Effects of Compound Herbal Formulary on Expression of Aquaporin 1 and Aquaporin 5 in the Model of Rats with Phlegm Obstruction Due to Lung-Deficiency Syndromes*

JIANG Yong, LI Xiao-bing△, LIU Xiao-hong, WANG Li-xin, REN Ming-neng, WU Qi-duan, XIE Yu-hui, WU Jian-qi

(First Affiliated Hospital of Guangzhou University of TCM，Guang Zhou, 510405, China)

ABSTRACT Objective: 1) To investigate the formative mechanism of phlegm obstruction due to lung-deficiency syndromes. 2) To investigate the dynamic changes of the above parameters by detecting the effects of the herbal compound that "invigorates the lung and promotes expectorant" on the expression of AQP1 and AQP5 in the phlegm obstruction due to lung-deficiency syndromes model rats. Methods: Male SD Rats were randomly divided into three groups: normal, model, and treatment (with herbal Rats were treated by "invigorate lung expectorant" herbal compound). The pathologic changes of lung were analyzed by microscopy with H&E staining. The mRNA and protein expressions of AQP1 and AQP5 were assayed by semi-quantitative RT-PCR and western blot respectively. Results: There was no expression of AQP1 mRNAs in the model and treated groups,while the expression of AQP5 mRNAs increased in the model group. Western blot analysis showed similar patterns of AQP5 protein being upregulated ($P<0.01$) while AQP1 downregulated ($P<0.05$). The expression of the AQP5 protein in the treated group decreased compared with that in the normal group ($P<0.05$). Conclusions: 1) One of the pathological mechanisms of the syndrome of "phelgm obstuction due to lung deficiency" is caused by the up-regulation of AQP5 and down-regulation of AQP1 gene and protein expression in lungs of the alveolar epithelium of rats. 2) The expectorant herbal compound treatment can regulate the gene and protein expression of AQP5 but can't regulate the AQP1 when the rats have this desease. These results suggest that the mechanism of this compound acting on this desease is related to the regulation of the expression of AQP1 AQP5.

Key words: AQP1; AQP5; Rat lung; Phlegm obstruction due to lung-deficiency syndromes; Invigorate lung expectorant complex

Chinese Library Classification: Q95-3, R256.1 Document code: A

Article ID: 1673-6273(2012)01-30-06

Introduction

Zhenghou research is the main content, nodus and breakthrough point in TCM theory. Going into zhenghou deeply is the necessary path in the modernization of TCM. It will demonstrate the uniqueness and superiority of TCM. The theory of Phlegm zheng is a unique component of TCM. It effectively directs clinical application. According to TCM, "phlegm" is the source of all kinds of diseases.

However, untill now there were no studies on the pathogenesis of phlegm. Recent study of tanzheng is focused on the following three aspects: First there is the nature of tanzheng and its objectivized index: character of the blood flow [1], saccharide, lipid and energy metabolism [2], free radical damage, immune function [3], autonomic nervous system function, gene expression [4]. The second aspect is the standardizing of syndrome differentiation [5]. The third aspect is the toxicity and pathogens of phlegm[6-7].

Aquaporins (AQPs) is a hot topic of research internationally. Cell volume regulation is dominated by transmembrane ions[8]. It is accomplished with passive transmembrane movement of water driven by osmotic pressure [9]. The efficiency of water transmembrane movements which was, determined by plasmalemma' s permeability to water can also affect the migratory behavior and related physiological as well as pathological processes in cells[10].

To investigate the formation mechanism of phlegm obstruction due to lung-deficiency syndromes, this research explain the role of changes of AQP1 and AQP5 expression in this syndromes and the effects of Compound Herbal Formulary on their expression.

1 Materials and methods

1.1 Herbal medicament preparation exerting ′invigorate lung & expectorant′

Prescription consists of ginseng 10 g, prepared arisaenatis 6 g, prepared pinellia 10 g, immature bitter orange 10 g, tangerine red epicarp 6 g, poria cocos 15 g, tatarinow sweetflag rhizome 10 g, bamboo shavings 10 g，prepared liquorice root 6 g.

1.2 Preparation techniques

After being sliced, ginseng was abstracted two times by refluxing process by using 95% ethanol for 2h every time and the extracting solution was combined. Recovery ethanol from this extraction until no ethanol flavor can be snuffed. The dregs of gin-

*Funded by: National Natural Science Foundation of China (30772687); Natural Science Foundation of Guangdong province (9251040701000001)

Author Introduction: JIANG Yong (1968-), Famale, Associate Researcher, Master's suporvison, Doctor Degree.

