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Calculus-related functional protein expression in ureteral calculus-adhered polyp

A preliminary study

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Abstract

To explore the expressions of calculus-related functional proteins in the ureteral calculus-adhered polyp tissues and investigate the role of these proteins in the formation of adhesions between the calculus and polyp.

Patients with ureteral calculi and polyps who underwent ureteroscopic lithotripsy for the excision of polyps between January 2019 and June 2019 were enrolled. Polyps obtained from each patient were divided into 2 groups using a matched pairs design: observation group (polyps adhered to calculus) and control group (polyps not adhered to calculus). Histopathological examination of polyps was performed using hematoxylin and eosin staining. Polyp tissues were immunohistochemically stained to assess the expressions of calculus-related functional proteins, that is, annexin A1, calcium-binding protein S100A9 (S100A9), uromodulin, and osteopontin. Furthermore, quantitative analysis was performed using the H-score of tissue staining; Pearson correlation analysis was performed for proteins with high expression.

Overall, 40 polyp specimens were collected from 20 patients with ureteral calculi combined with polyps (observation group, 20 specimens; control group, 20 specimens). Hematoxylin and eosin staining revealed obvious epithelial cell proliferation in polyps of both groups; crystals were observed in the epithelial cells of the polyp tissue in the observation group. The expression levels of annexin A1 and S100A9 in the observation group were significantly greater than those in the control group ($P < .05$). However, no obvious expression of osteopontin or uromodulin was observed in the polyp tissues of both groups. There was a strong correlation between the increased expressions of annexin A1 and S100A9 in the observation group ($R = 0.741$, $P = .022$).

We documented increased expressions of annexin A1 and S100A9 in the ureteral calculus-adhered polyp tissues. Annexin A1 and S100A9 may play an essential role in the adhesion of calculus and polyp and the growth of calculi.

Abbreviations: COM = calcium oxalate monohydrate, IHC analysis = immunohistochemical analysis, S100A9 = calcium-binding protein S100A9.

Keywords: annexin, calcium oxalate binding protein, osteopontin, ureteral calculus, ureteral polyp, uromodulin

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Data available statements: The datasets generated and analyzed during the current study are not publicly available due to the experimental data relates to other experiments that are progressing, but are available from the corresponding author on reasonable request.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Shandong Provincial Third Hospital Medical Ethics Committee (KYLL-2018004) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants included in the study.

The authors declare that they have no competing interests.

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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1. Introduction

There are 2 types of ureteral polyps: primary fibrous polyps and inflammatory polyps secondary to ureteral calculi. The present study focused on the latter, which is inflammatory polyps secondary to ureteral calculi. Renal calculi may get incarcerated in the physiological stricture of the ureter, and the resultant ureteral obstruction may cause rapid increase in renal pelvic pressure, leading to renal colic. This phenomenon is conducive to the timely detection, diagnosis, and treatment of the disease. However, lack of timely diagnosis or treatment of ureteral calculus may lead to prolonged incarceration of the calculus in the ureteral lumen in some patients. Incarceration of ureteral calculi in the ureter for over 2 weeks may induce mucosal edema and congestion and gradually lead to inflammatory polyp formation. The formation of ureteral polyps may further aggravate the degree of ureteral obstruction. Massive ureteral polyps can cause complete obstruction of the ureteral lumen, aggravate the degree of hydronephrosis, and worsen renal colic.

Basic research on urolithiasis conducted in recent decades has identified some proteins that are closely associated with the occurrence and growth of calculi, such as calcium-binding protein S100A9 (S100A9), annexin A1, osteopontin, and uromodulin, which were found to play an important role in nucleation, aggregation, and growth of calculi. Because of their specific molecular structure and function in epithelial tissues, these proteins can promote crystal aggregation and growth.[1–4] In our surgical practice, we commonly encounter intraoperative findings of large ureteral calculi combined with polyps. We wondered how do calculi adhere to polyps and do polyps promote the growth of calculi? Therefore, in the present study, we determined the expressions of calculus-related functional proteins in the ureteral calculus-adhered polyp tissues and investigated the role of these proteins in causing adhesion between calculus and polyp on the basis of previous research findings.

2. Materials and methods

2.1. Clinical data and scientific research design

The present study was conducted at the Shandong Provincial Center for the Prevention and Treatment of lithiasis from January 2019 to June 2019. According to the adhesive status of the polyps to the calculus, the excised polyp tissues were categorized into the observation (polyps adhered to calculus) and control (polyps not adhered to calculus) groups, with 20 polyp specimens in each group. Data on demographic and clinical characteristics, including sex, age, body mass index, occupation, residence, education level, maximum diameter of the calculus, location of the calculus, length of renal pelvis separation, serum creatinine level, course of disease, and operation time, were recorded. The size and location of calculi and the length of renal pelvis separation were determined by abdominal computed tomography. The study was registered (No. ChiCTR1800020208) with the Chinese Clinical Trial Registry, and its ethical document review (KYLL-2018004), safety examination, and course supervision were implemented by Shandong Provincial Third Hospital's Science and Education Department and the Medical Ethics Committee, which acted as independent official agencies. Informed consent was obtained from all individual participants included in the study.

