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Original Article

Aberrant differentiation of urothelial cells in patients with ureteropelvic junction obstruction

Teng Hou[1,2*], Xiong Yang[1*], Bo Hai[1], Bing Li[1], Wencheng Li[1], Feng Pan[1], Min Chen[1], Fuqing Zeng[1], Xiaomin Han[1]

[1]*Department of Urology, Union Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430022, China;* [2]*State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Collaborative Innovation Center for Cancer Medicine, Guangzhou, GD 510060, China.* [*]*Equal contributors.*

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Abstract: Aim: To investigate the urothelial changes in the pathogenesis of ureteropelvic junction obstruction (UPJ-O). Methods: A total of 12 patients of UPJ-O were respectively studied. The expression of Annexin A7, Annexin A11, EGFR, Keratin 5, uroplakin III, and SMA in the urothelium of obstructed UPJ segment and of the normal ureter below the obstructed segment were determined by immunofluorescence. Transmission electron microscopy was used to determine the morphological changes in UPJ epithelium in compared to normal ureteral epithelium. Results: We found that Annexin A7, Annexin A11, EGFR, Keratin 5, and SMA were upregulated, while uroplakin III was downregulated in the urothelium of UPJ-O patients. Furthermore, ultrastructural analyses showed that intercellular spaces between urothelial cells were dilated and the number of microvilli on superficial cells was increased in UPJ-O patients. Conclusions: We propose that a disrupted urothelial barrier in UPJ-O may results in urothelial inflammatory response and truncated differentiated urothelial cells, which may play an important role in the development and pathogenesis of UPJO.

Keywords: Differentiation, proliferation, urothelial cells, UPJO

Introduction

Ureteropelvic junction obstruction (UPJ-O) is a congenital defect of the urinary 8tract that causes a blockage where the ureter and renal pelvis meet. It is the most common urinary tract obstruction in children, occurring in 1/1000 to 1/2000 newborns [1] and could be caused by intrinsic disorganization or extrinsic compression from crossing vessels [2].

The exact pathophysiology of UPJ obstruction is still unknown. Previous studies have implicated that the histological alterations described for UPJ-O are defective innervations [1], increased collagen and elastin [3], local inflammation and fibrosis [4], and the decreased density of C-kit positive interstitial cells of Cajal [5]. It has also been revealed that the underlying mechanism of UPJ obstruction is highly associated with smooth muscle structural derangement. However, abnormality concerning the quantitative amount of smooth muscle in the obstructive segment compared with that in normal UPJ remains controversial. Kajbafzadeh et al. reported that smooth muscle apoptosis index and the content of elastin fibers were significantly increased at the site of ureteropelvic junction obstruction [6]. Murakumo et al. also reported the atrophy of muscle fibers and an increase of collagen fibers in the muscle layers of obstructed UPJs [7]. In contrast to these findings, Starr et al. indicated a increased proportion of smooth muscle cells in the stenotic portion [8].

Although histological studies of UPJ obstruction mostly focus on the changes in the intermuscular and intramuscular connective tissue, atypical changes in the urothelium were frequently observed in the obstructed segment in UPJ-O patients. Tadros et al. observed cytokine alterations in the hyperplastic urothelial cells of UPJ-O samples [9]. Chiou et al. reported infiltration of urothelial cells, as well as urothelial hyperplasia in UPJ-O segment [10]. Ruiz-Deya et al. suggested that NF-kB may participate in inflammatory responses in UPJ obstruction [4]. Takeyama et al. detected irregular mucosal folds characterized by fibroepithelial polyps projected into the lumen in the stenotic segment [11]. However, urothelial inflammatory in the pathogenesis of UPJ-O has not been well addressed. In addition,

Table 1. Clinical and immunofluorescence features of the uretopelvic junction obstruction (UPJO) patients enrolled in the study

Patient NO./Sex	Age (ms)	ANX7 U/N	ANX11 U/N	EGFR U/N	KRT5 U/N	UPKIII U/N
1/M	6	+/-	+/-	++/+	++/+	-/++
2/M	13	++/-	+/-	++/++	++/+	++/++
3/M	28	++/-	++/-	++/+	++/+	-/++
4/F	10	+/-	+/+	++/-	++/+	+/++
5/M	7	-/-	++/+	++/-	+/+	+/++
6/M	24	++/+	+/+	+/+	++/+	-/++
7/M	15	+/-	++/-	++/+	++/+	+/++
8/M	35	++/+	++/-	+/-	+/+	++/++
9/F	18	+/-	++/-	++/+	++/+	-/++
10/M	82	+/-	+/+	++/-	++/+	-/++
11/M	15	+/-	++/-	++/+	++/+	-/++
12/M	4	++/-	+/-	+/+	++/+	+/++

