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## Expression and significance of neutrophil extracellular network in nephridial tissues of severe Henoch–Schonlein purpura nephritis patients

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### ABSTRACT

The expression and significance of the neutrophil extracellular network (NET) in nephridial tissues of severe Henoch–Schonlein purpura nephritis (HSPN) patients were analyzed in this study. Citrullinated histone H3 as a marker and an immunological method were used to detect NET expression in 25 cases of HSPN patients, 15 cases of small lesions (MCD) patients, and 7 cases of healthy nephridial tissues. Analysis of patients with renal histological activity index and chronic index as well as detection of the infiltration of the corresponding part of the CD19 as a marker of B lymphocytes along with their relationships with NET were performed simultaneously. Results showed that NET expression was significantly higher in the glomeruli of the patients with severe HSPN than those of the healthy control group and the MCD group ( $1.146 \pm 0.903$ ,  $0 \pm 0$ ,  $0 \pm 0$ ,  $P < 0.01$ ). NET expression increased significantly in moderate and exuberant mesangial cells, cellular crescents, and renal tubular interstitium infiltrated with inflammatory cells. Meanwhile, glomerular NET expression in moderate and exuberant mesangial cells was significantly higher than that in other types, and the differences were statistically significant ( $P < 0.01$ ). We also found that, on the average, the number of NET-positive cells in moderate and exuberant mesangial cells and severe HSPN renal tubules were positively correlated ( $r = 0.558$ ,  $P = 0.011$ ). The number of NET-positive renal interstitial cells was also positively correlated with the chronic index of purpura nephritis pathology ( $r = 0.645$ ,  $P = 0.002$ ). Therefore, NET is widely expressed in the renal tissues of severe HSPN patients. The infiltrated NET possibly caused renal injury in the patients with severe HSPN.

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### KEYWORDS

Henoch-Schonlein purpura nephritis;  
Neutrophil extracellular network;  
B lymphocytes;  
The renal histological activity index;  
The renal histological chronic index.

## INTRODUCTION

Neutrophil extracellular network (NET) is a bactericidal mechanism of neutrophils. NET is a net-like structure composed of nucleic acid and protein particles (such as elastase, cathepsin G, myeloperoxidase, and so on) that is released from dead neutrophils. NET, which was found by Brinkmann et al. in 2004, can kill pathogens and has important functions as chemotactic, phagocytic, bactericidal, and so on<sup>[1]</sup>.

In autoimmune diseases, such as ANCA-associated vasculitis and allergic purpura, abnormal neutrophil activation and infection can induce or aggravate the illness. However, little research has been reported on the relationship between NET and Henoch-Schonlein purpura nephritis (HSPN). Furthermore, the neutrophil bactericidal efficiency of NET is much higher than the neutrophil phagocytic and bactericidal capacity of neutrophil granulocytes in the nonspecific immune system we previously studied<sup>[2]</sup>. Citrullinated histone H3 is an important marker of NET. We used the immunohistochemical method to study NET expression in renal tissues of severe HSPN and its relationship with patient conditions as well as to examine the possible pathogenic mechanism.

## MATERIALS AND METHODS

### General data

A total of 25 cases of patients with severe HSPN diagnosed by renal biopsy in our hospital from March 2008 to October 2011 were selected as the object. The patients included 22 females and 3 males, 15 to 53 years old with a mean age of  $33.2 \pm 13.1$ , which conforms to the HSPN diagnosis standard of WHO. Hormone and/or immunosuppressive therapy have not been previously received; infection and application of immunomodulatory drugs were also ruled out. The control group included 18 cases. A total of 15 cases had lesions (MCD), and no infiltration of neutrophils in renal tissue was found under a light microscope. Another three cases were of acute pyelonephritis, and a high degree of renal interstitial and tubular neutrophil infiltration were examined under a light microscope. The

healthy control group included seven normal renal tissues obtained from surgical or renal resection operations and had been confirmed with no abnormal change using light-microscope observation, immunofluorescence, and electron microscopy examination.

### Renal tissue pathology analysis

All patients underwent renal biopsy, and the biopsy specimens were subjected to HE, PAS, PASM, and Masson staining. The renal histological activity index and chronic index scoring method used are as follows<sup>[3]</sup>: (1) activity index: glomerular cells (endothelial cells, mesangial cells, and infiltrated mononuclear cells) proliferation; nuclear fragmentation and necrosis; cellular crescents or cell fibrous crescents; hyaline thrombus; leukocyte infiltration; interstitial inflammatory cell infiltration; (2) chronic index: glomerular sclerosis; renal tubular atrophy; fibrous crescents; interstitial fibrosis. Each item was given scores of 0, 1, 2, or 3 (0 score: none, 1 score: mild or <25%, 2 score: moderate 25%-50%, and 3 score: severe >50%). The score was multiplied by 2 in cases of fibrinoid necrosis, nuclear fragmentation, and cellular crescent, and then all scores were added to obtain the total result. The highest activity index was 24 points, and the highest chronic index was 12 points. The degree of mesangial cell proliferation was classified as follows<sup>[4-5]</sup>: (1) mild: mesangial cell proliferation and/or mesangial matrix does not exceed the capillary diameter, and capillary lumen are not crushed; (2) moderate: mesangial cell proliferation and/or mesangial matrix exceeds the capillary diameter, and the capillary lumen is extruded; (3) severe: mesangial cell proliferation and/or mesangial matrix damages the capillary loops, and the adjacent capillaries disappear in sclerotic segment state.