2.2. Patient selection criteria

The inclusion criteria include patients with solitary ureteral calculus (largest diameter, $\leq$20 mm), with concomitant presence of polyp tissue adhered to the ureteral calculus, aged between 18 and 60 years, with confirmed calculus composition of COM.

The exclusion criteria include patients with primary ureteral polyp, history of ureteral trauma, or history of ureteral surgery.

2.3. Ureteroscopic polypectomy

Surgery was performed under general anesthesia: the patient was placed in a lithotomy position on the back. (If preoperative examination suggested a significant tortuous ureter, the patient was placed in the Trendelenburg position so that the ureter could be straightened properly and an ureteroscope could enter the cavity.) The lower limb of the affected side was properly lowered to facilitate surgical operation. A zebra guidewire was inserted into the ureter to lead the procedure. The action was gentle enough to not cause any damage to the ureter, and intake pressure was well controlled to prevent separation of the ureteral calculus from the polyp. A ureteroscope was inserted into the ureter. When the front end of the ureteroscope reached the ureteral calculus bed, the zebra guidewire was inserted into the pelvis through the gap between the ureteral calculus and the ureteral wall. If the gap was not wide enough for the zebra guidewire, lithotrity was carefully and patiently conducted to unchoke the cavity, ensuring that the fragile polyp was uninjured and did not bleed, and then the zebra guidewire was inserted into the pelvis. In the case of a ureteral calculus-adhered polyp, the ureteral calculus was gradually broken from the edge away from the polyp. The residual calculus separated from the polyp was taken out, and then the ureteral calculus-adhered polyp was excised (Fig. 1B and F). The integrity of polyp tissue was ensured as much as possible. The removed ureteral calculus-adhered polyp was kept in formalin solution. In contrast, if there was no adhesion between the polyp and the ureteral calculus, the polyp was removed first and kept in formalin solution, and then the ureteral calculus was handled so as to prevent severe damage to the polyp, which might affect the subsequent molecular trial. The ureteroscope we used in the operation was a product of the German brand Richard Wolf (PFORZHEIMER STRASSE 32, 75438 Knittlingen, Germany) (Model #8703.533, 12°, 8/9.8 Fr, 315 mm WL), and the holmium laser system was Holmium Nd: YAG Dual Wavelength Laser System (VersaPulse 80/100 W PowerSuite) from the Lumenis Inc (Yokneam Industrial Park, Hakidma 6, P.O.B. # 240 Yokneam 2069204 Israel).

Ultrasound of the urinary system was performed 6 months after operation to confirm whether there was recurrence, residual stone fragments, or pyelectasis. Intravenous pyelogram should be performed with a suspicion of pyelectasis induced by ureteral stenosis. All patients underwent ureteroscopy a year after operation to observe whether localized narrowing ("annular" stricture) occurred in the original operating region in the ureteral lumen.

2.4. Antibodies

Antibodies specific for annexin A1 (Item No. ab214486), calcium-binding S100A9 (Item No. ab227570), uromodulin (Item No. ab207170), and osteopontin (Item No. ab214050) were obtained from Abcam (Cambridge, UK).