ANX7: annexin a7, ANX11: annexin a11, KRT5: keratin 5, UPKIII: uroplakin III; For ANX7, ANX11, EGFR, UPKIII: ++, most superficial cells labelled; +, about half of superficial cells labelled; –, no labelled cells; for KRT5: ++, most basal cells labelled; +, about half of basal cells labelled; –, no labelled cells.

whether the urothelial cells in the obstructive segment undergo abnormal differentiation has not been documented.

In the present study, we examined the inflammatory and differentiative changes in the urothelium of obstructed UPJs. We observed a truncated differentiation of the urothelial cells in the obstructive segments by uroplakin immunostaining and electron microscopy. We therefore hypothesized that the aberrant differentiation of urothelial cells might contribute to urothelial inflammatory responses by positive feedback loops in UPJ-O. Our study may shed light on the pathogenesis of ureteropelvic junction obstruction.

Materials and methods

Patients and tissue specimens

This study was conducted on a total of 12 paraffin-embedded UPJ-O samples, which were collected immediately after surgical resection at Wuhan Union Hospital from 2012 to 2013. Samples used in this study were approved by the committes for ethical review of research involving human subjects at Wuhan Union Hospital. Clinical information of the samples is summarized in **Table 1**. The 12 patients included 10 males and 2 females from 4 to 82 months (mean, 21.4 months). Preoperative urine cultures samples were collected to confirm the absence of UTIs. The UPJ tissues from all patients were divided into two parts: the obstructed UPJ, and the normal ureter below the obstructed segment as a control.

Immunofluorescence

Immunofluorescence analysis was performed to study altered protein expression in 12 paraffin-embedded UPJ-O samples. The procedures were carried out according to manufacturer's instructions. In brief, paraffin-embedded specimens were cut into 4 lm sections and baked at 65 C for 30 min. The sections were deparaffinized with xylenes and rehydrated. Sections were submerged into ethylenediaminetetraacetic acid antigenic retrieval buffer and microwaved for antigenic retrieval. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% fish skin gelatin to block the non-specific binding. Tissue sections were incubated overnight with polyclonal rabbit antibody against EGFR (Santa Cruz Biotechnology, USA; 1:100), polyclonal goat antibody against Annexin 7 (Santa Cruz Biotechnology, USA; 1:50), polyclonal goat antibody against Annexin 11 (Santa Cruz Biotechnology, USA; 1:50), polyclonal rabbit antibody against Keratin 5 (Convance, USA; 1:500), monoclonal rabbit antibody against uroplakin III (Abcam, USA; 1:200), monoclonal mouse antibody against SMA (Abcam, USA; 1:200). After washing, sections were incubated for 30 min at room temperature with fluorochrome-coupled secondary antibodies (Alexa Fluor, 1:200). Finally, washed slides were coverslipped with DAPI (Invitrogen).

Transmission electron microscopy

For transmission electron microscopy, ureter samples were cut into small pieces (<1 mm^2), fixed with 2.5% glutaraldehyde in 0.1 M sodium caco-dylate buffer, pH 7.4, post-fixed with 2% (wt/vol) osmium tetroxide, and embedded in Epon 812 (Polysciences, Inc.).

Results

Immunofluorescence features

To investigate the differentiation status of the urothelial cells in UPJ-O patients, immunofluorescence was performed in 12 UPJ-O samples.