Be engaged in molecular biology, pharmacology. E-mail: drjiangyong@163.com, Tel: 020-36591640

△Corresponding Author: Li XB Tel：+86-020-3659 1640 E-mail: lixb139@139.com

(Received: 2011-05-30 Accepted: 2011-06-23)

seng and the other herbal medicines in this prescription were decocted two times. Combine the water extract and stored at 4 ℃ overnight. Filter and concentrated the water extract. Ginseng abstracting solution was added into it, adjusted the solution concentration to 200 %.

1.3 Model preparation

Male Sprague Dawley rats, weighing 200-250 g, were supplied by Guangdong Medicine Laboratory Animal Research Center (GDMLAC). They received standard rat chow with free access to tap water. Forty rats were subjected to cold wind with a wind chill effect of 5 ℃ lower than their normal surrounding for 10 min. then they were put into a smog cabinet with 0.2 g sulfur powder was besprinkled on Mugwort moxa stick liberally in it. Emblaze moxa stick. After enkindling them for 2 h, the rats were taken out. Twice a day, continued for 40 days.

1.4 Route of administration

At the 26 d after model mading, twenty rats were lavaged (10 ml/kg/d) with 'invigorate lung expectorant' herbal compound, while the normal and model groups were lavaged with normal saline (0.15 M) for 14 d.

1.5 Isolation and purification of total RNA from rat lung tissues

Animals were killed by cervical dislocation. Their lungs were rapidly removed and frozen on a bed of powdered dry ice and stored at -80 ℃. Total RNA was isolated from rat's lungs, they were then frozen and pulverized in liquid nitrogen. RNA was extracted by using Trizol Reagent (GIBICOBRL) according to the manufacturer's instruction. Purity and quality of RNA was quantified by spectroscopy at 260nm and tested by electrophoresis in 1.4 % agarose gel with GoldView NucleicAsid Stain respectively. Samples were aliquoted and stored at -80 ℃ until further processing.

1.6 **RT-PCR**

The first cDNA strands were synthesized with the Easyscript First-strand cDNA Synthesis Supermix Kit (GIBICOBRL) according to the suggestion of the manufacturer. In each PCR reaction, 0.15 μg total RNA was used as template. The amplifications were performed with genes' specific primers listed in table 1. The PCR reaction was carried out in a 25 μl volume of buffer also containing: 0.2 μM each primers and 12.5 μl 2× EasyTaq PCR SuperMix. Standard PCR cycle conditions were 94 ℃ for 30 s, 25 cycles of 92 ℃ for 10 s,60 ℃ for 30 s and 72 ℃ for 30 s. Followed by 72 ℃ for 10 min. Beta-actin was amplified for each sample to serve as an internal control, and the observed mRNA expression was normalized to the expression of β-actin. Amplification products were analyzed by electrophoresis in 1.4 % agarose gels. Each reaction was repeated three times.

Table 1 Primers

Gene	Primer, 5'-3'(for and rev)	Product size(bp)
AQP1	TCCGGCATCACCTCCTCCCT	319
	TGAATGGTCCCACCCAGAAA	
AQP5	CAAGGCGGTGTTCGCAGAGTTCC	730
	CCTCTCGAAGATCTTCCCAGTCC	
β- actin	GACCTGACTGACTGACTACCTCAT	542[11]
	TCGTCATACTCCTGCTTGCT	

1.7 **Western blotting**

Protein extracted from rat lung was isolated by Cyto Buffer. Protein was collected and protein concentrations were detected by using the Bradford method (Bio-Rad). Fifty micrograms of lung protein was dissolved in sample buffer and boiled for 5min prior to loading onto a 12% acrylamide gel. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5 % BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST), and incubated with mouse anti-polyclonal antibody to AQP1 and goat anti-polyclonal antibody to AQP5 1h at 1:200. After washing with TBST, blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence kits. Films were scanned with a Quantiti-one and the bands of AQP1, AQP5 and beta-actin were quantified with Quantiti-one software. The intensity of bands was normalized to the intensity of beta-actin.