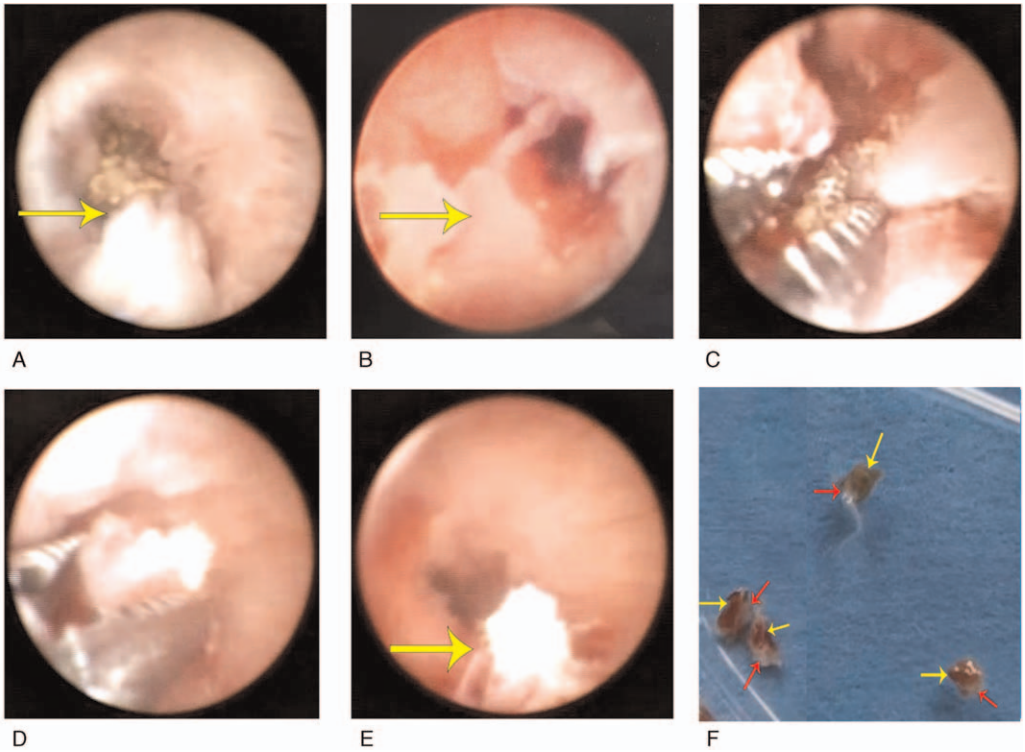

Figure 1. Treatment of calculus adhesion polyps with ureteroscopy. (A) The polyps are closely adhered to the calculus. The yellow arrow is pointing to the polyp–calculus adhesion. (B) The yellow arrow is pointing to a simple polyp that is joined to another polyp above the calculus. (C) A calculus plier is used to remove the calculus adhering to the polyps. (D) The calculus plier picks up some polyp tissue. (E) The yellow arrow is pointing to the laser fiber emitting laser light to remove polyp tissue. (F) The calculus is removed from the ureter, and the polyps are attached to the surface. Yellow arrows point to calculi, and red arrows point to polyps.

2.5. Polyp histological investigation

Ureteral polyp tissue specimens were fixed in a 4% formaldehyde solution for 48 hours, dehydrated in graded ethanol solutions, and then embedded in paraffin before being cut into 5-μm-thick sections. Morphological analysis of ureteral polyp was conducted by light microscopy after hematoxylin and eosin staining.

2.6. Immunohistochemical (IHC) analysis

We detected the expression of annexin A1, S100A9, uromodulin, and osteopontin localization in polyp samples using semiquantitative IHC analysis. The 5-μm-thick serial sections of ureteral polyp tissues were incubated with appropriate primary antibodies overnight at 4 °C before being incubated with appropriate secondary antibodies for 25 minutes at room temperature. Next, the sections were treated with peroxidase-marked streptavidin/peroxidase before being examined under an Olympus BX-51 microscope (Olympus, Japan). A semiquantitative scoring system was used to grade the immunoreaction intensity.[5] The cases were identified pathologically by a senior uropathologist blinded to the clinical outcome using the semi-quantitative H-score (0–300), including the intensity score (0 for negative, 1+ for weak, 2+ for moderate, and 3+ for strong). The intensity score was classified into 2 categories (negative or positive) to examine the concordance rate of the expressions of markers between different sites of the same organ.

2.7. Statistical analysis

All data were presented as the mean ± standard deviation unless otherwise stated. Statistical analysis was performed by a blinded investigator using SPSS Statistics 19.0. (Almonk, New York, USA.) Measurement data were analyzed using paired t test. Associations with annexin A1 and S100A9 were tested using nonparametric Pearson correlation coefficient tests. $P < .05$ was considered statistically significant. All experiments were repeated independently at least thrice.

3. Results

3.1. Clinical data

From January 2019 to June 2019, a total of 267 cases of minimally invasive surgery were performed for ureteral calculi. Among them, 34 patients were assessed for eligibility and 14 patients were excluded for a variety of reasons (Fig. 2). Out of the 20 patients, 15 were men and 5 were women; the age of patients ranged from 32 to 60 years (46 ± 6.53 years). Thirteen patients had left ureteral calculi, while 7 had right ureteral calculi. The maximum diameter of calculi ranged from 8 to 20 mm (12 ± 3 mm). The length of renal pelvis separation ranged from 15 to 40 mm (23 ± 6.98 mm). The disease course ranged from 2 to 6 weeks (3.5 ± 2.18 weeks). The operation time ranged from 31 to 100 minutes (46 ± 14.68 minutes). See Table 1 for details.

No recurrence of calculi, residual calculi, and hydronephrosis were found in all patients during the ultrasound examination 6 months after operation. All patients underwent ureteroscopy a year after operation, which exhibited full recovery of the mucosa in the original operating region in the ureteral lumen without "annular" stricture.

