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Original Article

Changes of bladder mucosal inflammatory factors and prognosis in cystitis glandularis

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Abstract: *Objective:* To investigate the relationships of bladder mucosal inflammatory factors, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) with the occurrence and development of cystitis glandularis (CG), and their effects on the prognosis. *Methods:* A total of 61 patients with CG from January 2010 to 2014 were randomly selected. Tissue specimens of postoperative patients were collected. 16 cases of normal bladder mucosa during the same period were collected as a control group. Blood specimens and fresh tissue specimens were collected from 6 patients with CG. The messenger ribonucleic acid (mRNA) levels of IL-1, IL-6 and TNF-α were detected via reverse transcription polymerase chain reaction (RT-PCR). The protein levels of IL-1, IL-6 and TNF-α in serum of patients with GC and normal controls were detected via an enzyme-linked immunosorbent assay (ELISA). The protein expressions of IL-1, IL-6 and TNF-α were detected via immunohistochemistry (IHC), and their relationships with the clinical features and prognosis of GC were analyzed. A Cox proportional hazard regression model was used for multivariate analysis on the prognostic factors of CG, and all the tests were performed with 95% confidence interval (CI). *Results:* The protein expressions of IL-1, IL-6 and TNF-α in patients with CG were obviously higher than those in normal group. The mRNA levels of IL-1, IL-6 and TNF-α in the serum of patients with CG were also significantly higher than those in normal group ($P<0.05$). The expressions of IL-1, IL-6 and TNF-α in CG were positively correlated. TNF-α is an independent prognostic factor of CG. Conclusion: IL-1, IL-6 and TNF-α are associated with the occurrence and development of CG. TNF-α presents as an independent prognostic factor of CG that can be used for the diagnosis of cystitis glandularis.

Keywords: CG, IL-1, IL-6, TNF-α, prognosis

Introduction

Cystitis glandularis (CG) is a kind of abnormal hyperplasia of the bladder, which is considered as a potential precancerous lesion. The incidence has been increasing gradually recently. The disease is common in middle-aged and old people and occurs more commonly in females than males. CG causes bladder urethral epithelial cell lesions that are predominantly found in chronic inflammation and in about 13% of asymptomatic bladders [1]. A recent study suggests that CG is caused by chronic irritation of the bladder epithelium and usually does not result in clinical symptoms [2]. The main histopathological feature of the disease is metaplasia of the glandular epithelium of the bladder transitional cells [3]. In 1761, Morgagni et al. [4] described the disease for the first time, but in clinical practice, the natural history of CG is still unclear. Chronic inflammation plays an important role in the occurrence and development of CG [5]. In addition, other cell events and physiological processes, including excessive cell proliferation, inhibition of cell apoptosis, abnormal cell differentiation and abnormal cell signal transduction, are also involved. However, the molecular mechanism and potential key regulatory networks of CG are still being explored.

Rosin et al. [6] studied and found that the oxygen free radicals produced by bladder transitional cells under the stimulation of chronic infection for a long time can damage chromosomes and result in chromosomal translocation, thus transforming the cells into cancerous cells. The expressions of p53 and p21 in CG

Table 1. Clinicopathological data of patients

		Sex		Age (years old)
		Male (n)	Female (n)	
CG	Non-recurrence group (group A)	11	23	52.5±1.2
	Recurrence group (group B)	8	19	55.7±1.9
Control group	Group C	4	12	51.9±2.1

with cell structure disorder and/or atypical hyperplasia are as high as those in bladder cancer tissues. In addition, chronic inflammation is a risk factor for malignant transformation of CG. Tumor necrosis factor (TNF-α), a proinflammatory cytokine mainly produced by monocytes and macrophages, can mediate β-catenin nuclear translocation. This mechanism plays a key role in the occurrence and development of epithelial metaplasia in Barrett's esophagus with precancerous lesions. CG is etiologically and histologically similar to Barrett's metaplasia, and its two subtypes (typical and intestinal/colonic) have an uncertain malignant potential [7]. Both interleukin-1 (IL-1) and interleukin-6 (IL-6) are important inflammatory cytokines. IL-1 plays an important role in a series of processes such as maturation, activation and proliferation of immune cells and immunoregulation. IL-6 can induce B cell differentiation, produce antibodies, induce T cell activation, proliferation, and differentiation, and participate in the immune response of the body, which is a stimulator of the inflammatory response [8].

