

Study on the expression of Toll-like receptor 4 and matrix metalloproteinase-9 in patients with chronic obstructive pulmonary disease and their clinical significance

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Abstract. – **OBJECTIVE:** To investigate the expression of Toll-like receptor 4 (TLR4) and matrix metalloproteinase-9 (MMP-9) in patients with chronic obstructive pulmonary disease (COPD) and their clinical significance.

PATIENTS AND METHODS: Paracancerous tissues from 48 patients with lung squamous cell carcinoma harvested during pulmonary lobectomy were studied. Twenty-four cases of COPD were chosen as the observation group and 24 cases of non-COPD as the control group. The degree of lung inflammation was observed; the ratio of the thickness of the wall to the external diameter of the pulmonary arterioles (WT%), and the ratio of the area of the wall to that of the pulmonary arterioles (WA%) were calculated. Immunohistochemistry was used to evaluate the expression of TLR4, MMP-9, and proliferating cell nuclear antigen (PCNA) in vascular smooth muscle cells, and the expressions were correlated with lung revascularization.

RESULTS: (1) Compared with the non-COPD group, the degree of inflammatory cell infiltration, WA%, and WT% of the COPD group were significantly increased ($p < 0.05$). Additionally, the expression of TLR4, MMP-9, and PCNA in vascular smooth muscle cells was significantly increased ($p < 0.05$). (2) Correlative analysis revealed that the expression of TLR4 and MMP-9 had significant positive correlation with the degree of inflammatory cell infiltration, WA%, WT%, and PCNA expression ($p < 0.05$). Multivariate regression analysis showed that, compared with the smoking index and inflammation score, TLR4 and MMP-9 expression were the strongest factors affecting the parameters of lung revascularization (WA% and WT%) ($p < 0.05$).

CONCLUSIONS: High expression of MMP-9 and TLR4 in patients with COPD may promote

inflammatory cell infiltration, induce proliferation of smooth muscle cells, degrade extracellular matrix, and play an important role in lung revascularization.

Key Words:

Chronic obstructive pulmonary disease, Toll like receptor 4, Matrix metalloproteinase-9, Proliferating cell nuclear antigen.

Introduction

Chronic obstructive pulmonary disease (COPD) is a frequently occurring disease characterized by airflow limitations and being incompletely reversible with treatment. Furthermore, it is often progressive. The basic pathological features of COPD include vascular remodeling caused by inflammatory cell infiltration of the pulmonary vasculature, and proliferation and reparation of smooth muscle cells^{1,2}. Remodeled pulmonary vasculature results in pulmonary hypertension and chronic pulmonary heart diseases, which severely effect human health³. Toll-like receptor 4 (TLR4) is an important pattern recognition receptor that recognizes lipopolysaccharides of the bacterial cell wall. Previous evidence⁴ has demonstrated that TLRs play important roles in the occurrence of inflammatory diseases such as COPD. Related to vascular remodeling and injury, as well as reparation and airway inflammation, matrix metalloproteinases (MMP) are important for degradation of the extracellular matrix (ECM)⁵⁻⁷. It was previously reported⁸ that TLR4 can regulate the expression of MMP-9 by

activating nuclear factor κ B (NF- κ B). In the present study, we collected lung tissue samples adjacent to lung cancer tissue and used immunohistochemistry (IHC) to detect the expression of TLR4, MMP-9, and proliferating cell nuclear antigen (PCNA), and analyzed the correlation between the expression levels of these proteins and vascular remodeling.

Patients and Methods

Lung Tissue Sample Collection

Lung tissue samples were collected from 48 males with highly differentiated squamous cell carcinoma of the lung who were admitted to the Department of Thoracic Surgery in our hospital from February 2015 to March 2016. For these patients, pulmonary functions were examined, smoking indexes were counted, and surgical treatment was carried out following admission. Samples were divided into the COPD group and the non-COPD group (control group) according to pulmonary functions, with 24 cases in each group. In the COPD group, patients were in the stable phase and did not have other systemic diseases. There were no significant differences in age among patients in the two groups. During surgery, normal peripheral lung tissues without infiltration that were over 5 cm away from tumors were collected and diagnosed by senior pathologists. Normal lung tissues were fixed, dehydrated, cleared, waxed, embedded, and sliced into 3- μ m paraffin sections. All patients signed the informed consent. The investigation has been approved from the Ethical Committee of our Hospital.