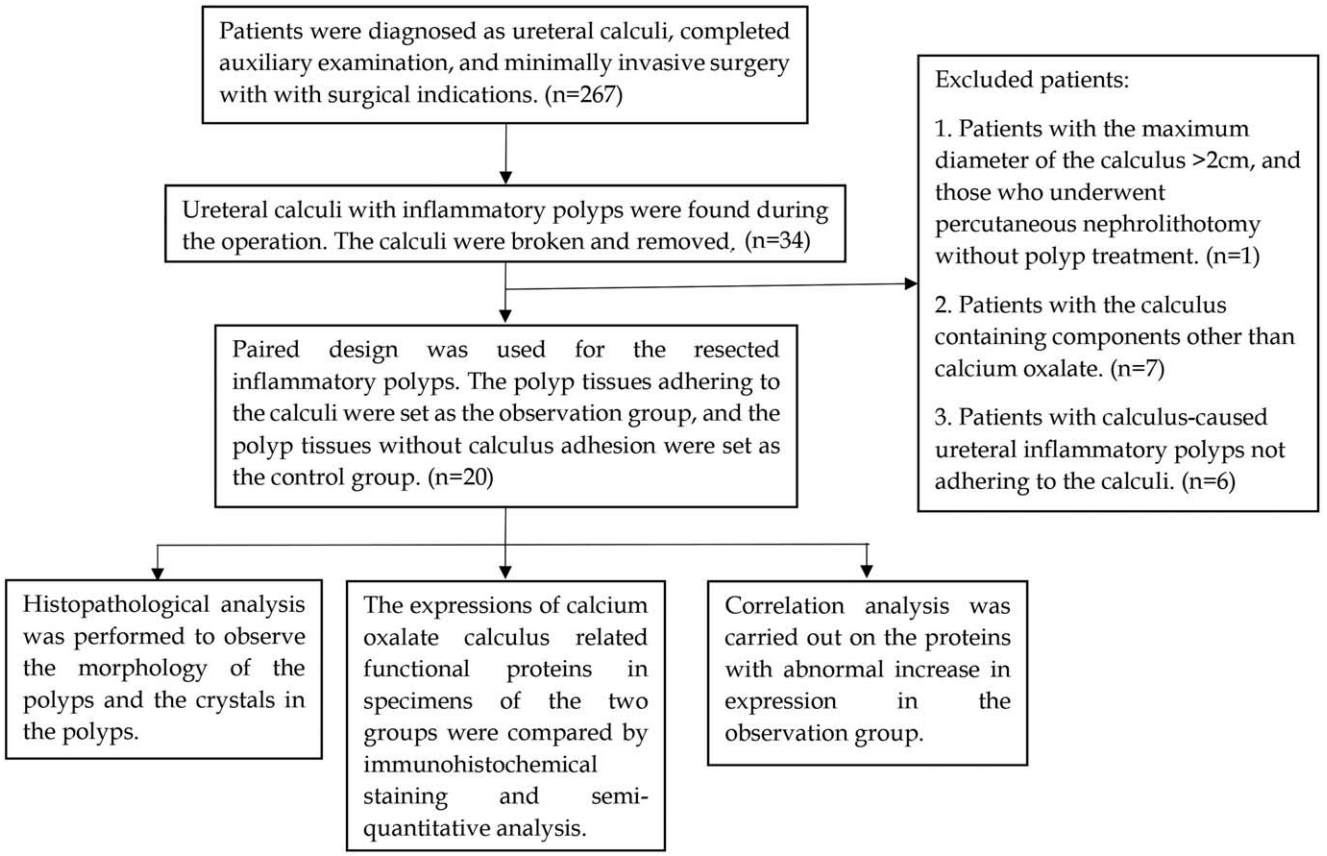

Figure 2. Patient flow.

3.2. Pathologic analysis of polyp tissues

Pathologic findings revealed chronic/acute ureteritis and local urothelial proliferation in both groups. In the observation group, we saw obvious crystal components on hematoxylin and eosin staining, and the crystals were wrapped in epithelial cells (Fig. 1A). In both groups, epithelial cells proliferated and gathered on the polyp mucous layer; interstitial tissue was loose in the structure, presenting the outlook of edema; and massive inflammatory cell infiltration were present. In addition, inflammatory cells in the observation group were significantly more compared with the control group (Fig. 3).

Table 1

Demographic and clinical characteristics of patients.