At present, although the role of related factors to bladder mucosa in bladder autoimmunity has been illustrated, there is little information about the roles of IL-1, IL-6 and TNF-α in CG, especially their correlations with the prognosis of CG. Therefore, we studied the changes of IL-1, IL-6 and TNF-α levels in patients with CG and their influences on prognosis.

Materials and methods

Clinical data

Tissue specimens and clinical data of patients with CG admitted in our hospital from January 2010 to January 2014 were randomly included in this study. In all, 61 tissue specimens were obtained. At the same time, 16 normal bladder mucosa tissue specimens were collected as control group. The fresh CG tissue specimens collected were each divided into 6 pieces, which were quickly stored in liquid nitrogen and stored at -80°C for later use. Blood specimens were collected from 6 patients with CG and were stained with hematoxylin and eosin (HE). Paraffin sections were made and immunohistochemistry (IHC) was performed to analyze the semi-quantitative expressions of the proteins. Ribonucleic acid (RNA) was extracted to detect the messenger RNA (mRNA) expression level of the genes. Blood specimens were used for an enzyme-linked immunosorbent assay (ELISA). At the same time, 6 samples of serum were collected from each control group case (**Table 1**).

Quantitative real-time polymerase chain reaction (PCR)

TRIzol reagent was used for total RNA extraction (Invitrogen, Carlsbad, CA, USA). Complementary deoxyribonucleic acid (cDNA) was synthesized using TaKaRa PrimeScript™ Kit (Bio Inc., Otsu, Japan). The target genes and β-actin gene sequences were obtained via GenBank. Primers were designed using the primer design software Primer-Blast from the National Center of Biotechnology Information (NCBI) website. Primer sequences were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The reaction system was 20 μL. The reaction conditions are as follows: predenaturation at 95°C for 2 minutes, followed by 40 cycles, including 95°C for 15 seconds, sequential at 60°C for 60 seconds. Relative mRNA expression calculation: $2^{-\Delta Ct}$ [ΔCt = Ct (target gene)-Ct (β-actin)], multiple of changes between different treatments were calculated according to $2^{-\Delta\Delta CT}$, where ΔΔCT = ΔCT (experimental group)-ΔCT (control group). The specific primer sequences are as follows: IL-6: Forward: 5'-GACAAAGCCAGAGTCCTTCAGAGAG-3', Reverse: 5'-CTAGGTTTGCCGAGTAGATCT-3'; TNF-α: Forward: 5'-ATGAGCACAGAAAGCATGATC-3', Reverse: 5'-TACAGGCTTGTCACTGGAATT-3'; IL-1: Forward: 5'-ATGGCAGAAGTACCTAAGCTC-3', Reverse: 5'-TTAGGAAGACACAAATTGCATGGTGAACTCAGT-3'.

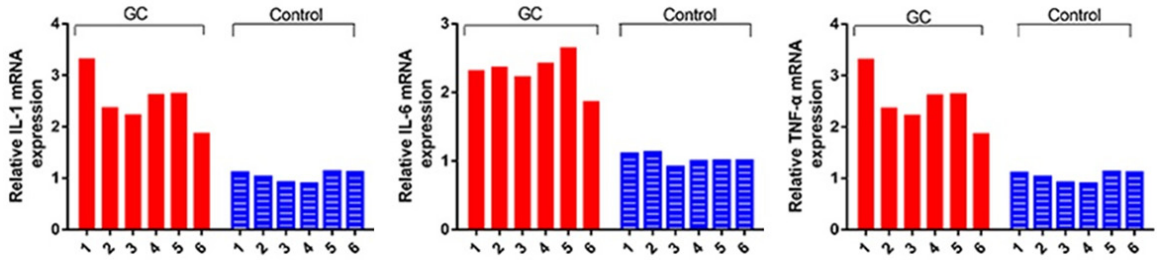

Figure 1. mRNA expressions of IL-1, IL-6 and TNF-α in CG. The expressions of tissue samples from 6 patients with CG and 6 normal controls respectively were detected by RT-PCR. The average levels of IL-1, IL-6 and TNF-α in patients with CG were significantly higher than those in the normal control group (P<0.05).

