<td>23.95±15.46</td>
<td>17.94±5.19</td>
<td>0.538</td>
</tr>
</tbody>
</table>

*Values are represented as mean ± S.E., P<0.05 was considered significant.
Induction and handling of sputum was conducted as previously reported [13-16]. Prior to sputum induction, patients with a FEV1 <80% were administered inhaled beta2-agonist (salbutamol, 200~400 µg, GSK, catalogue number: KR0160) to prevent secondary bronchospasm during the procedure, a FEV1 testing was reapplied. If the FEV1 is still <80%, the sputum induction was canceled. Sputum induction was performed using an inhalation of atomized hypertonic sterile saline (3%) at 4 ml per minute, during a total 20 minutes. Procedures were immediately interrupted if patients felt uncomfortable. Under the guidance of physicians, patients were encouraged to actively cough up sputum at 5, 10, 15 and 20 minutes following inhalation of hypertonic saline. Sputum plugs weighing over 0.3 gram were collected with sterile forceps and deposited in a dry and sterile vial, to which fresh sputum-lysis buffer was added (4 times larger in volume). Specimens were then incubated for 15 minutes at 37°C and vortexed to homogeneity. Cells and supernatant were subsequently separated by centrifugation under 4°C, at 3000 g for 10 minutes; supernatant was removed and stored at -80°C until further measures of AQP1, AQP3, AQP4, AQP5 and MUC5AC using an ELISA assay, while IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, IL-172 were subsequently measured using a Cytometry Bead Array (CBA kit).

Cell pellets were resuspended with PBS and viability measured with the Trypan Blue exclusion method. Sputum samples with cell viability >50% were processed for further analysis and underwent cell count. Samples were then further centrifuged for 5 minutes at 500 g and processed for cytological examination. Cell smears were made and stained with hematoxylin and eosin. At least 400 non cubic epithelial cells were counted and classified under light microscope. Epithelial cells, alveolar macrophages, neutrophils, squamous cells, eosinophils and basophils were classified and counted under microscope. Samples with >20% squamous cells were discarded as large number of squamous cells indicates pollution.

Pulmonary function test

Spirometry was performed using pulmonary function impulse oscillometry (Impulse Oscillation Detector (Master Screen IOS) for Pulmonary Function, Jaegere, Germany). Pulmonary capacity and flow-volume loop were determined and FEV1 value and FEV1% of predicted normal value were recorded, as well as the PEF% of predicted normal value.

Statistical analysis

Statistics were analyzed with MP Pro 10.0. Mean values are presented with their corresponding standard errors, and mean values between patients and controls were tested with t-test. For multiple comparisons, F-test was firstly applied and, if SD was not uniform, Welch test was applied to test for significance. In all instances, tests with P-values under α=0.05 were considered statistically significant.

We analyzed the correlation between eosinophils, AQP1, AQP5, MUC5AC and IFN-γ. MUC5AC was included as it is an important readout for mucus hypersecretion in the pulmonary tracts and associated with chronic obstructive pulmonary disease [17-19]; IFN-γ was included because it showed significant difference between asthma patient and healthy controls in our primary analysis (Table 1).

Results

Subjects and sample selection

During the study period, 110 subjects were invited to participate in the study. Among those, 76 subjects met the exclusion criteria and 34 patients with adequate samples were accepted for final analysis (Figure 1).
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Table 2. Summary of correlation among eosinophils, AQP1, AQP5, MUC5AC and IFN-γ

<table>
<thead>
<tr>
<th></th>
<th>Eosinophils</th>
<th>Sputum AQP5</th>
<th>Sputum AQP1</th>
<th>MUC5AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum AQP5</td>
<td>-0.386*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum AQP1</td>
<td>-0.201</td>
<td>0.498*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum MUC5AC</td>
<td>-0.279</td>
<td>0.529*</td>
<td>0.661*</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>0.114</td>
<td>-0.145</td>
<td>0.075</td>
<td>0.155</td>
</tr>
</tbody>
</table>

*P<0.05.

![Figure 2](image-url)  
Figure 2. Scatter plot and linear-regression analysis of correlation between sputum AQP1 and sputum AQP5 (R²=0.248).

Physiological parameters

We measured several physiological parameters (Table 1). There was no statistically significant difference in gender, age and BMI between asthma patients and healthy controls. Among those multiple parameters we measured, asthma patients had significantly higher counts of both eosinophils (6.56 vs. 0.50, P=0.024), neutrophils (48.79 vs. 27.45, P<0.001), and IFN-γ (2.22 vs. 1.64, P=0.029) while macrophages were significantly lower in the asthma group (42.03 vs. 67.64, P<0.001).

Correlation among eosinophils, AQP1, AQP5, MUC5AC and IFN-γ

Results of the correlation analysis between eosinophil counts, AQP1, AQP5, MUC5AC and IFN-γ are presented in Table 2. Sputum AQP5 was inversely correlated with eosinophil counts. Sputum AQP1 showed positive correlation with sputum AQP5, sputum MUC5AC was positively correlated with both sputum AQP5 and sputum AQP1. No correlation was found between IFN eosinophil counts, sputum AQP5, sputum AQP1 or MUC5AC.

For a more comprehensive analysis, we fitted linear regression models to evaluate the association between measured levels of MUC5AC, sputum AQP1 and sputum AQP5. Results were also consistent with the summary analyses showing that sputum AQP5 was positively correlated with sputum AQP1 (Figure 2) and sputum MUC5AC was positively correlated with sputum AQP1 (Figure 3) and sputum AQP5 (Figure 4).