Variable	Data analysis or presentation
Sex (male/female)	15/5
Age, y*	46 ± 6.53
Body mass index*	24.4 ± 4
Occupation (staff/freelance work)	14/6
Residence (local residents/foreign residents)	18/2
Education level (University/Senior/Junior)	6/8/6
Stone position (upper/middle/lower)	18/1/1
Maximum diameter of stone,* mm	12 ± 3
Length of renal pelvis separation,* mm	23 ± 6.98
Serum creatinine level,* μmol/L	97 ± 21.74
Course of disease,* wks	3.5 ± 2.18
Duration of operation,* min	46 ± 14.68

* Mean ± SD.

3.3. IHC analysis of annexin A1, S100A9, uromodulin, and osteopontin in polyp samples

Annexin A1 and S100A9 were localized in urothelial cells. Their staining intensities were significantly increased in the observation group compared with the control group, indicating high expression of both proteins in the ureteral calculus-adhered polyp epithelial mucosa. However, uromodulin and osteopontin were not significantly expressed in either group (Fig. 4). The H-scores of the 4 proteins also confirmed that annexin A1 and S100A9 expression significantly increases in the observation group, whereas uromodulin and osteopontin expression is not significantly different between the observation and control groups (Fig. 5). Pearson correlation coefficient tests to verify the correlation between annexin A1 and S100A9 showed a significant positive correlation between annexin A1 and S100A9 H-scores ($R = 0.741$; $P = .022$).

4. Discussion

Ureteral polyps are classified as primary and secondary polypsits pathogenesis is still uncertain. When analyzed from epidemiologic perspective, the disease may attack all age groups, but those aged 30 to 50 and men are more risky than other age groups and

