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Expression of Acid-Sensing Ion Channels in Renal Tubular Epithelial Cells and Their Role in Patients with Henoch-Schönlein Purpura Nephritis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ADEFG 1 **Li-ping Yuan**
BC 2 **Yan Bo**
A 1 **Zhang Qin**
BF 1 **Hua Ran**
F 1 **Wang Li**
F 1 **Yu-fei Li**
A 1 **Gui Ming**

1 Department of Pediatrics, First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, P.R. China
2 Department of Medical Technology, Anhui Medical College, Hefei, Anhui, P.R. China

Corresponding Author: Li-ping Yuan, e-mail: yuanliping3986@sina.com

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Background: Acid-sensing ion channels (ASICs) are ligand-gated cation channels activated by extracellular protons. However, the role of ASICs in kidney diseases remains uncertain. This study investigated ASICs expression in kidney tissues and their role in the development of Henoch-Schönlein purpura nephritis (HSPN).

Material/Methods: The expression of ASIC subunits was examined by immunochemical techniques in the kidney tissue from HSPN patients. Acid-induced ASICs expression in cultured renal tubular epithelial cells was determined by quantitative RT-PCR analysis. The expression of K7 and K18 protein in renal tubular epithelial cells was used to evaluate acid-induced cell injury. In addition, we observed the effect of blocking ASICs on acid-induced cell injury to assess the role of ASICs in renal tubular epithelial cell injury.

Results: The results showed that ASIC1, ASIC2, and ASIC3 proteins were obviously expressed in renal tubular cells from HSPN patients. ASIC1 expression and 24-h urine protein level were higher in the pathological grade ISKD III group than in the ISKD II group. ASIC1, ASIC2, and ASIC3 mRNA, and K7 and K18 protein expression in cultured renal tubular epithelial cells were increased when exposed to pH 6.5. K7 and K18 protein expression was closely related to ASIC1 expression, and ASICs blockers reduced K7 and K18 protein expression in tubular epithelial cells.

Conclusions: These findings suggest ASICs are most highly expressed in renal tubular cells of HSPN patients, which is closely related to renal tubular injury. ASICs might be involved in the development of HSPN.

MeSH Keywords: **Acid Sensing Ion Channels • Acidosis, Renal Tubular • Purpura, Schoenlein-Henoch**

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Background

Henoch-Schönlein purpura (HSP) is the most common systemic vasculitis in children and involves inflammation of the small vessels of the skin, joints, gut, and kidney [1]. Renal involvement, known as Henoch-Schönlein purpura nephritis (HSPN), which most commonly presents with hematuria and/or proteinuria, is the most serious complication of HSP and often determines the prognosis of HSP. It has been reported that approximately 30–50% of children with HSP develop nephritis within 4 to 6 weeks after disease onset [2]. The extent of renal injury is important in evaluating HSPN prognosis and early individualized therapeutic strategies [3,4]. The pathological findings of HSPN include mesangial cell proliferation, crescent formation in epithelial cells, and mesangial IgA deposition [5]. However, the exact pathogenesis of HSPN is unknown. In the present study, we found high ASIC1a and ASIC3 expression in the vascular endothelium of HSP patients [6].

Acid-sensing ion channels (ASICs), which are cationic channels that belong to the degenerin/epithelial Na⁺ channel (DEG/ENaC) superfamily, are activated by extracellular acidosis [7]. Tissue acidosis associated with ischemia is caused by an accumulation of lactate and local or systemic lactic acidosis, which is the common feature of the diseases, such as tumors, ischemic injury, and inflammation [8–10]. Renal tubular acidosis due to ischemic tubular injury has been observed in kidney transplant recipients [11], but whether ASICs might be involved in the development of HSPN has not been reported. The present study is the first to investigate ASICs expression in the kidneys of HSPN patients and to explore the potential function of ASICs involved in the development of HSPN.

Material and Methods

Patients

We screened 13 HSPN patients (mean age 20.08±6.96 years, range 8-29 years) undergoing renal biopsy at the First Affiliated Hospital of Anhui Medical University between January 2011 and June 2015. The patients were enrolled when they were first diagnosed with HSPN at presentation. We also recruited 7 patients with minimal change nephrotic syndrome (mean age 25.27±3.58 years, range 10–31 years) as normal controls when the pathological changes in their renal biopsies were normal. HSPN was diagnosed according to the criteria recommended by the Nephrology Group of the Chinese Medical Society in November 2000 [12]. The diagnosis of HSP was based on the criteria defined by the European League against Rheumatism, the Pediatric Rheumatology International Trials Organization, and the Pediatric Rheumatology European Society (UULAR/PRINTO/PRES) in 2010 [13]. Informed consents were obtained

and the study was approved by hospital Institutional Review Board (Quick-PJ 2016-5-12).