Equipment

Mouse anti-PCNA and rabbit anti-human MMP-9 polyclonal antibodies, and test kits for PV-900 two-step immunohistochemistry were from ZSGB-Bio (Beijing, China); rabbit anti-human TLR4 polyclonal antibody was from Bioss (Beijing, China) and used at the working dilution

of 1:100. The automatic Hematoxylin and Eosin (H&E) dyeing machine was from Leica (Wetzlar, Hesse, Germany); the optical microscope and image acquisition system were from Olympus (Tokyo, Japan); SPSS 18.0 software was from SPSS Inc. (Chicago, IL, USA).

Morphological Observation of H&E Staining

Pulmonary arteries from ϕ 100-500 μ m were selected for observation. Morphological changes were observed with an optical microscope, and the degree of infiltration in the arterial wall and adjacent inflammatory cells was semi-quantitatively evaluated. Five pulmonary arterioles with round and complete cross-sectional structure were randomly chosen. Images were acquired, and the thickness and area of the walls, and the inside and outside diameter of pulmonary arteriolar walls were measured. The ratio of wall thickness to the outside diameter (WT%) and the ratio of cross-sectional area of the vascular wall to the total vascular area (WA%) were calculated. The average WT% and WA% in each group were recorded. The scoring criteria are shown in Table I.

IHC-Mediated Detection of TLR4, MMP-9, and PCNA

The protocol was followed in strict compliance with the instructions of the PV-900 two-step immunohistochemistry test kit. Five pulmonary arterioles from ϕ 100-500 μ m (400 \times magnification) were randomly selected and diagnosed by two senior pathologists. The average optical density of the pathological sections was taken after counting. If MMP-9 staining appeared brown in the cytoplasm, the cell was considered positive; if the cell membrane and/or the cytoplasm stained brown-yellow for TLR4, the cell was considered positive. The ratio of the number of MMP-9 and TLR4-positive smooth muscle cells of the pulmonary artery to the total number of smooth muscle cell was calculated. If brown-yellow granules were observed in the nucleus, PCNA staining was positive. The positive

Table I. Scoring criteria for the degree of inflammation of pulmonary arteries.

	0 points	1 point	2 points	3 points	4 points
The degree of infiltration of inflammatory cells	None	Minor	Moderate, but with uneven distribution	Moderate, and with even distribution without gathering into a mass	High, with gathering into a mass

Table II. Basic parameters and pulmonary functions of patients in the two groups ($\bar{x}\pm s$).

Groups	Cases	Age	Years of smoking (pack/year)	FEV ₁ (% pred)	FEV ₁ /FVC (%)
Control group	24	62.8±5.2	24.6±7.5	98.3±10.4	84.2±6.0
COPD group	24	63.1±6.0	32.3±7.6	69.9±16.7	61.5±6.7
<i>t</i> -value	-	-0.701	-4.012	9.156	13.121
<i>p</i> -value	-	>0.05	<0.01	<0.01	<0.01

Table III. Comparisons of the degree of vascular inflammation and remodeling ($\bar{x}\pm s$).

Groups	Cases	The degree of inflammation	WT%	WA%
Control group	24	0.2±0.5	15.3±1.9	26.0±3.3
COPD group	24	1.6±0.9	27.5±3.1	44.2±6.7
<i>t</i> -value	-	-8.102	-18.524	-14.426
<i>p</i> -value	-	<0.01	<0.01	<0.01

rate of PCNA in smooth muscle cells of the pulmonary arteries (the ratio of positive cells to the total number of cells) was counted. The overexpression of PCNA suggested active cell proliferation and decreased cell apoptosis.

Statistical Analysis

SPSS18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are presented as mean ± standard deviation ($\bar{x}\pm s$). Comparisons of two samples were analyzed by *t*-test, and related indicators were analyzed by Pearson correlation analysis and multiple linear regression. $\alpha=0.05$ was taken for examination, and $p<0.05$ was considered statistically significant.