1.8 **Statistical analysis**

All data were analyzed by using the StatView program (Abacus Concepts). The effect of drug stimulation on the expression of AQP mRNAs was analyzed by using multivariate analysis of variance (MANOVA), where there were two variables. When statistical significance was observed, one-way ANOVA with Scheffe's post-hoc test was carried out for each set of variables. P-values less than or equal to 0.05 were considered significant.

1.9 **Histology staining**

The regular pieces of each lung was dehydrated with alcohol in the usual matter, then clarified with toluene and embedded in paraffin. Each tissue block was microtomed into serial sections of

4 μm thickness, stained with hematoxylin-eosin, the light microscope was used to both examine and photomicrograph lung tissues. Values are means± S.E.M., n≥ 20 from 20 rats each group, *P<0.05 vs. model group.

2 Results

2.1 Effects of treatment on the expression of AQP1 and AQP5 mRNA

Semi-quantitative expression data showed that AQP1 mRNA was only found in the normal lung. There was no mRNA in model and treated lungs (Fig.1). The AQP5/β-actin ratio was significantly higher for the treated group and normal group than for the model group (Fig.2, P<0.01).

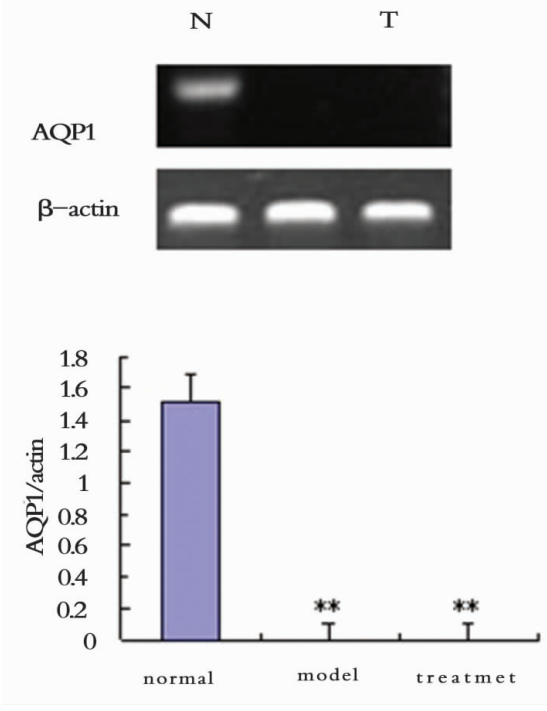

Fig.1 AQP1mRNA expression

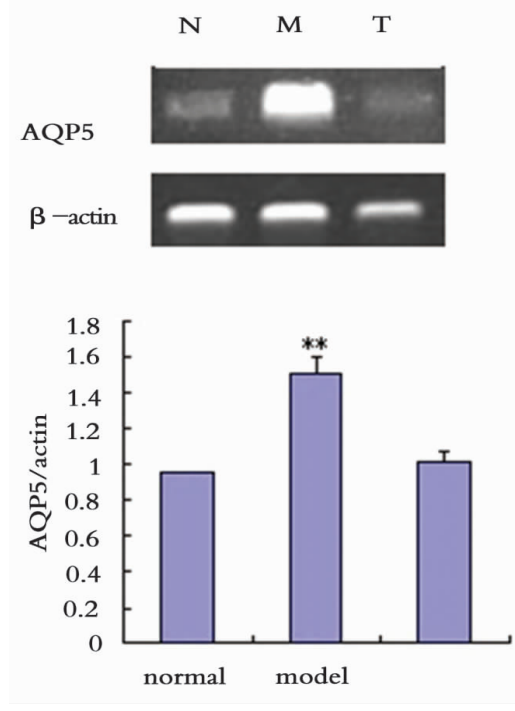

Fig.2 AQP5 mRNA expression

2.2 The impact of herbal treatment on the induced expression of AQP1 and AQP5 at the protein levels

The protein levels of AQP1 and AQP5 were analyzed by western blotting. There was AQP5 protein bands with molecular weights of 35 kDa. AQP5 in the model group was remarkably higher than that in the normal (P<0.01) and treated group (P<0.05) (Fig.4). AQP1 was 28 kDa and in the model and treated group, decreased remarkably when compared with that the normal group(P<0.05) (Fig.3).