### Histopathological analysis

#### Materials

Rabbit anti-human citrullinated histone H3 antibody (Calendon Canada, xl69193), mouse anti-human CD19 antibody (labeled B lymphocyte, with resistance), a general two-step immunohistochemical detection kit (PV9000), and diaminobenzidine (DAB) color liquid (Shanghai Xinran) were used for the experiments.

#### NET detection

A sample slice with a thickness of 3  $\mu\text{m}$  was thrice

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dewaxed and dipped in xylene for 5 min. Then, the specimen was washed in 100%, 95%, 80%, and 50% ethanol for 5 min for dehydration and washed in PBS for 2 min. The slice was repaired in EDTA (pH 8.0) under microwave at high heat for 1 min and at low heat for 2 min and cooled naturally. The sample slice was washed thrice in PBS for 2 min, and endogenous peroxidase was blocked in a closed system using 3% H<sub>2</sub>O<sub>2</sub> at room temperature away from light for 10 min. The sample was then washed in PBS for 2 min and kept in a closed system with 1% human serum albumin at room temperature for 30 min. Then, the first antibody (1:50) was added and placed in a refrigerator at 4 °C overnight. The slice was washed thrice in PBS for 2 min and then the second antibody (PV9000 Kit) was added and incubated in a 37 °C constant temperature box for 20 min, after which the slice was washed thrice in PBS for 2 min. DAB staining was performed for 30s until the water showed no further coloration, followed by PAS staining, oxidation in periodate solution for 1 min in water, and full immersion in Schiff reagent while avoiding light for 1 min. Color change in water was observed and the sample was fully washed with flowing water after the solution turned red. The nucleus was double stained and washed in water thrice after treatment with hematoxylin for 1 min. Then, the nucleus was fully washed in water after dipping in 1% ammonia alcohol for 5 s, followed by gradient ethanol dehydration. Xylene transparent and neutral resin sealing were performed successively. A negative control was observed using 0.01 mmol/L PBS instead of the first antibody while the other steps remained the same.

### B lymphocyte detection

A mouse anti-human CD19 antibody was dripped followed by the remaining steps of the NET-staining procedure described above. Lymphoma tissue was used as positive control.

### Pathology analysis

Each specimen was divided into the glomerular region and the renal interstitial region. Positive glomerular, renal interstitial, and tubular staining were counted. The number of positive glomerular-stained cells was based on the number of positive staining found in

each glomerular cell. For the renal interstitial positive staining cell count, we adopted the single blind method. Each specimen was observed at high magnification ( $\times 400$ ) in 10 consecutive non-overlapping renal cortical fields. In the fields excluding the glomerular vascular and renal tubular areas, we counted positive staining cell number. The average number in 10 fields was recorded as the infiltration number for the cell of each specimen. Renal tubular positive staining cell count was taken as the average positive staining cells in the renal tubules of each patient.

### Data processing

Measurement value was represented by  $\bar{x} \pm s$ , the differences between groups was determined using the t-test. Kruskal-Wallis rank sum test was used to analyze parametric differences between groups. Spearman rank correlation analysis was performed for correlation analysis. Data were analyzed using SPSS17.5 software.

## RESULTS

### NET expression

NET expression was not found in the renal tissues of the patients in the healthy control group and the MCD group. However, NET was widely expressed in the renal interstitium and tubules of the patients with acute pyelonephritis and HSPN. In the renal tissues of the HSPN patients, glomerular NET was distributed mainly in the middle, severe mesangial cell hyperplasia and peribulbar, renal interstitial infiltration of inflammatory cells, cell area crescent, and renal tubules. Part of the roughly normal glomerulus also indicated positive NET expression. Infiltrated NET was most commonly observed in severe proliferated glomerular and mesangial cells. NET expression levels were  $1.146 \pm 0.903$  and  $0.470 \pm 0.461$  respectively in the renal glomerulus and interstitium in the HSPN group and were contrasted with the healthy group and the MCD group. The difference was statistically significant ( $P < 0.01$ ,  $P < 0.05$ ). The difference between severe mesangial infiltrated cell and other types was statistically significant, as shown in TABLE 1.