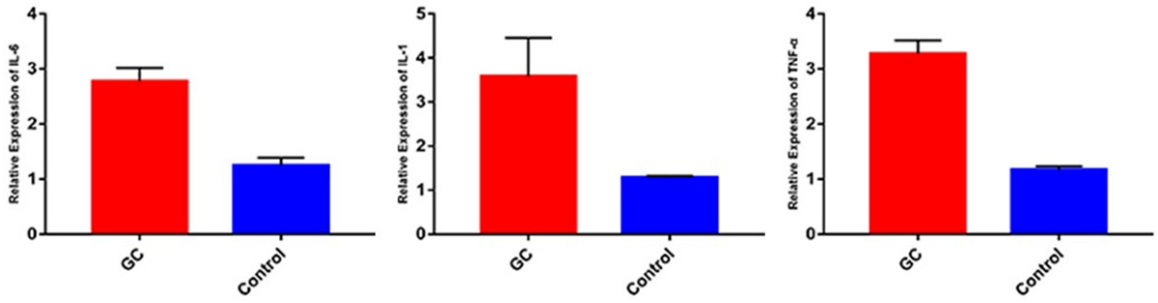

Figure 2. Protein expression of IL-1, IL-6 and TNF-α in the serum of patients. A significant difference of IL-1, IL-6 and TNF-α between CG and the control is shown, as are the levels of these three factors of patients with CG (P<0.05).

Determination of protein levels of IL-1, IL-6 and TNF-α in serum of patients via enzyme-linked immunosorbent assay (ELISA)

After 5 mL of fasting whole blood was collected from patients with CG and healthy subjects and kept for 1 hour at room temperature, the serum was separated by centrifugation at 1000 g. According to the corresponding standard, the serum was diluted at 1:50 in the small test tube provided by the kit. The reagent was mixed well when diluted, and bubbles should be avoided during the operation as far as possible. The BrdU-ELISA kit was purchased from Roche (Indianapolis, IN, USA). According to the instructions provided, the protein levels of IL-1, IL-6 and TNF-α in serum samples were detected, respectively, and this experiment was repeated for three times to take the average value.

IHC detection

The tissues of patients with CG and the corresponding control normal tissues were fixed with formaldehyde and embedded in paraffin. IL-1 polyclonal antibody (1:200, Abcam, Cambridge, MA, USA), IL-6 polyclonal antibody (1:1000, Beverly, MA, USA) and IL-6 polyclonal antibody (1:2000, Abcam, Cambridge, MA, USA) were stained via IHC streptavidin-peroxidase (SP) assay. Positive control: In reference to Abcam and CST's official websites, a strong positive expression could be produced by detecting known tissues containing a test antigen. Negative control: The results were negative when the first antibody was replaced by a phosphate buffered solution (PBS). All the positive signals were yellow, brown-yellow or tan. Five high-power fields (10×40) were randomly observed under the microscope (Olympus, Shinjuku, Tokyo, Japan). The proportion of positive cells with yellow, brown-yellow or tan signals and the intensity of the signals were taken as a determination standard.

Prognosis analysis

The medical records were reviewed, including imaging data, the patients' general conditions, and their treatment plans. Patients were followed up for 6 months to 4 years. Among them, the recurrence group included disease progression and cancerization. The deadline for follow-up was January 2018.