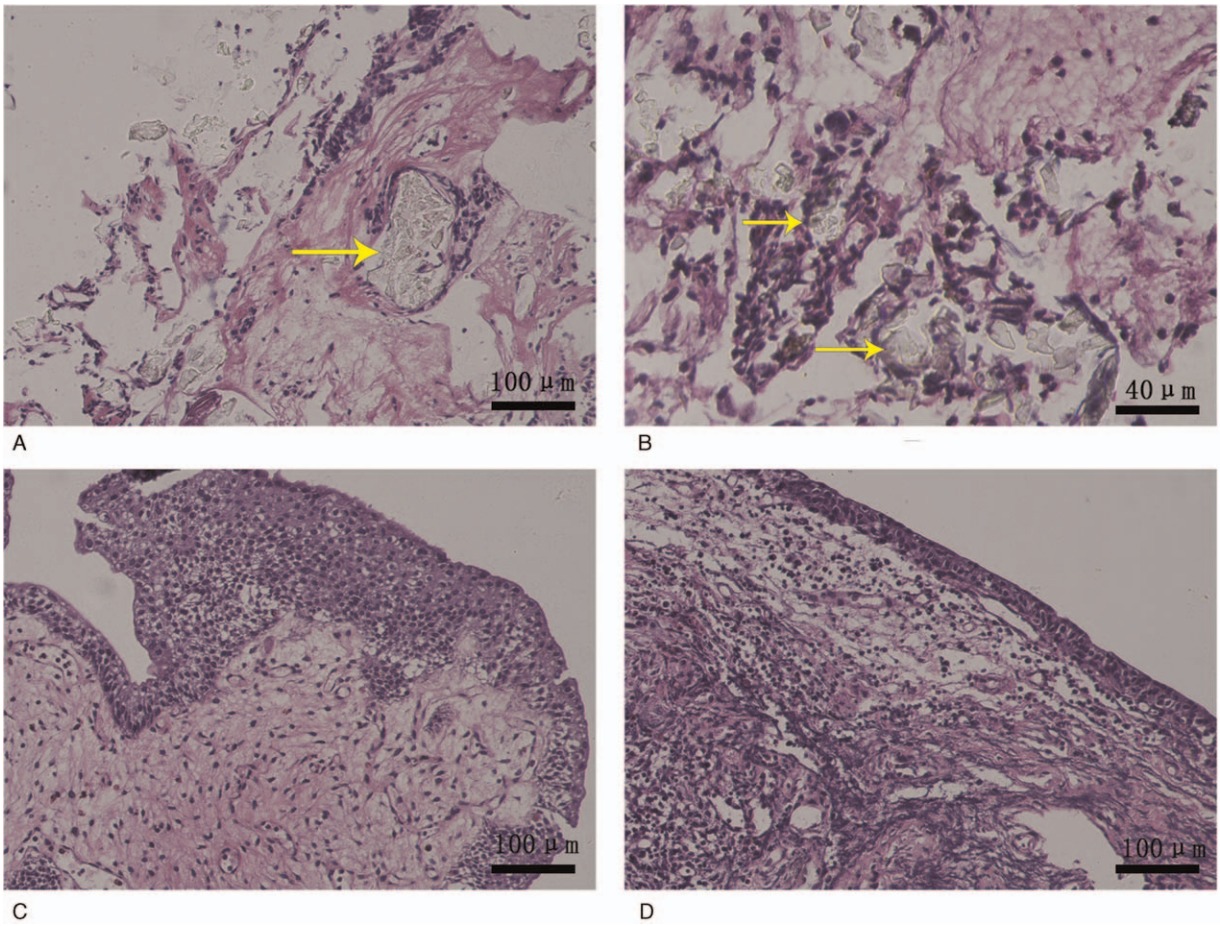

Figure 3. H&E staining of polyp tissue. (A) Under a 200× magnification microscope, the crystalline component of the interstitial tissue of the polyp was observed. Epithelial cells enveloped the calculus crystal. (B) Crystallization was in intimate contact with the apical surface of epithelial cells of the polyp under a 400× magnification microscope. (C) Epithelial cells of the polyp increased significantly in the observation group, which was consistent with the proliferation performance. (D) Polyps in the control group were consistent with the pathological manifestations of chronic ureteritis. H&E = hematoxylin and eosin.

women (at a ratio of 1.5–1), respectively.[6] As reported by existing literature, the polyp length ranges from 0.4 to 17 cm (4 cm in average). The major clinical manifestations of polyp include abdominal pain with or without visible gross hematuria.[7] Secondary polyps are generally attributed to infection, trauma, and calculus, as well as other internal and external oncogenic factors.[8] This study focuses on secondary polyps caused by ureteral calculi obstruction and the consequent ureteral mucosal proliferation. The corresponding pathogenetic mechanism is associated with ureteral epithelial mucosal injury, infection, and release of inflammatory factors locally. It is generally believed that incarceration of ureteral calculi in the ureter for >2 weeks stimulates the urinary epithelium to form polyps. In this study, microscopic examination revealed epithelial cell proliferation in the observation group along with the presence of crystalline components in the intercellular space and inflammatory cell infiltration in the intercellular space. On ureteroscopic examination, ureteral calculi were found closely adhered to the polyps, which is liable to further aggravate the degree of ureteral obstruction, resulting in increased pressure in the renal pelvis. Moreover, prolonged hydronephrosis may induce nephron loss, atrophy of renal parenchyma, and eventual impairment of renal function. Physical stimulation caused by the ureteral calculus induces mucosal edema and epithelial proliferation leading to the development of mucosal polyps. Moreover, we speculated that the chemical constituents of the calcium oxalate calculus can also act on urothelial cells, regulate the expression of specific intracellular functional proteins, and play a unique role in causing adhesion between calculi and polyps, as well as inducing the growth of calculi.

In recent decades, proteomics analyses have demonstrated the association of multiple proteins with urolithiasis. For example, annexin A1, S100A9, uromodulin, and osteopontin were shown to play an important role in the occurrence and development of urolithiasis. These proteins can be expressed in epithelial cells and play a role during various stages of calculus formation. For instance, uromodulin was found to inhibit crystal agglomeration and calculus growth in rat calculus model of COM, while osteopontin expression was detected in the kidney of patients with urolithiasis; both these phenomenon were considered attributable to the interaction between the crystals and cells.[9] Accordingly, we assessed the expressions of these proteins in ureteral polyps. IHC analyses revealed enhanced expressions of annexin A1/S100 A9 in ureteral calculus-adhered polyp tissue. However, no significant between-group differences were observed with respect to the expressions of uromodulin or