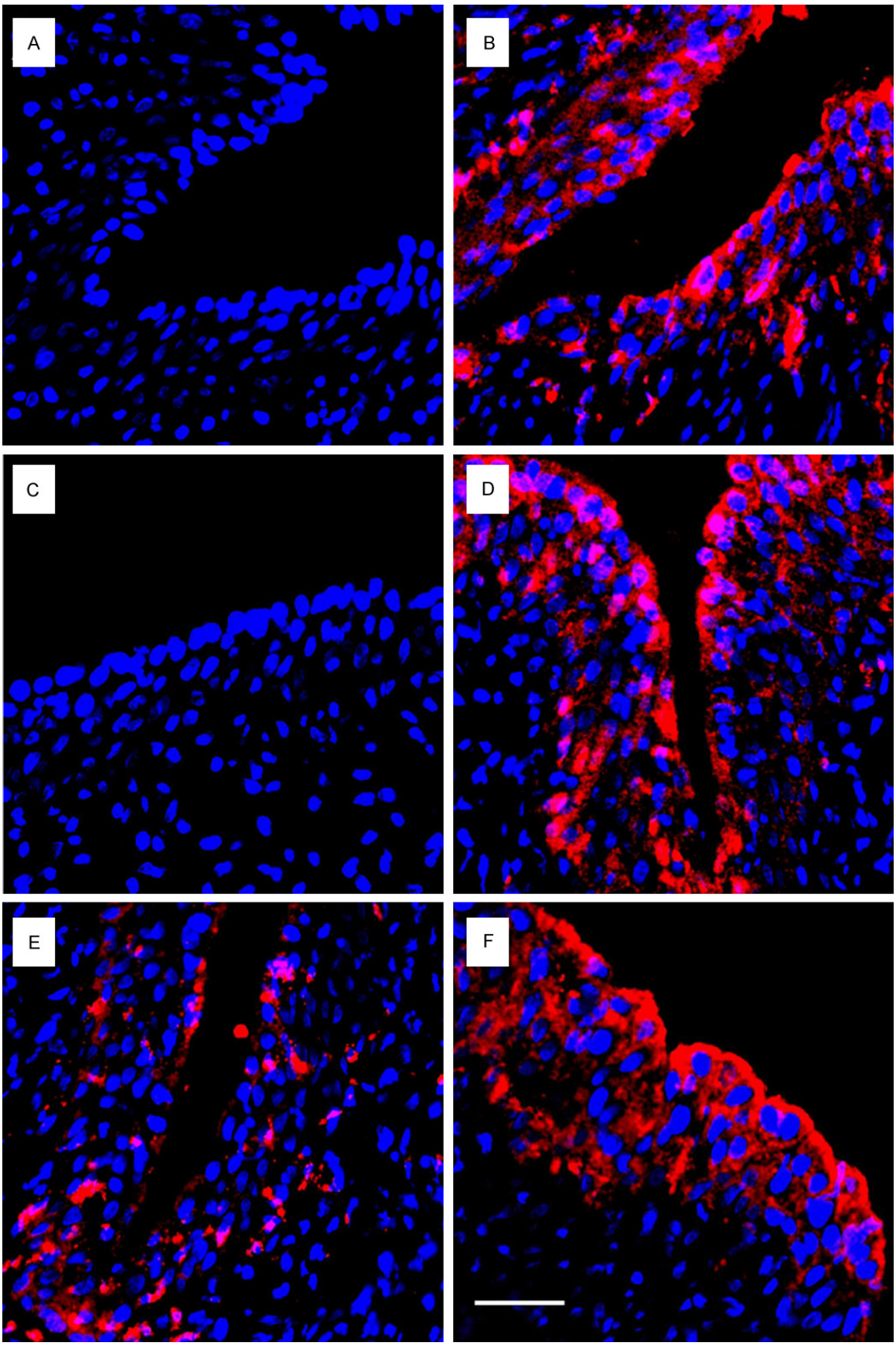
A
B
C
D
E
F

Figure 1. Representative images of Annexin 7 (A, B), Annexin 11 (C, D), and EGFR (E, F) from immunohistochemistry assays in the urothelium of obstructed UPJ segment (A, C, E) and of the normal ureter below the obstructed segment (B, D, F). Bar, 50 um.