Immunohistochemical analysis

The ASIC1, ASIC2, and ASIC3 protein levels in kidney tissue of HSPN patients were examined using immunohistochemical staining. The kidney tissue was fixed and embedded in paraffin blocks. Slides of the tissues were routinely prepared and the staining was performed according to a streptavidin-biotin complex (SABC) immunohistochemical assay (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Based on the SABC staining technique, brownish-yellow granules in the cytoplasm indicated positive cells. We also performed an SABC immunohistochemical assay using rabbit anti-human ASIC1, ASIC2 and ASIC3 antibodies (Santa Cruz Biotechnology, Inc) as the primary antibodies. Brownish-yellow granules in cytoplasm indicated positive cells.

Results were analyzed using the percentage of the positive area of ASIC1, ASIC2, and ASIC3 expression.

Clinic manifestations and pathological grading of kidney tissue in HSP patients

Clinical classification in HSPN patients included 13 patients with hematuria and proteinuria, 5 with acute nephritis, and 8 with chronic nephritis.

Tissues from the renal biopsy were examined by light microscopy, glomerular lesions were classified using International Society for Kidney Disease Community (ISKDC) guidelines, and HSPN was classified into levels I–VI. Pathological grading of kidney tissue showed 7 patients with ISKDC and 6 with ISKDC .

Human renal tubular epithelial cell culture

Human renal tubular epithelial cells were supplied by MDL Biotech Co., Ltd (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). Cells were treated with different pH levels (pH 7.4, pH 7.0, pH 6.5, pH 6.0, pH 5.5) solution for 3 h. After treatment, the cells were kept in normal media for about 4 h and then collected for the analysis of ASIC1, ASIC2, and ASIC3 mRNA expression and K7 and K18 protein expression. Subsequently, in another set of experiments, the cells were exposed to solutions with pH 7.4, pH 6.0, and pH 6.0+Amiloride (100, 50, and 25 μmol/L) for 3 h and used to assess K7 and K18 protein expression.

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR analysis was performed to analyze ASIC1, ASIC2, and ASIC3 mRNA expression. Total RNA was extracted

Table 1. Primer sequences used for real-time quantitative PCR.

Symbol	Primer F	Primer R
ASIC1	CGAAGCAGGCATCAAAGT	AATCCAATCCGAGTCCAT
ASIC2	GGGTTCCAGACCTTTGTG	CAATCTACAGGCGGTGA
ASIC3	GGGCGATTGCAGTTCAGC	GAGCCACGTAGCGGGTTT
β-actin	GACAGGATGCAGAAGGAGATTACT	TGATCCACATCTGCTGGAAGGT

using Trizol reagent. cDNA was produced using the GeneAmp RNA PCR kit. Gene expression was performed using the one-step RT-PCR kit with SYBR green in the ABI 7900 qPCR detection system. PCR reactions were performed with reverse transcription at 50°C for 15 min, denaturation and reverse transcriptase inactivation at 95°C for 2 min, followed by 40 cycles (20 s each) of denaturation at 94°C, and annealing and extension at 65°C for 20 s. Melt curve analysis was performed to confirm the specificity of PCR products. PCR amplification data of each gene were normalized to Ct value of internal housekeeping gene (β-actin) from the same sample and the fold-changes in gene expression were calculated using the delta-delta Ct method. A list of forward and reverse primer sequences used for qRT-PCR analysis is given in Table 1.

Western blot analysis

The treated cells were harvested, and total cellular protein lysates were prepared using RIPA lysis buffer. Protein concentration was determined using the Bradford method. Protein samples (50 μg) were subjected to SDS-PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk in TBST for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies: Keratins K7 (1: 500) and Keratins18 (1: 500). Lastly, the membranes were incubated with an HRP-conjugated secondary antibody (1: 1000) for 60 min at 37°C, which were then detected by an ECL luminescent detection system. The intensity of protein bands was analyzed using Image J software and normalized to expression of β-actin.

Statistical analysis

Values are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS software. Student's t-test was used to analyze the statistical significance in the differences between 2 groups. The relationship of ASIC expression with keratins7 and keratins19 expression was analyzed using Pearson correlations. For all comparisons, a P-value less than 0.05 were considered statistically significant.

Results

ASICs expression in the kidneys of HSPN patients

Immunohistochemical analysis of sections revealed positive ASICs subunits expression in kidneys of HSPN patients. The ASIC1, ASIC2 and ASIC3 expression, as brown or yellow granules, was clearly shown in the cell cytoplasm of renal tubular epithelial cells from HSPN patients (Figure 1A, 1C, 1E), whereas ASIC1, ASIC2, and ASIC3 expression was rarely observed in normal kidneys (Figure 1B, 1D, 1F). The image analysis of the positive area of ASIC1, ASIC2, and ASIC3 using semiquantitative methods confirmed that the protein expressions of ASIC2 and ASIC3 were significantly different between the normal controls and patients with HSPN (Table 2).

Furthermore, we found that ASIC1 expression and 24-h urine protein level were higher in the pathological grades ISKD III group than that in the ISKD II group (Table 3).