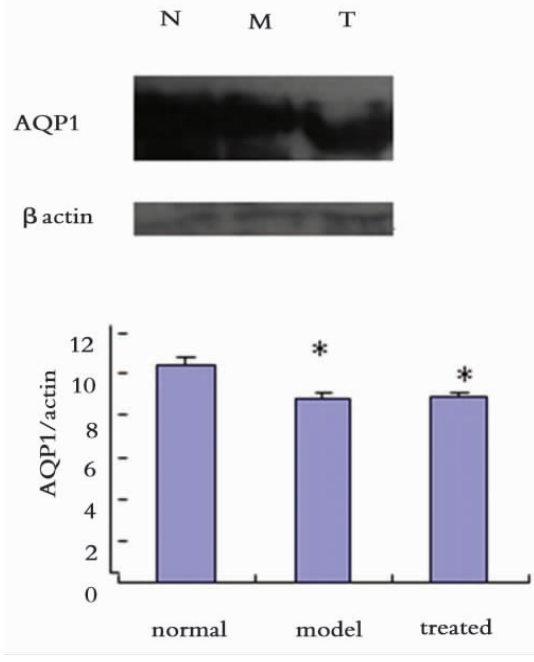

Fig. 3 Protein expression of AQP1

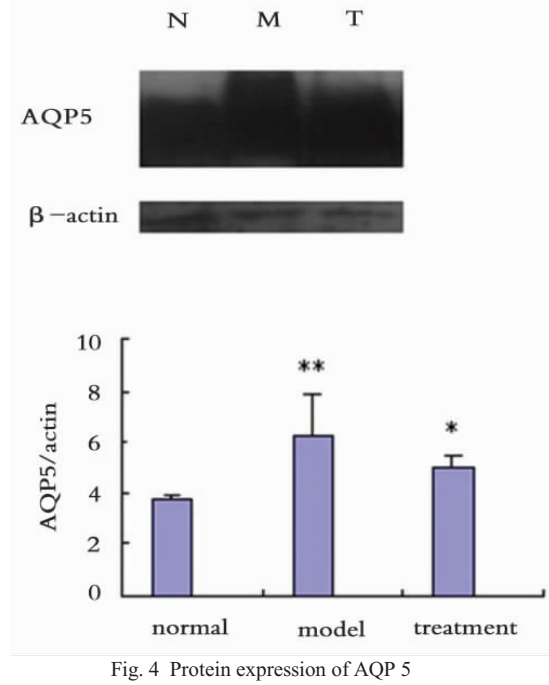

Fig. 4 Protein expression of AQP 5

2.3 **Histological observation**

In the normal group, the pulmonary alveoli were quite uniform in dimensions, with even thickness of the alveolar septa. There was no sign of engorgement and inflammatory infiltrate, no sign of fibroplasia and no inflammatory exudate in the alveolar lumen. Bronchial and bronchiolar epithelia and cilia were intact, and there was no obvious inflammatory infiltration. In the model group, lymphocyte and plasmocyte infiltration was observed in the bronchioles. Cilia of some bronchiolar mucosa were collapsed. Epithelial cells lost; alveolar septa were thickened, their small vessels congested and chronically infiltrated. As for the treatment group, there was significant relief of symptoms of the model group, and there was no difference when compared with that in the normal group (Fig.5).

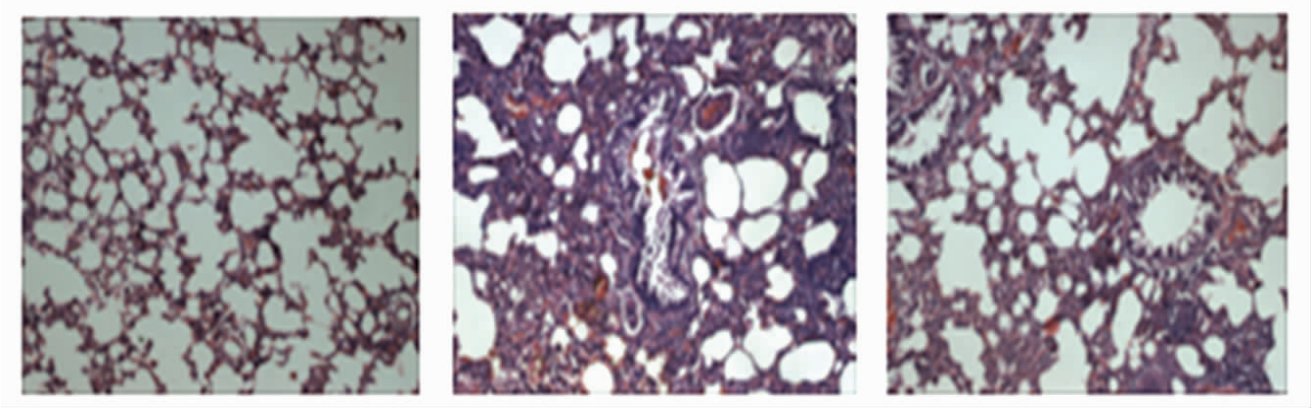

Fig. 5 Pathology detection for rat lungs tissue (100×) :A: norma group;B: model group;C: treated group groupgroup