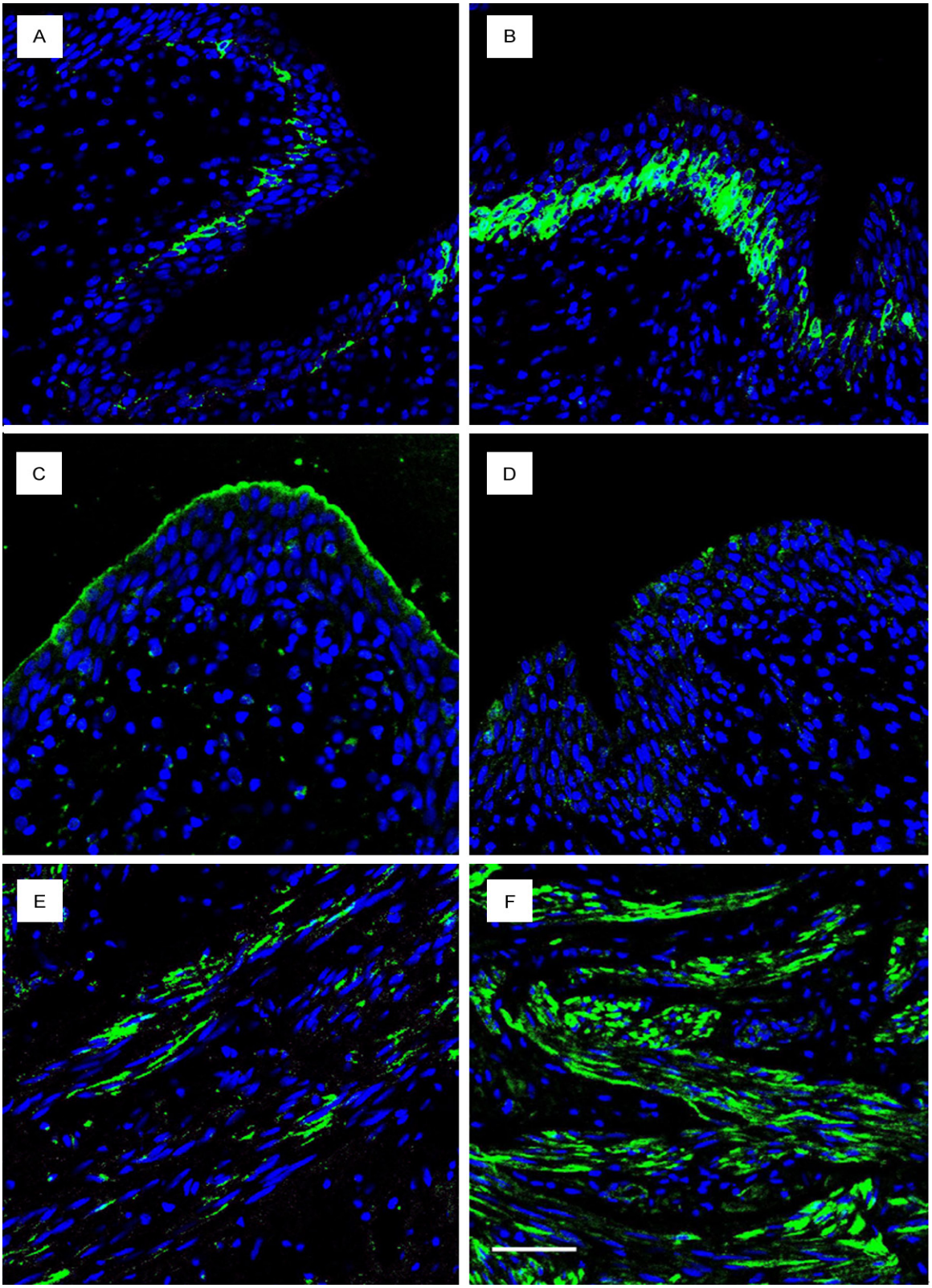

Figure 2. Representative images of KRT5 (A, B), uroplakin III (C, D), and SMA (E, F) from immunohistochemistry assays in the urothelium of obstructed UPJ segment (A, C, E) and of the normal ureter below the obstructed segment (B, D, F). Bar, 50 um.