Results

Basic Parameters and Comparisons of Pulmonary Functions

There was no statistically significant difference in the age of patients in the two groups ($p>0.05$). In the COPD group, pulmonary functions, the ratio of forced expiratory volume in one second to the predicted value (FEV₁%) and the ratio of forced expiratory volume in one second to forced vital capacity (FEV₁/FVC%) decreased significantly compared with the control group ($p<0.01$) (Table II).

Histological Analysis of Lung Tissues

By light microscopy, in the COPD group, a large number of monocytes and lymphocytes

infiltrated the pulmonary vascular wall and the surrounding area; the pulmonary vascular wall was obviously thickened; there was lodging and shedding of the cilia of bronchial epithelial cells; there was expansion of pulmonary alveoli; alveolar walls became thinner, or even fused; alveolar septa were destroyed; and the integrity of the structure of lung tissue disappeared. In the control group, there was no pulmonary vascular wall thickening and they were complete; there was little to no inflammatory cell infiltration; the bronchial mucosa was complete; and the structure

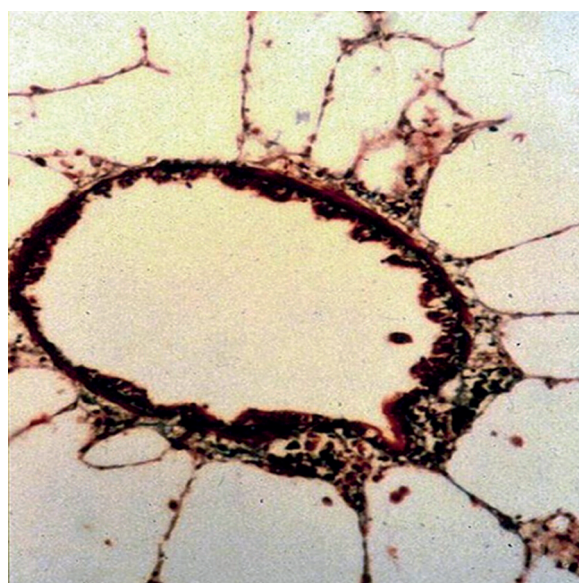


Figure 1. Representative airway smooth muscle of a non-COPD patient (original magnification x 400).

Table IV. Immunohistochemical results of patients in the two groups ($\bar{x}\pm s$).

Groups	Cases	TLR4 (%)	MMP-9 (%)	PCNA (%)
Control group	24	4.6±4.2	6.4±6.0	9.6±5.1
COPD group	24	32.1±16.2	41.8±23.9	28.2±16.3
<i>t</i> -value	-	-7.96	-7.01	-5.63
<i>p</i> -value	-	<0.01	<0.01	<0.01

Table V. Correlation analysis of the expression of TLR4, MMP-9, and PCNA and indexes of vascular remodeling (r value).

Indexes	TLR4 (%)	MMP-9 (%)	PCNA (%)
WT%	0.562	0.721	0.547
WA%	0.648	0.590	0.613
PCNA (%)	0.481	0.379	1.000
Inflammatory scores	0.453	0.604	0.411

Note: $p < 0.01$.

of pulmonary alveoli was normal. The inflammatory scores in the COPD group were significantly higher than in the control group ($p < 0.01$), and inflammatory cell infiltration in the lumen of the pulmonary artery and surrounding area was apparent. Additionally, WT% and WA% of patients in the COPD group were significantly higher than those in the control group ($p < 0.01$), which suggested that proliferation of smooth muscle cells and collagen deposition led to arterial wall thickening and narrowing of the lumen (Table III, Figure 1, and Figure 2).

Analysis of Immunohistochemical Results in the Two Groups

According to our results, when MMP-9 staining was positive, the cytoplasm appeared with brown-yellow granules, which were distributed in smooth muscle cells of pulmonary vessels, epithelial cells of bronchiole alveolus, and macrophages of pulmonary alveoli, neutrophils, and fibroblasts. When TLR4 staining was positive, cell membranes and/or the cytoplasm stained brown-yellow. Staining was distributed in the endothelial cells of pulmonary vessels and airway epithelial cells. PCNA staining was positive when nuclei appeared with brown-yellow granules. Positive PCNA staining implied there was cell proliferation. In the COPD group, the expressions of TLR4, MMP-9, and PCNA in lung tissue were significantly higher than in the control group ($p < 0.01$) (Table IV).