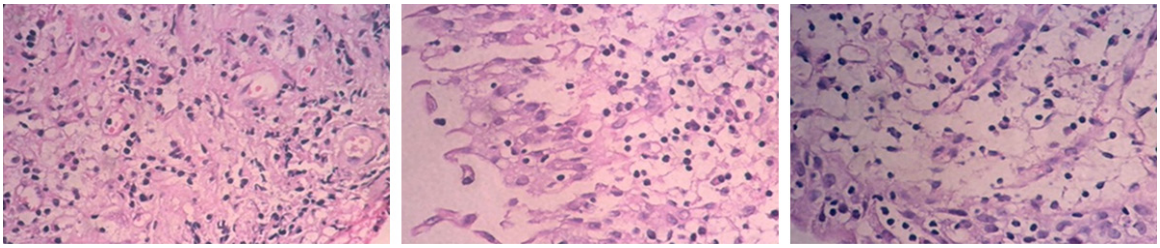

Figure 3. CG bladder mucosa. IL-1, IL-6 and TNF-α proteins were expressed in the cytoplasm and stained brown-yellow. The positive expression rates of these three cytokines in the tissues of patients with CG were than calculated.

Table 2. Positive expressions of IL-1, IL-6 and TNF-α proteins

Group	n	IL-1 [n (%)]	IL-6 [n (%)]	TNF-α [n (%)]
Group A	34	23 (67.6)	27 (79.4)	29 (85.3)
Group B	27	25 (92.6)	23 (85.2)	22 (81.5)
Group C	16	6 (37.5)	8 (50.0)	5 (31.3)
p		0.012	0.031	0.042

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 software was used for statistical analysis. Data in this study were presented as mean ± standard deviation. Continuous data from multiple groups were analyzed by using one-way analysis of variance (ANOVA), with the Tukey's post hoc test. Correlations of the expression levels of IL-1, IL-6 and TNF-α with clinicopathological characteristics were analyzed by chi-square test. Survival and prognosis were analyzed by Kaplan-Meier curve and Log-rank test. The Cox proportional hazard regression model was used to analyze independent prognostic factors of the patients. All the test methods were bilateral distributed, and P<0.05 suggested that there was statistical significance.

Results

Detection of expressions of IL-1, IL-6 and TNF-α in CG via reverse transcription PCR (RT-PCR)

The mRNA expressions of bladder mucosal inflammatory factors in 6 patients with CG and normal control group were detected via real-time RT-PCR. The results showed that the levels of IL-1, IL-6 and TNF-α in patients with CG were significantly higher than those in the control group (P<0.05) (**Figure 1**).

Determination of protein levels of IL-1, IL-6 and TNF-α in serum of patients via ELISA

To further explore the roles of IL-1, IL-6 and TNF-α in CG, the protein levels of IL-1, IL-6 and TNF-α in the subjects' serum were detected via ELISA. The results revealed that the levels of IL-1, IL-6 and TNF-α in serum of patients with CG were significantly higher than those in control group (P<0.05) (**Figure 2**).

Detection of protein expressions of IL-1, IL-6 and TNF-α via IHC

IL-1, IL-6 and TNF-α proteins were expressed in the cytoplasm and stained brown-yellow (**Figure 3**). The positive expression rates of these three cytokines in the tissues of patients with CG were higher than those in the control group, and the expression levels were the highest in the recurrence group (92.6%, 85.25% and 81.5%, respectively) (P<0.05) (**Table 2**).

Correlations among expressions of IL-1, IL-6 and TNF-α

A Spearman rank correlation analysis was used to analyze the correlations among the expressions of the three cytokines in CG. The results showed that in CG, there were significant positive correlations between IL-1 and IL-6 expressions (r=0.625, P<0.001), IL-1 and TNF-α expressions (r=0.549, P<0.001), and IL-6 and TNF-α expressions (r=0.552, P<0.05) (**Table 3A-C**).