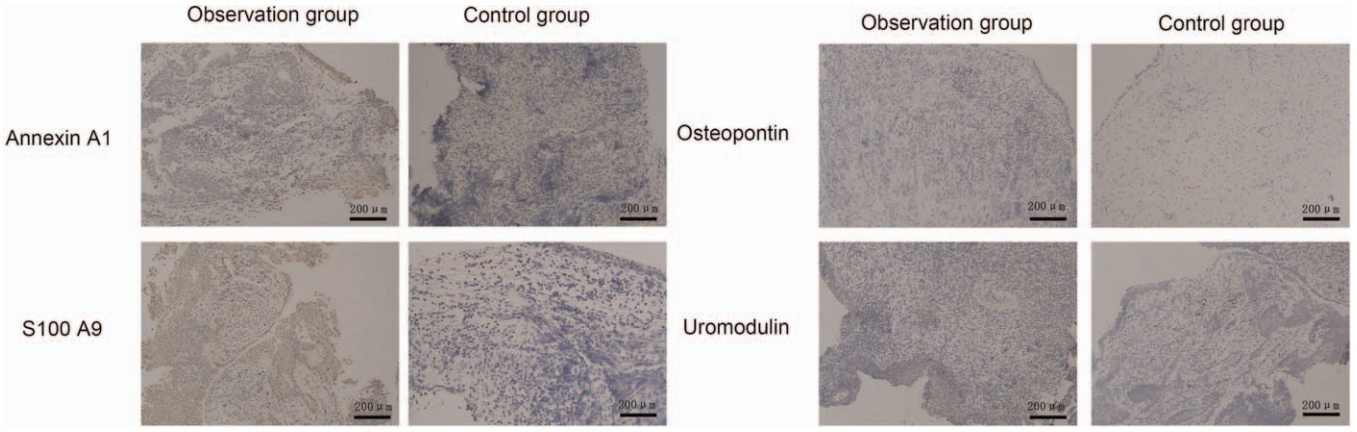

Figure 4. In the observation group, epithelial cells of the polyps showed obvious staining of annexin A1 and S100A9, which was in sharp contrast with interstitial components. In the control group, annexin A1 and S100A9 staining was not obvious, and there was no significant difference from interstitial components. IHC staining of osteopontin and uromodulin showed no significant staining in epithelial cells in either group. IHC = immunohistochemical.

osteopontin. These results suggested a potential role of annexin A1/S100 A9 in the interaction between the calculus and polyp.

Firstly, annexin A1/S100 A9 may be involved in causing adhesion between calculi and polyps. In the present study with matched pairs design, annexin A1/S100 A9 expression was enhanced in ureteral calculus-adhered polyp tissues. Of note, the increased expression of these 2 proteins in this study was not an isolated phenomenon. Both annexin A1 and S100A9 proteins have been found to be involved in the inflammatory signaling pathway in experiments involving skin tumor mouse model, experimental autoimmune encephalomyelitis mouse model, and rat model of septicemia, as indicated by proteomics or bioinformatics analysis. Actually, the proposed inflammatory pathways can promote the proliferation and repair of epithelial