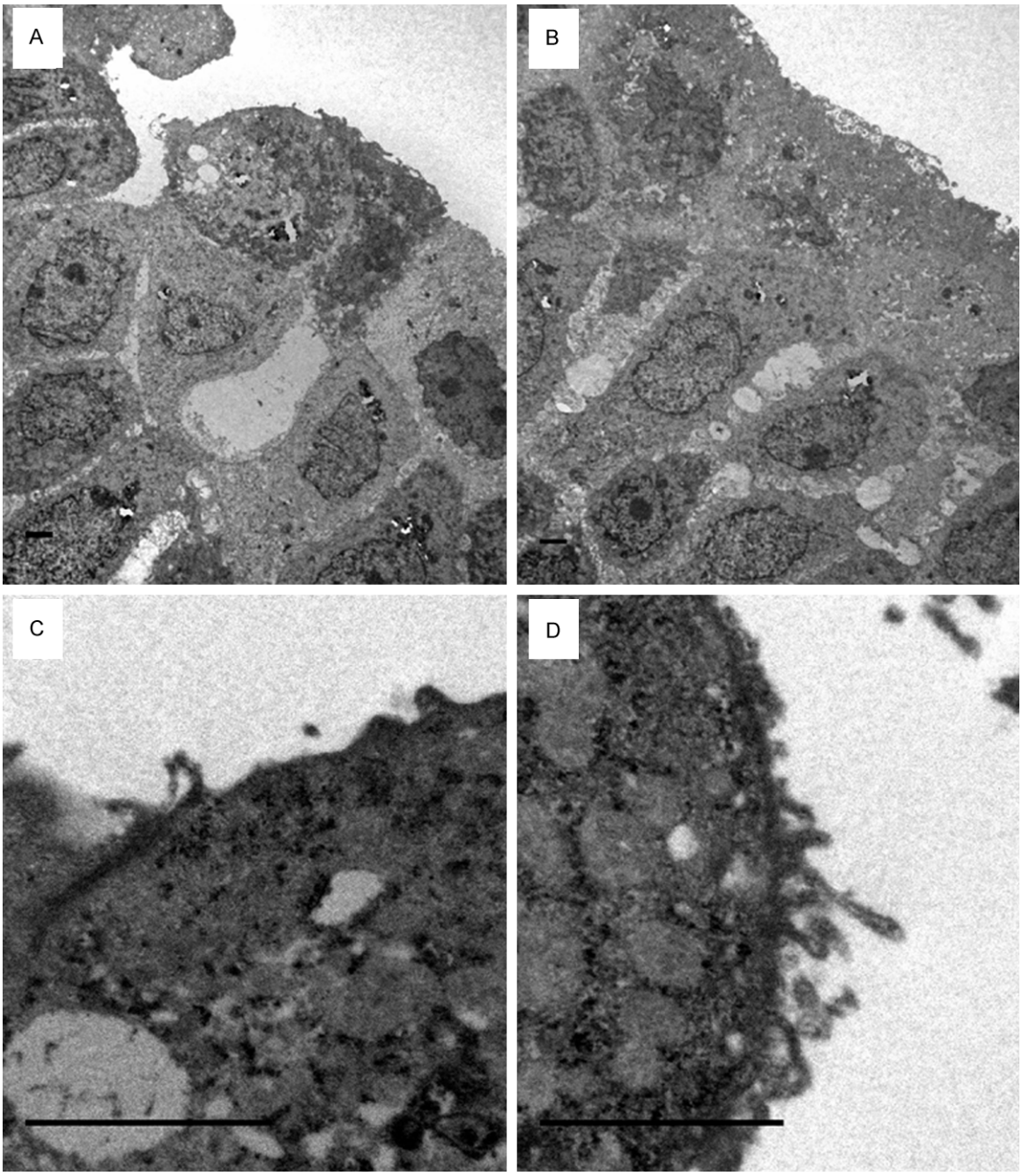

Figure 3. Ultrastructure of urinary epithelium from patients with UPJ-O. The intercellular spaces between urothelial cells were dilated in the urothelium of obstructed UPJ segment (B) in compared with normal ureteral epithelium (A). Microvillis were frequently observed in superficial cells of obstructed UPJ epithelium (D) in compared with normal ureteral epithelium (C). Bar, 2 um.

The immunofluorescence features of UPJ-O patients are shown in **Table 1**. The representative immunostaining of UPJ-O was shown in **Figures 1**, **2**. The expression of Annexin 7, Annexin 11, and EGFR were significantly increased in the urothelium of UPJ segment

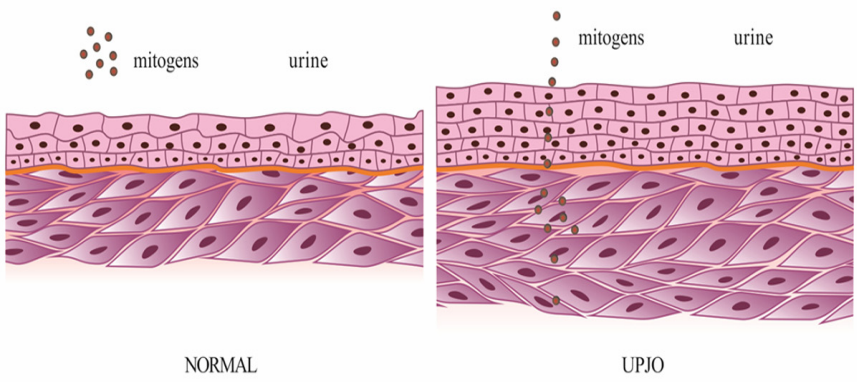

Figure 4. Proposed model. urothelial inflammatory may have critical roles in the development of UPJ-O. When the superficial cells of ureteral epithelium are incompletely differentiated, the urothelial barrier is impaired, and mitogens in the urine penetrate into the urothelial basal layer to stimulate epithelial and mesenchymal proliferation.