Cox proportional hazard regression model

The prognostic factors that may significantly affect the prognosis were identified by using the univariate survival curve comparison method, including patients, namely, age, sex, and expressions of IL-1, IL-6 and TNF-α. Notably, multivariate analysis showed that the expres-

Table 3A. Relationship between IL-1 and IL-6 in CG

		IL-1		*r*	*p*
		High expression	Negative and low expression		
IL-6	High expression	47	3	0.625	0.00004
	Negative and low expression	2	9		

Table 3B. Relationship between IL-1 and TNF-α in CG

		IL-1		*r*	*p*
		High expression	Negative and low expression		
TNF-α	High expression	42	2	0.549	0.00006
	Negative and low expression	4	13		

Table 3C. Relationship between TNF-α and IL-6 in CG

		IL-6		*r*	*p*
		High expression	Negative and low expression		
TNF-α	High expression	39	4	0.552	0.034
	Negative and low expression	7	11		

Table 4. Cox proportional hazard regression model analysis of independent prognostic factors of CG

Parameter		Multivariate prognostic analysis	
		HR (95% CI)	*p*
Age	≤55 years old	1	
	>55 years old	1.457 (0.970-2.289)	0.062
Sex	Male	1	
	Female	2.570 (1.348-3.814)	0.202
IL-1 expression	Low expression	1	
	High expression	0.952 (0.662-1.618)	0.051
IL-6 expression	Low expression	1	
	High expression	0.871 (0.576-1.455)	0.056
TNF-α expression	Low expression	1	
	High expression	1.406 (0.671-1.597)	0.031

sion of TNF-α was an independent factor affecting the prognosis of patients with CG (**Table 4**).

Discussion

The cause of CG remains unclear. At present, most people believe that CG is results from acute and chronic inflammation and other chronic irritation due to chronic bladder infection, urinary obstruction, urinary dysfunction, etc. Generally, inflammation is a protective response of the body to external injury, as well as a primitive reaction. The way that the body fights against inflammation includes an endogenous anti-inflammatory mechanism, which protects the body from excessive tissue damage and promotes the recovery of tissue structure and function [9]. The uncontrolled development of inflammation, and the excessive release and interactions of proinflammatory mediators and various inflammatory factors are the basic pathological mechanisms of histocyte proliferation, metaplasia, injury, malignancy, etc., in various inflammation-related diseases [10]. Multiple studies demonstrated that proinflammatory cytokines play important roles in anti-tumor regeneration by inducing the production of different tumor cells and tumor-associated leukocytes [11, 12]. More importantly, the regulatory networks of these inflammation-related diseases involve a wide variety of biomolecular elements, such as genes, non-coding, proteins and metabolic small molecules, as “network nodes”. Through complex interactions among one another, biomolecular elements form a multidimensional and dynamic “Internet”, which regulates the balance of inflammation between controllability and non-controllability and determines the biological behavior and characterization of complex inflammation-related diseases [13, 14]. CG, as an inflammatory proliferative lesion and precancerous lesion,

can be described as a typical manifestation of uncontrollable inflammation.

IL-1 can be regulated by a variety of inflammatory mediators and is one of the main inflammatory cytokines secreted by macrophages [15, 16]. IL-6 is a kind of cytokine secreted by Th17 cells. It has a powerful inflammatory effect and has special functions in autoimmunity [17, 18]. Some studies have found that the uncontrollable inflammation mediated by IL-6 may play an important role in the occurrence and development of CG, but its specific mechanism is not clear [19, 20]. CG is associated with complicated clinical symptoms, can possibly develop into bladder adenocarcinoma, and can also coexist with adenocarcinoma. Therefore, more and more attention has been paid to the role of tumor-associated inflammatory factors in CG. In this study, we found that TNF-α was closely related to the occurrence and development of CG and represented an independent prognostic factor of CG. At the same time, the results of this study suggested that the expressions of bladder mucosal factors (IL-1, IL-6 and TNF-α) in CG were increased, the levels of which were positively correlated. Moreover, the levels of these three inflammatory factors were the highest in the canceration group, indicating that IL-1, IL-6 and TNF-α can be used not only as the diagnostic bases of CG, but also as potential predictors of prognosis.

Conclusion

Our data demonstrate that the increasing levels of IL-1, IL-6 and TNF-α are associated with the occurrence and development of CG. TNF-α is an independent factor for the prognosis of patients with CG, which provides new insights for the future diagnosis of CG in clinical practice.

Disclosure of conflict of interest

None.

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