Correlation Analysis

Correlation analysis of the expression of TLR4, MMP-9, and PCNA and the indexes of vascular re-

modeling is shown in Table V. Smoking indexes were negatively correlated with FEV₁% and FEV1/FVC% ($r = -0.315, -0.324, p < 0.05$); they were positively correlated with inflammatory scores and the expression of TLR4 and MMP-9 in the smooth muscle of pulmonary vessels ($r = 0.418, 0.297, 0.332, p < 0.05$); they were also positively correlated with remodeling of pulmonary vessels (WT% and WA%) ($r = 0.352, 0.483, p < 0.01$). Multiple analysis suggested that smoking indexes, inflammatory scores, and the expression of TLR4 and MMP-9 had statistically significant effects on WA% and WT% ($p < 0.05$). It was found that MMP-9 (%) and

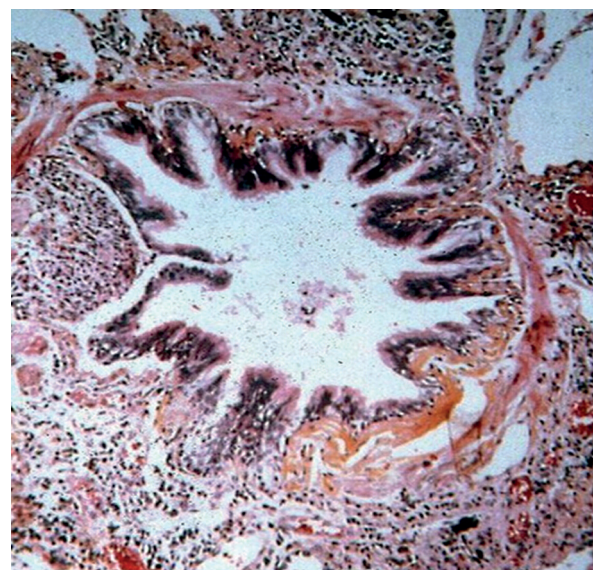


Figure 2. In COPD patients, there is thickening of airway smooth muscle with inflammatory cell infiltration (original magnification x 400).

Table VI. Multiple linear regression analysis of influential factors of WA%.

Variable	Regression coefficients	Standard error	Standardized regression coefficient	t-value	p-value
TLR4 (%)	12.34	4.08	0.29	3.03	<0.01
MMP-9 (%)	19.12	4.44	0.42	4.81	<0.01
Inflammatory scores	16.74	6.63	0.23	2.66	<0.05
Smoking indexes	11.17	4.32	0.26	2.49	<0.05

Table VII. Multiple linear regression analysis of influential factors of WT%.

Variable	Regression coefficients	Standard error	Standardized regression coefficient	t-value	p-value
TLR4 (%)	8.42	2.59	0.27	3.13	<0.01
MMP-9 (%)	13.01	2.47	0.49	5.72	<0.01
Inflammatory scores	8.64	3.93	0.20	2.28	<0.05
Smoking indexes	7.86	2.53	0.25	2.87	<0.05

TIMP-1 (%) had the greatest effects on WA% and WT% by referring to a standardized regression coefficient ($p<0.01$). See Table VI and VII.

Discussion

The basic pathological feature of COPD is the reconstruction of airways and vascular walls. Pulmonary vascular remodeling is affected by different forms of injury (i.e. hypoxia, inflammation, and shear forces from strong blood flow). Blood vessels of the ECM change structure, ECM increases, and smooth muscle cells proliferate, which causes thickening of the pulmonary arterial wall, narrowing of the lumen, increased the elasticity of blood vessels, and poor compliance⁹.

Inflammation is an important factor in pulmonary vascular remodeling. Inflammation can result in the activation of inflammatory cells, mainly T- and B-lymphocytes, monocytes, and macrophages, which further induce the expression of cellular factors such as interleukin (IL)-1, IL-6, and TNF- α . Based on the proliferation of interstitial cells, the structure and function of blood vessels change, which influences pulmonary vascular remodeling¹⁰. O'Shaughnessy et al¹¹ found that the number of inflammatory cells in lung tissue was related to the degree of damage to the lung. Furthermore, if the number of inflammatory cells is reduced, the degree of airflow limitation will improve. In the present work, we found that