compared with that of the paired normal ureter (**Figure 1A-F**). Keratin 5 was strongly expressed in the basal and intermediate layer of UPJ epithelium, while it was diffusely expressed in the basal layer of normal ureteral epithelium (**Figure 2A**, **2B**). The superficial urothelial cells or normal ureter showed uroplakin staining, whereas loss of expression was observed in UPJ urothelium (**Figure 2C**, **2D**). Expression of SMA in mesenchymal layers of ureter was oberserved to increase in UPJ segment in compared to normal ureter (**Figure 2E**, **2F**).

Abnormal urothelial apical surface in UPJ-O ureter

The ureteral epithelium of patients with UPJ-O frequently showed dilatations of intercellular spaces that connected the neighbouring cells (**Figure 3A**, **3B**). Moreover, the UPJ-O patients showed numerous relatively undifferentiated superficial cells covered with microvilli, which were not frequently observed in normal terminally differentiated cells in the ureter below the obstructed segment (**Figure 3C**, **3D**).

Discussion

At present, the role of urothelial inflammatry in the development of UPJ-O is unclear. In this study, we report that Annexin A7 (ANX7), Annexin A11 (ANX11), EGFR, Keratin 5 (KRT5), and smooth muscle antigen (SMA) were overexpressed, while the expression of uroplakin III was decreased in the urothelium of UPJ-O patients. Moreover, ultrastructural analyses showed dilated intercellular spaces and enriched microvilli in the urothelial cells of UPJ-O patients, which are rarely present in (control) normal urothelium. These findings may indicate a mechanism by which the aberrant barrier function of urothelium can contribute to the urothelial inflammatory, and in turn to the development of ureteropelvic junction obstruction.

There is growing evidence to suggest that inflammation is an essential factor in epithelial hyperproliferation [12], and the role of pathogen or chemical induced inflammation in the pathogenesis of urothelial hyperplasia has been accepted. It was observed that bladder epithelial inflammation was induced by repeated instillation of Escherichia coli (E. coli), which was followed by urothelial hyperplasia [13]. Similarly, E. coli was administrated intravenously to induce hydronephrosis and urothelial hyperplasia [14]. In addition, oral administration of 4-ethyl sulfonyl naphthalene-1-sulfonamide, acetazolamide and oxamide caused epithelial regeneration and a reversible hyperplasia of the transitional epithelium, which was subsequent to urothelial inflammation [15].

Studies regarding urothelial inflammation mostly focus on eosinophil infiltration and eosinophil-associated cytokines and chemokine [10]. In this study, we described an increased expression of epidermal growth factor receptor (EGFR) in the urothelium of UPJ-O compared with that of poststenotic region. EGFR is a 170-kDa membrane glycoprotein expressed on the surface of many cells. It is known to play roles in proliferation and differentiation of epithelial cells [16]. Previous findings have suggested that EGFR upregulation may play important roles in epithelial inflammation [17]. EGFR can be activated by the binding of specific ligands, which may be derived from inflammatory cells such as eosinophils and neutrophils. Our study described an increased EGFR expression in UPJ-O urothelium, which was in consistent with the previous studies.

Although several EGFR ligands have been found to be increased in UPJ-O, and the increase has been thought to be responsible for the pathogenesis of UPJ-O [18], the exact role and importance of EGFR and its ligands in modulating the urothelial inflammation need to be explored.

Annexins are a unique class of proteins that provide a link between $Ca2^{+}$ signaling and membrane functions and that have pivotal roles in the regulation of many cellular processes in all eukaryotic organisms. Annexin A7, the first annexin to be described, was described in regulation of cell growth and differentiation. The ANX7 gene acts as a tumor suppressor. Expression studies demonstrated that ANX7 is highly correlated with late stage of prostate cancer and with poor cellular differentiation of gastric cancer [19]. Whether ANX7 is involved in the pathogenesis of the urothelial inflammatory process need further investigation.