3 Discussion

Phlegm is the pathologic product of the obstructed metabolism of body fluid. The normal maintenance of body fluid depends on the coordination of viscera function. The viscera are related closely to body fluid metabolism of the zang-fu. Of the five zang organs three are more closely related to fluid metablolism: lung, spleen and kidney. Their functional disorders can lead to body fluid metabolic disorders, lead to the accumulation of dampness to form phlegm, cumulating to be a tangzheng[12-14].

Phlegm in this research is caused by the deficiency syndrome of the lung. Invigorate lung expectorant complex is aimed at Phlegm obstruction due to lung-deficiency syndromes and treating both manifestation and root cause of disease. This prescription come from added erchen decoction. Ginseng is a key reagent in treating qi deficiency of both lung and spleen by tonifying middle-Jiao and Qi. The character of pinellia is Xinwen and is the key reagent of eliminating dampness and resolving phlegm. Poria cocos is able to clearing damp and promoting diuresis. Tangerine red epicarp can promote the circulation of qi and eliminating dampness and resolving phlegm. Tatarinow sweetflag rhizome can clear damp and Kaiwei, eliminating phlegm from the heart to restore to consciousness. Arisaenatis have the damp-drying and phlegm-eliminating ability. Bitter orange, relieving stagnant Qi and resolving accumulation, eliminating phlegm and a lump in the abdomen. Bamboo shavings have the ability of clearing heat and eliminating phlegm, restless and stopping vomiting. Liquorice is in harmonious proportion of every elements in this prescription, which can promote every elements work together to enhance function of tonifying lung and eliminating phlegm.

Aquaporins (AQPs) are a family of subcellular channels which are expressed ubiquitously in biological membranes. They are highly selective in transport of water and are a highly specialized group of channels. Untill now, AQP1,AQP3, AQP4, AQP5, AQP8, AQP9 are found to be expressed in lung tissues [15]. AQP1and AQP 5 are highly permeable to water but not to other solutes. They not only participate in fluid transport under physiological conditions but are also associated with the imbalance of fluid transport in pathology conditions [16]. The permeability of water in AQP1 and AQP5 knockout mice lung decreased significantly. As for the syndrome of 'lung-deficiency leading to phlegm obstruction", because of lung asthenia, the functions of dispersing and descending functions of lungs are lost.

The clinical condition of the commonly seen visible phlegm of respiratory diseases was examined and respiratory disease modelling was used to investigate the mechanisms of phlegm forming. The theory is based on the principle of that the lungs are the upper source of phlegm which has the responsibility to regulate water pathways. The model combines the disease with the syndrome in the form of animal models of chronic bronchitis. It can be supposed that the active water transport function of alveolar epithelium is carried out by lung to regulate water passage. They are the

physiological foundation of water's upper source. Abnormal water active transport function is one of the mechanisms of the syndrome of lung deficiency leading to phlegm obstruction. We further select the investigational targets of AQP that can reflect the water active transport function of alveolar epithelium at the molecular level.

By detected the expression of both mRNA and protein of AQP1 and AQP5 in the lung, it was found that their activities and related gene expression. Measurement of such parameters and dynamic changes both before and after administrations of TCM combination formularies, the relationship between pathogenesis of this syndrome and the perspective of lung epithelium's active water transport function can be explored. In this study, the intrapulmonary gene expression of AQP1 was not expressed in the model and treated group and the protein expression of AQP1 was significantly attenuated compared with that in the normal group. The reason may be that AQP1 gene was silenced while the half-life of protein is longer than that of gene. AQP1 and AQP5 expressed in lung provide the principal route for osmotic driven water transport [17]. When AQP1 absence, the water couldn't be transported facilely from the plasma membrane which led to pulmonary edema. So, the disease may play an important role in regulating the expression of AQP1 but the treatment has no effect on this disease by regulating it. The relevance and role of AQP1 in human diseases is being discussed in various researches [18-20]. This research is only a complementation in this field with TCM feature.