Assessment of AQP1 and AQP5 in asthma diagnosis

We further assessed whether AQP1 and AQP5 level in induced sputum could be used as a diagnostic marker for asthma by analyzing their receiver operating characteristic curve (ROC, Figure 5A and 5B). The AUC value for AQP1 and AQP5 was 0.729 and 0.745 (Table 3), respectively, indicating either of them could serve as moderate markers in the diagnosis of asthma (AUC>0.7, [20]). The best cutoff value for diagnostic purposes was 0.69 for AQP1 and 419.23 for AQP5 (Table 3).

Discussion

By analyzing blood and induced sputum samples from adult-onset asthma patients, we carried out the first study to monitor a variety of contents, including different cell types, related proteins and cytokines, and compared their differences between patient and healthy people. We found that, compared to healthy controls, asthma patients had significantly higher counts
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IFN-γ concentrations increased significantly in asthma patients. Furthermore, sputum AQP5 were negatively correlated with eosinophil counts, sputum AQP1 were positively correlated with sputum AQP5, and sputum MUC5AC showed positive correlation with both sputum AQP5 and sputum AQP1. However, IFN did not show correlation with either eosinophil counts, sputum AQP5, sputum AQP1 or MUC5AC. Moreover, we assessed whether AQP1 and AQP5 could be used as diagnostic marker for asthma in clinics using receiver operating characteristic (ROC) assay. We found that AQP1 and AQP5 could be used as moderate markers of asthma, suggesting that their level can assist in the diagnosis of asthma in the clinical setting; the optimal cutoff values were estimated at 419.23 for AQP1 and 0.69 for AQP5.

Our results not only provide direct evidence of correlation between asthma and both AQP1 and AQP5, but also indicate that these two proteins could be used as clinical markers of disease.

Water homeostasis plays an important role in maintaining adequate fluid transportation within the lung and is involved in the pathogenesis of asthma [3-5, 7-9, 11]. AQP1, AQP3, AQP4 and AQP5 have been found to be expressed in mammalian pulmonary tissue [5, 21]. In 2001, Kreda et al. documented the distribution of AQPs in human airways using in situ hybridization and immunohistochemistry staining [22]. Moreover, King et al. further showed that vascular permeability was impaired in AQP1 knockout mice and that congenital AQP1 deficiency leads of both eosinophils and neutrophils while macrophages were significantly lower. Meanwhile,
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induced sputum supernatant. Secondly, AQP5 is involved in glandular secretion, fluid clearance in airway and pulmonary tissue and in maintaining a normal fluid surface in airways. AQP5 decrements can lead to decreased fluid secretion and elevated mucoprotein concentrations in airway. Furthermore, elevated inflammatory factors and cytokines may also lead to airway injury and asthma progression via the downregulation of AQP5 [25, 28, 29]. Those studies all suggest that AQP dysfunctions likely contribute to the pathogenesis and progression of asthma.

Although we have found that the sputum concentration level of several cell types were altered in asthma patients, AQP expression was also higher but we did not observe a statistically significant change in AQPs. It is possible that because we included patients suffering from both mild and moderate asthma in the primary analysis, could have underestimated, or diluted any difference possibly seen in patients with moderate asthma. However, we were unable to evaluate AQP expression in patients suffering from severe asthma, as sputum induction is contraindicated in patients. We believe, however, that differences in AQP expression should be more obvious in patients suffering from severe asthma and that any changes in AQP expression would be significant among this group. Also, our sample size consisting of 34 patients was likely too small and insufficient to less thickening of blood vessel walls following intravenous saline perfusion in human [23] indicating that AQP1 is required for maintaining permeability of pulmonary vasculature. Our findings are consistent with this hypothesis, as asthma also led to decreased expression of AQP1 in our patients compared to healthy controls. However, how AQPs contributes to the pathogenesis asthma is not clear, but there are indications that AQPs may influence pulmonary physiology in a variety of ways and that their dysfunction can contribute to pulmonary pathogenesis. Firstly, AQP1 is the predominant type of AQPs found in pulmonary tissue; it mediates water transportation between airway and pulmonary microvasculature. Upregulation of AQP1 in asthma may increase the water level in pulmonary tissue [24], promote the allergic response and secretions in airway, and promote leakage of inflammatory corpuscle [25]. It can also lead to pathological angiogenesis and promote airway remodeling, a characteristic feature of asthma [26, 27]. Those effects are consistent with our findings that AQP1 shows significant positive correlation with MUC5AC in

Table 3. Summary for AUC, cutoff value, sensitivity and specificity of AQP5 and AQP1

<table>
<thead>
<tr>
<th>AQP</th>
<th>AUC</th>
<th>Cutoff</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP5</td>
<td>0.745</td>
<td>419.23</td>
<td>1</td>
<td>0.441</td>
</tr>
<tr>
<td>AQP1</td>
<td>0.729</td>
<td>0.69</td>
<td>0.882</td>
<td>0.714</td>
</tr>
</tbody>
</table>

Figure 5. Receiver operating characteristic (ROC) of AQP1 (A) and AQP5 (B).
our study was underpowered to detect differences and a larger sample size is needed for future studies to draw convincing conclusions.

In summary, we have characterized cell types and counts, cytokine and aquaporin expression in induced sputum from adult-onset mild to moderate asthma patients. We found that aquaporin expression showed a tendency towards correlation with asthma. Furthermore, we found that the level of aquaporins could be used as moderate diagnostic marker for asthma.

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Disclosure of conflict of interest
None.

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