in samples from the COPD group, a large number of inflammatory cells infiltrated the pulmonary vascular wall and surrounding areas, and there was obvious thickening of the pulmonary vascular wall. In addition, smooth muscle cells were hypertrophic and hyperplastic, which led to narrowing of the lumen and pulmonary vascular remodeling. Inflammatory scores, WT%, and WA% in the COPD group were significantly higher than those in the control group ($p<0.01$). Additionally, inflammatory scores were positively correlated with WT% and WA% ($p<0.01$). The study by Peinado et al^{12,13} suggested that patients with mild COPD without anoxic reactions and long-term smokers had evident inflammation in the pulmonary vessels and had abnormal vascular structures, which resulted in pulmonary vascular remodeling. Inflammation of pulmonary vessels may participate in pulmonary vascular remodeling during the early stage of COPD.

TLRs were first identified in *Drosophila*¹⁴. As primary receptors of the innate immune and inflammatory response, they recognize pathogenic microorganisms and transmembrane proteins of their cell walls. Initially named by Janeway, TLR4 is encoded by the human hTOLL gene. A study on lung tissue in rats suggested that TLR4 is mainly expressed in endothelial cells, airway epithelial cells, and smooth muscle cells in pulmonary vessels^{15,16}. After TLRs bind their ligands, intracellular signals are transmitted via MyD88-dependent and independent pathways to promote

the secretion of inflammatory factors, activation of dendritic cells, and the expression of interferon^{17,18}, which further mediates no tolerant inflammatory responses of blood vessels. The chronic inflammatory response of COPD is closely related to inflammatory factors produced by the highly expressed TLR4 in lung tissues and by interferon. TLR4 induces aggregation of inflammatory cells, changes the permeability of pulmonary vessels, and promotes the expression of MMPs and other active substances, which exacerbate injury and reparation of lung tissues. Inflammatory factors produced by different immune cells and lesions of vascular endothelial cells, smooth muscle cells, fibroblasts, and other cell types are the main causes of pulmonary vascular remodeling. The results of this study suggest that in the COPD group, with high expression of TLR4, an inflammatory cell infiltration was observed. Additionally, the expression of TLR4 was positively correlated with WA% and WT%, which suggests that TLR4 not only participates in the inflammatory responses of COPD, but also is closely related to pulmonary vascular remodeling.

MMPs, which are secreted by cells such as macrophages, belong to the family of endopeptidases, and can degrade ECM components in the presence of zinc and calcium ions¹⁹. MMPs participate in many physiological and pathological processes, including embryo formation, wound healing, tissue remodeling, blood vessel formation, and invasion and metastasis of tumors²⁰. MMP-9 is also known as gelatinase B. It is mainly synthesized and secreted by monocytes, macrophages, and endothelial cells. It specifically binds its substrates including type IV collagen and gelatin and degrades the basement membrane and extracellular components of the vascular wall. Flamant et al²¹ found that vascular remodeling in the early stage of hypertension was closely related to the expression of MMP-9. In the present study, our results showed that for patients in the COPD group, MMP-9 expression in lung tissue was positively correlated with the expression of PCNA, which suggests that MMP-9 may induce the proliferation of smooth muscle cells. There were positive correlations between smoking indexes and inflammatory scores and the expression of MMP-9 in pulmonary vascular smooth muscle ($p < 0.05$), which suggested that pulmonary vascular remodeling of patients with normal lung function began to occur. The expression of MMP-9 was positively correlated with the indexes of vascular remodeling (WT% and WA%). Multi-analysis suggested that the expression of

MMP-9 is the main influential factor of WA% and WT%, and high expression of MMP-9 is closely related to pulmonary vascular remodeling.

Conclusions

The etiology and pathogenesis of pulmonary vascular remodeling in COPD are not entirely clear. The present data shows that inflammation and vascular remodeling in COPD may be associated with the expression of TLR4 and MMP-9. As important factors for pulmonary remodeling, high expression of TLR4 and MMP-9 can exacerbate the inflammatory responses of the pulmonary artery, induce the proliferation of smooth muscle cells, and degrade ECM. Inhibiting the expression of TLR4 and MMP-9 may be significant for prevention and treatment of COPD, which can slow the progression to pulmonary hypertension and pulmonary heart disease.

Conflict of interest

The authors declare no conflicts of interest.

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