The mRNA level of AQP5 is higher in the model group than in the normal group. When treated this disease, the level can be lowered than the model group so did AQP5 protein. The regulation of AQP5 is affected by different surroundings, stages of lung development [21], physiological condition and cell phenotype [22]. The AQP5 is a target of intrapulmonary diseases in other than the mechanisms cAMP dependent phosphorylation [23-24]. In the process of treating patients with pulmonary diseases suggests potential roles of AQP5 in the regulating some disease for example regulating airway hyper secretion. In phlegm Obstruction Due to Lung-Deficiency syndromes situation, AQP5 may by upregulated by this stress. The role of AQPs in this research has not yet been clearly defined. Furthermore, they may provide attractive therapeutic targets.

Acknowledgements

Thanks given to Laboratory of clinical Foundation of Chinese Medicine, the National Key Discipline of Guangzhou University of Chinese Medicine and Dept. of Immunology and Molecular Biology of Guangzhou University of TCM for their equipment supporting.

References

[1] Wang Y, Ye JN. Experimental Study on the Relationship Between Hyper Lipidemia and Phlegmint Radi-Tional Chinlse Medicine [J]. Chinese J Basic Med TCM, 1995, 1(1): 52-54

[2] Guo HC, Niu XY, Zhang XD. Analysis of the Relaionship Between Syndrome of Phlegm Turbidity and Lipid [J]. Henan J TCM Pharm, 1998, 13(6):18-19

[3] Li XB, Lin CS. The Observision on T Lymphocyte Subgroup of Cardio-/Cerebrovascular Disease Phlegm Syndrome Patient [J]. Sponsored Gubei College TCM, 2000, 22(1):13-14

[4] Wang DS, Yuan ZK, HUANG XP,et al. Relationship Between Syndrome Differentiation Of Phlegm & Blood Stasis And PDGF-A Mrna Expression Of Peripheral Blood Mononuclear Cell [J]. J Anhui TCM College, 2003, 22(2):14-17

[5] Fang YQ, Wei G, Li XB. Quantization of the Distinguish Phlegm Syndrome[J]. J Liaoning TCM, 1995, 22(11): 490-492

[6] Wang P, Wu XY, Zhong ML. Expression of NF-κ B and COX-2 mRNA in rats with phlegm obstruction due to lung-deficiency[J]. J Chin Integr Med, 2005, 3(2):119-122

[7] Wang P, Tian DZ, Zhang ML, et al. The Function of Macrophage and Expression of CD11b on Rats of Asthenic Lung and Phlegm Blocking [J]. Chin Archiv TCM, 2004, 22(7):1167-1195

[8] Saadoun S, Papadopoulos MC, Hara-Chikuma et al. Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption [J]. Nature, 2005, 434(7034):786-792

[9] Borok Z, Verkman AS.Lung edema clearance:20 years of progress: invited review: role of aquaporin water channels in fluid transport in lung and airways[J]. J Appl Physiol, 2002, 93(6):2199-2206

[10] Papadopoulos MC, Saadoun S,Verkman AS. Aquaporins and cell migration[J]. Pflugers Arch, 2008, 456(4): 693-700

[11] Nudel U, Zakut R, Shani M, et al. The nucleotide sequence of the rat cutoplasmic beta-actin gene [J]. Nucleic Acids Res, 1983, 11: 1759-1771

[12] Wang B. An annotated suwen of Huangti Internal Classic [M]. Beijing: The People's Medical Publishing House,1979,139,246,341

[13] Zhang ZJ. Medical treasures of the golden chamber [M]. Beijing: Incunabula Publishing Company of TCM, 1997,18,37

[14] Jin HM. Pathophysiology [M]. 5th edition, Beijing: The People's Medical Publishing House, 2001,18,56,223

[15] Carbrey JM, Agre P. Discovery of the aquaporins and development of the field [J]. Handb Exp Pharmacol , 2009, 190: 3-28

[16] Magni F, Chinello C, Raimondo F, et al. AQP1 expression analysis in human diseases: implications for proteomic characterization [J]. Expert Rev Proteomics, 2008, 5(1):29-43

[17] Ben Y, Chen J, Zhu R, Gao L, et al. Upregulation of AQP3 and AQP5 induced by dexamethasone and ambroxol in A549 cells [J]. Respir Physiol Neurobiol, 2008, 161(2): 111-118