The calcium-dependent phospholipid-binding protein annexin ANX11 is expressed in a wide range of tissues. Anti-ANX11 antibodies have been found in association with a number of chronic inflammatory diseases including Sjogren syndrome, polymyositis, and rheumatoid arthritis [20]. A recent study has implicated annexin ANX11 as a highly associated susceptibility locus for sarcoidosis, which is a systemic immune disorder characterized by abnormal collections of chronic inflammatory cells form as nodules in various organs [21]. In addition, ANX11 is likely to play an important role in the regulation of cell apoptosis, proliferation and differentiation [22]. We have demonstrated a significant increase in ANX7 and ANX11 expression in the urothelium of UPJ-O segment, which indicates their involvement in the pathogenesis of UPJ-O. In addition, Annexin A1 and A6 are thought to be important regulators of EGFR signaling pathway in the regulation of critical physiological processes including proliferation, differentiation, inflammation and cell migration [23]. It would be interesting to investigate whether ANX7 and ANX11 participate in the crosstalk with the EGFR signaling pathway in the future.

The intermediate filament protein KRT5 is a basal cell marker of keratinocytes. It is expressed in mitotically active keratinocytes of all stratified squamous epithelium. Normally it is expressed at a low level in normal urothelium, while it is upregulated in hyperplastic urothelium [24]. We observed an appreciable increase of KRT5 expression in both basal and intermediate layers of urothelium in UPJ-O segments, which indicates that the urothelium of UPJ-O segments exhibited substantial hyperplasia. In the light of the above information, it's possible that urothelial hyperproliferation in the UPJ-O segment may arise from urotheial inflammatory reactions regulated by interacting proteins such as annexins and EGFR ligands.

The major function of urothelium is to provide an impermeable barrier, which is maintained in part by terminally differentiated umbrella cells in the outermost layer. No relationship between the pathogenesis of the obstructed portion in UPJ-O and urothelial differentiation has been shown in the literature previously. In this study, we found that patients with UPJ-O had only a few of their superficial urothelial cells stained positively with uroplakins antibodies, suggesting a significant defect in urothelial differentiation. Moreover, we found that increased intercellular spaces and microvilli-enriched superficial cells in UPJ-O epithelium, indicating a defective urothelial barrier. Our study was supported by Romih et al., who proposed that the exposure of undifferentiated cells to the luminal surface may contribute to defective urothelial permeability [25]. It is possible that the high concentrations of EGF-related mitogens in the urine penetrate into the urothelial basal layer to stimulate cell proliferation [26]. Besides, the fully assembled urothelial plaques that are known to trigger certain growth-inhibiting signals are now absent in the uroplakin-deficient urothelium, which may lead to urothelial hyperplasia as well. Thus, we speculate that in patients with UPJ-O the barrier is disrupted, followed by a inflammatory response resulting in urothelial hyperproliferation and incompletely differentiated superficial cells, which amplify the urothelial inflammatory response in a positive feedback loop (**Figure 4**).

Although urothelial layers are seperated from stroma by basement membrane which directly contact them, urothelial dynamics are strictly connected to the underlying stromal phenomena. Early study showed that urotheial-mesenchymal interactions are necessary in the development of bladder [27]. It has also been suggested that urotheial-mesenchymal interactions may be important in ureteric bud morpho-

genesis and collagen synthesis, as well as in smooth muscle proliferation [28]. Urothelium-derived factors or signals were presumed to play vital roles in mediating detrusor smooth muscle contractility [29], and in promoting mesenchymal proliferation and smooth muscle differentiation [2]. In contrast, urothelial proliferation and differentiation might also be regulated by stromal cells-derived factors [30]. In the present study, we also observed that SMA was overexpressed in UPJ-O patients. Taken together, we speculate that the urothelial-mesenchymal interactions become active and trigger stromal remodeling and smooth muscle proliferation in the pathogenesis of UPJ-O.

In conclusion, we describe a previously unreported association of abnormal differentiation of urothelial cells and UPJ-O. Further studies are necessary to define the biological processes and the molecular mechanisms responsible for this pathology. Our finding may shed lights on a better understanding of the pathology of UPJ-O.

Disclosure of conflict of interest

None.

Address correspondence to: Xiaomin Han, Department of Urology, Union Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430022, China. Tel: +86 27-8575-1625; Fax: +86 27-8577-6343; E-mail: hanminmin@126.com

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