### Expression of B lymphocytes

B lymphocyte expression was not observed in

healthy controls or MCD patients. In the renal tissues of the HSPN patients, B lymphocytes were mainly distributed around the sclerotic glomeruli and severe mesangial proliferated glomeruli. Low amounts of B lymphocytes were positively expressed around the cell fibrous crescents and fibrous crescent glomeruli. In the renal interstitial inflammatory cell infiltration area, they mostly clustered together. Nearly no expression was found in the glomerulus, as shown in TABLE 2. B lymphocyte expression levels were  $1.201 \pm 1.395$  and  $5.879 \pm 7.033$  respectively in the renal glomeruli and interstitium in the HSPN group and were contrasted with the healthy group and the MCD group. The difference was statistically significant ( $P < 0.01$ ,  $P < 0.05$ ). The number of positive B lymphocytes around the glomeruli in severe mesangial proliferated cells was significantly higher than that in other glomerular types, as shown in TABLE 1.

**TABLE 1 : Number of NET-positive cells in renal tissues of purpura nephritis and B lymphocyte ( $\chi^2 \pm s$ )**

Project	NET	B lymphocyte
Normal glomeruli (n=108)	0.306 $\pm$ 0.408	0
Severe mesangial proliferated glomeruli (n=136)	1.335 $\pm$ 1.246 <sup>#</sup>	0.892 $\pm$ 1.105 <sup>+</sup>
Sclerotic glomeruli (n=5)	0	0.871 $\pm$ 0.930
Renal interstitium (/HPF)	0.485 $\pm$ 0.403	5.96 $\pm$ 6.40
Fiber crescentic glomeruli (n=3)	0	0.285 $\pm$ 2.013
Fiber cell crescentic glomeruli (n=4)	0	0.202 $\pm$ 0.775
Cell crescentic glomeruli (n=15)	0.531 $\pm$ 1.88	0
Renal tubules	7.12 $\pm$ 7.27	0 <sup>+</sup>

**n: number of glomeruli**

### Correlation analysis

The average number of NET positive cells in severe mesangial proliferated glomerular and renal tubule were positively correlated ( $r=0.573$ ,  $P=0.013$ ). Chronic index and renal interstitial-positive B lymphocyte number were not related. The number of renal interstitial NET-positive cells were positively correlated with the number of positive B lymphocyte and the number of renal tubular NET-positive cells and chronic index ( $r=0.573$ ,  $P=0.007$ ;  $r=0.435$ ,  $P=0.046$ ;  $r=0.632$ ,  $P=0.003$ ). No correlation was found with activity index. Renal tubular NET masculine cell number and activity index, chronic index, and renal interstitial B lymphocyte numbers were not related.

## DISCUSSION

Peptidylarginine deiminase (peptidylarginine deiminases 4, PAD4) catalyzed neutrophil nuclear core histones H2A, H3, and H4 positively charged arginine into electrically neutral citrulline, thereby removing the negative charge of chromatin and setting chromatin loose. Citrullinated histone H3, as an important marker of NET components, was undetected during neutrophil apoptosis [6]. At present, only a few reports are available on NET and LN activity damage correlation, NET expression in different glomerular types, and whether NET as antigen can stimulate B lymphocytes to produce antibodies. The results of this study showed that no NET expression was found in healthy people and patients with mild disease in renal tissues. NET was expressed in the glomeruli and renal interstitium of severe HSPN patients and was mainly distributed in mesangial cells, severe proliferated cellular crescent glomeruli, and renal tubular interstitium with inflammatory cells. NET expression was mainly located at the injured renal tissues. The results suggested that NET may be involved in the renal tissue injury of patients with severe purpura.

NET has a reticular structure composed of granule protein released by nucleic acid substances and neutrophils. The characteristics of NET enable it to solidly adhere to the vascular endothelium, which contains myeloperoxidase protein components that can damage the small vascular endothelium. Such damage can cause acute injury of the vascular wall. A previous study confirmed that NET can adhere to the vascular endothelium when sepsis occurs, causing acute injury of the small vessel wall<sup>[7]</sup>.

A related research involving severe HSPN patients reported the infiltration of focal neutrophils into the renal interstitium, most of which were located around vascular lesions. Most scholars believe that the significance of neutrophil infiltration is closely related with the severity of renal injury,

Inflammatory cells can release a net-like structure composed of nucleic acid and particulate proteins, such as elastase, cathepsin G, myeloperoxidase, and so on, in the infiltration process. Cytokine activation and tubular epithelial cell transdifferentiation are then promoted, thereby inducing or exacerbating renal fibrosis. Vascular injury releases angiotensin II and endothelin, and the

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latter may promote infiltration of inflammatory cells in the renal interstitium<sup>[8]</sup>. Renal pathological changes in patients whose NET could not be cleared in time were much worse than the control group. Renal histological activity index, chronic index, and the severity of glomerular mesangial cell proliferation were found to be statistically significantly correlated with NET.

In summary, NET may be involved in the pathogenesis of HSPN and related with its activity. Anti-NET antibody removal by ELISA from patients using endonuclease column and selective NET decomposition may be a mode of treatment for HSPN in the future.

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