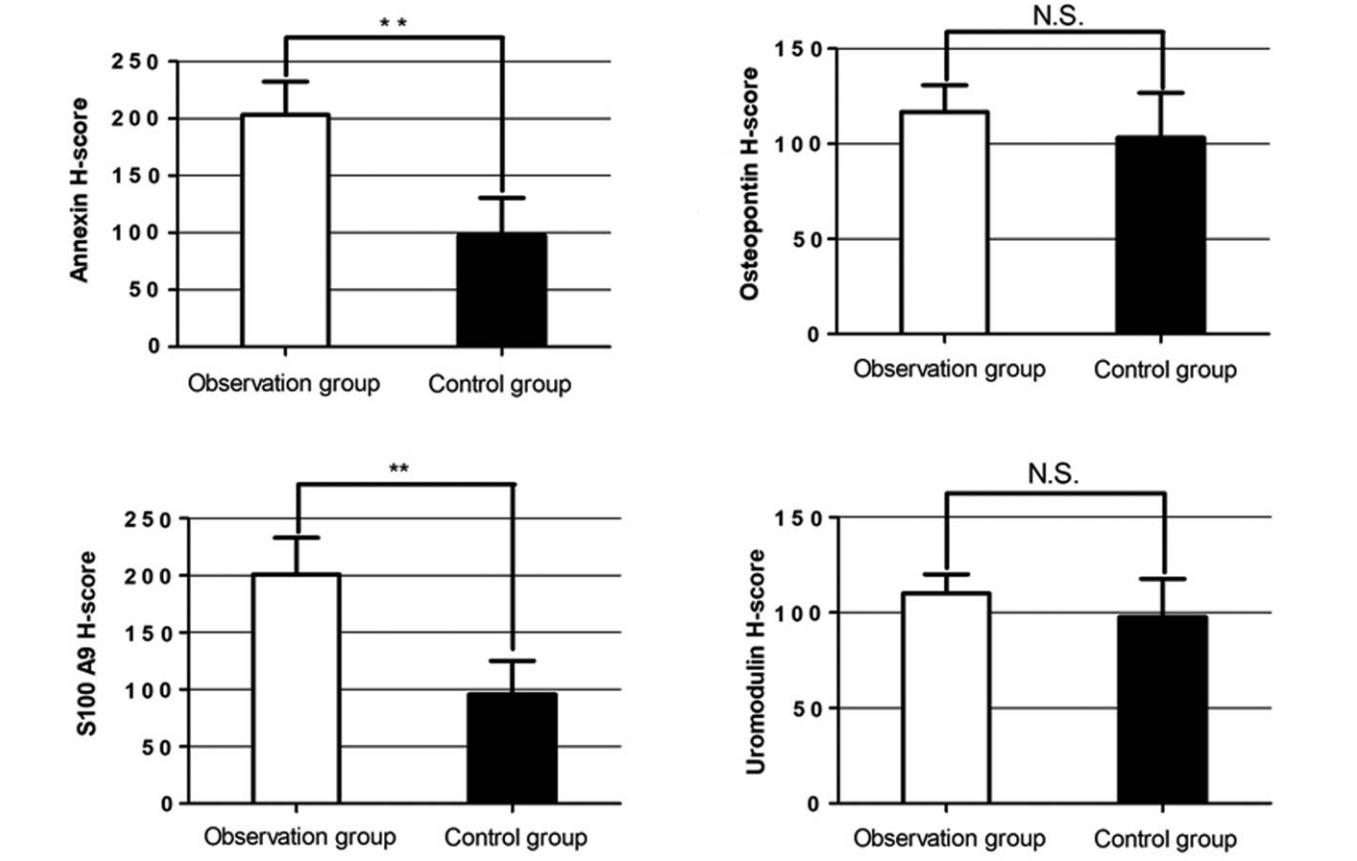

Figure 5. H-scores of annexin A1 and S100A9 in the observation group were significantly higher compared with the control group. However, uromodulin and osteopontin were not significantly expressed on the urothelial mucosa in either group. Values are expressed as mean ± SD; n = 20 in the observation group and n = 20 in the control group, $^{**}P < .01$; N.S. $P > .05$. SD = standard deviation.

cells, among which annexin A1 plays a key role in promoting tumor growth.[10–12] In another research related to nasopharyngeal carcinoma, proteomics analysis of annexin A1 in cancer cells revealed S100A9 as a key protein; further protein–protein interaction analysis revealed that annexin A1/S100 A9 can promote cell proliferation and invasion.[13] In this regard, we speculated that the expressions of annexin A1/S100 A9 would be upregulated in the setting of close contact between calculus and polyp. The 2 proteins may further enhance the invasiveness and proliferation of epithelial cells that wrap the crystals or promote some epithelial cells to enter into the calculus pores, finally leading to the fusion of calculi and polyps. This may be one of the reasons of the adhesion between the calculus and polyp tissue.

In addition, annexin A1/S100 A9 in the polyp tissue may also contribute to the growth of ureteral calculi. Annexin A1 has been shown to be a COM crystal-binding protein. As a receptor of COM crystal, annexin A1 expressed on the renal tubular epithelial cell membrane can promote the adhesion of COM crystals to the surface of renal tubular cells and their gradual deposition on the surface of renal tubule to form calculus.[14–17] In the microenvironment of long-term contact with urothelial mucosa, the ureteral calculi can stimulate the mucosa to form polyps. Annexin A1 that is highly expressed in epithelial cells of polyps can bind COM crystal in the urine to the surface of calculi through its transmembrane domain.[18,19] Alternatively, it can bind oxalic acid and calcium ions in the urine through its target protein S100 A9, which may be realized by EF-hand motifs in the S100 A9 domain.[4,20] In our study, correlation analysis of the H-score of annexin A1/S100 A9 indicated a strong correlation between the expressions of the 2 proteins; this suggested a synergistic relationship between annexin A1 and S100 A9 in binding COM crystal.

We were careful and conservative in our approach when handling ureteral polyps during surgery, and only the polyps associated with ureteral calculi in the following situations were treated: adherent polyps affecting the field of vision during surgery and the lithotripsy and calculi removal were performed only after the partial removal of polyps; polyps closely adhering to the ureteral calculi were completely removed only after the partial removal of the polyps. In addition, as already mentioned in the manuscript, we only removed the raised portion of the inflammatory ureteral polyps to meet the needs of the molecular experiment in this study without damaging the base of the polyps and the neighboring mucosa. All patients in the study underwent ureteroscopy at 1 year of surgery, and none developed ureteral stenosis.

This study had a few limitations. Firstly, the number of patients enrolled was small. Therefore, some observational data still need to be further examined. Secondly, the present study failed to demonstrate the causal relationship between annexin A1 and S100 A9. Despite the supportive findings from previous studies, the regulatory mechanisms mediating the interrelation between protein expressions in ureteral polyps are not well characterized. Thirdly, it is believed that there may be complex mechanism between the calculus and polyps. Therefore, additional calculus-related proteins and adhesion molecules hence need to be included to enhance the effectiveness of our description on the internal mechanism of the interaction between calculi and inflammatory polyps.

Author contributions

Investigation: Mingjie Li, Heng Zhang, Deqi Jiang, Lihua Li, Xia Liu, Xiaoxiao Zhao, Dandan Ye.

Project administration: Mingjie Li, Lihua Li, Xia Liu, Yongliang Ni.

Writing – original draft: Mingjie Li, Heng Zhang, Deqi Jiang.

Writing – review & editing: Yongliang Ni.

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