[18] Verkman AS. Dissecting the roles of aquaporins in renal pathophysiology using transgenic mice[J]. Semin Nephrol , 2008, 28(3): 217-226

[19] Cao CS, Yin Q, Huang L, et al. Effect of angiotensin II on the expression of aquaporin 1 in lung of rats following acute lung injury [J]. Chin Critic Care Med,2010, 22(7): 26-29

[20] Nishino T, Devuyst O. Clinical application of aquaporin research:

aquaporin-1 in the peritoneal membrane[J]. Pflugers Arch, 2008, 456 (4): 721-727

[21] Kopantzev EP, Monastyrskaya GS,Vinogradova TV, et al. Differences in gene expression levels between early and later stages of human lung development are opposite to those between normal lung tissue and non-small lung cell carcinoma [J]. Lung Cancer, 2008,62 (1): 23-34

[22] Woo J, Lee J,Chae YK,et al. Overexpression of AQP5, a putative oncogene, promotes cell growth and transformation [J]. Cancer Lett, 2008 264(1): 54-62

[23] Woo J, Lee J,Chae YK, et al. Overexpression of AQP5, a putative oncogene, promotes cell growth and transformation [J]. Cancer Lett, 2008, 264(1): 54-62

[24] Woo J, Chae YK, Jang SJ, et al. Membrane trafficking of AQP5 and cAMP dependent phosphorylation in bronchial epithelium [J]. Biochem Biophys Res Commun, 2008, 366(2): 321-327

肺虚痰阻证模型大鼠肺组织水通道蛋白 1 和 5 分子表达与补肺化痰方对其影响 *

江 湧 李小兵△ 刘小虹 王丽新 任明能 吴启端 谢宇晖 吴建奇

(广州中医药大学第一附属医院 广东 广州 510405)

摘要 目的：1) 从肺泡上皮水主动转运功能的角度探讨肺虚痰阻证的发生机理。2) 通过观察肺虚痰阻证模型的 AQP 的活性及其相关基因、蛋白的表达和补肺化痰中药复方治疗前、后的对比，观察这一过程中上述指标的变化情况。方法：将雄性 SD 大鼠随机分为正常组、模型组、中药治疗组。模型组和治疗组造模 40 天，治疗组在造模 26 天后，药物灌胃治疗 2 周。采用组织化学染色法，对大鼠肺进行病理分析；RT-PCR 的方法检测大鼠肺组织中 AQP1、AQP5 基因表达；western blot 法检测大鼠肺组织中 AQP1、AQP5 蛋白水平。结果：1) 与正常组相比，模型组局部出现明显炎症反应 (P<0.01)，治疗组局部炎症反应减轻 (P<0.05) 。2) mRNA 结果显示，AQP1 在正常组有表达，在模型组和治疗组未见表达。AQP5 模型组与正常组相比，表达量显著增高 (P<0.01)；治疗组与模型组比较，表达量显著降低 (P<0.01)，但与正常组无显著差异。3) 蛋白水平上，AQP1 在模型组和治疗组与正常组相比差异显著 (P<0.05)，表达下降。AQP5 模型组与正常组相比，显著升高 (P<0.01)；治疗组与模型组比较，显著下调 (P<0.05)；正常组表达低于治疗组，差异显著(P<0.05)。结论：1) AQP1 和 5 基因及蛋白表达量变化是肺虚痰阻证的病理机制之一。2) 补肺化痰中药复方可调节肺虚痰阻证模型大鼠肺组织 AQP 5 基因及蛋白表达。提示补肺化痰中药复方治疗肺虚痰阻证其作用机制与调节 AQP5 有关。

关键词：AQP1；AQP5；大鼠肺；肺虚痰阻证；补肺化痰中药复方

中图分类号：Q95-3, R256.1 **文献标识码**：A **文章编号**：1673-6273 (2012) 01-30-06

* 基金项目：国家自然科学基金资助项目[30772687]；广东省自然科学基金面上项目[9251040701000001]

作者介绍：江湧 (1968-)，女，副研究员，硕士生导师，博士，从事分子生物学，药理学。

△通讯作者：李小兵 (1965-)，男，研究员，硕士生导师，硕士，从事中西医结合基础。Tel (020) 36591640 E-mail lixb139@139.com

(收稿日期：2011-05-30 接受日期：2011-06-23)