Effect of extracellular acidosis on ASICs expression in renal tubular epithelial cells

To observe the effect of pH on ASICs expression in renal tubular epithelial cells, we measured ASIC1, ASIC2, and ASIC3 expression with real-time quantitative polymerase chain reaction. Figure 2 shows that ASIC1, ASIC2, and ASIC3 expression was significantly increased at lower extracellular pH, and the change in ASIC1 expression was most notable with pH fluctuations *in vitro*. When extracellular pH only drops from 7.4 to 7.0, ASIC1 expression had already reached the maximum level.

Effect of extracellular acidosis on K7 and K18 protein expression in renal tubular epithelial cells and correlation with ASIC1 expression

To observe the effect of different pH on renal tubular epithelial cell injury, we investigate the expression of keratins, which are sensitive markers of renal tubular cell stress. We found K7 and K18 protein expression was obviously upregulated at lower pH. Moreover, extracellular pH transients (varying from 7.4 to 7.0) *in vitro* significantly stimulated K7 and K18 protein expression, as shown in Figure 3. In addition, K7 and K18 protein

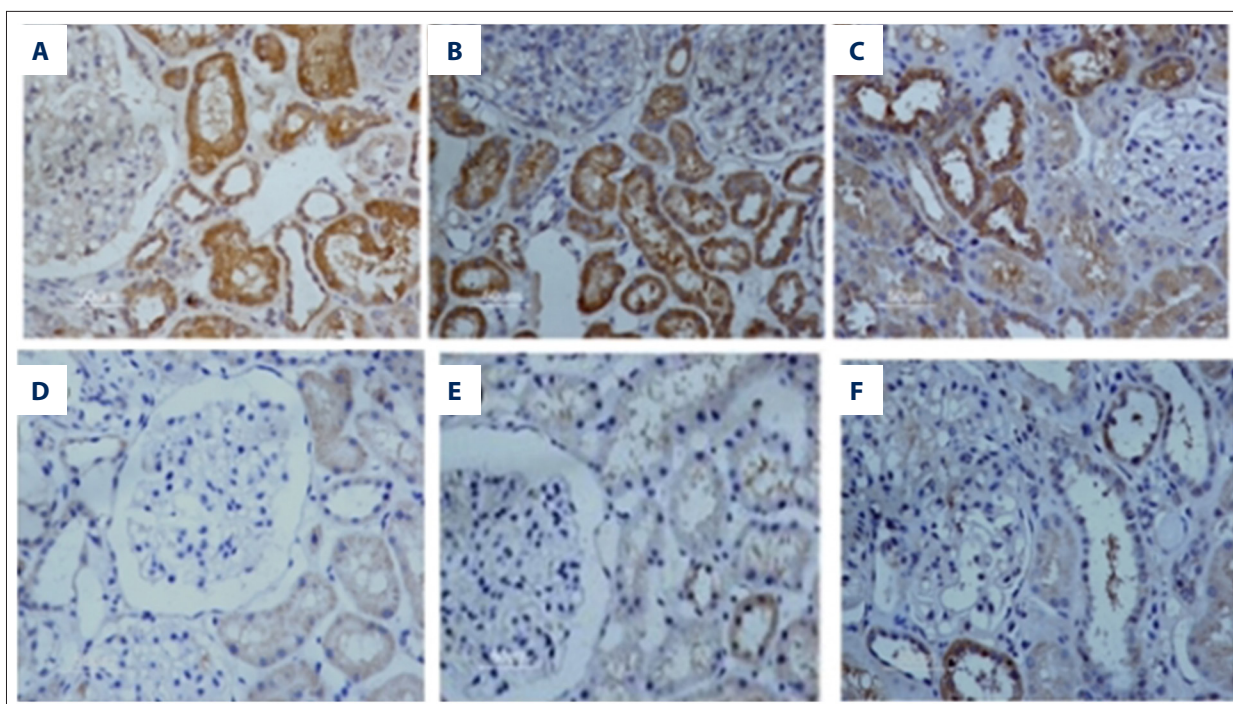


Figure 1. Expression of acid-sensing ion channel (ASIC) 1, ASIC2, and ASIC3 in kidney tissue from HSPN patients examined by immunohistochemical analysis (SP×400).

Table 2. ASICs expression in kidney tissue from different groups ($\bar{x}\pm s$).

Groups	n	Positive area (%)		
		ASIC1	ASIC2	ASIC3
Normal	7	10.28±0.05	23.03±0.07	14.33±0.04
HSPN	13	73.24±16.24*	61.45±13.82*	65.81±11.58*
t		10.41	7.46	11.94
P		<0.01	<0.01	<0.01

* P<0.01, compared with the normal control group.

Table 3. ASICs expression in kidney tissues of different pathological grading and 24h urine protein in HSPN patients ($\bar{x}\pm s$).

Group	Pathological grading	n	Positive area (%)			24 h urine protein
			ASIC1	ASIC2	ASIC3	
HSPN	ISKD II	7	48.61±18.02	53.37±12.36	63.31±10.51	0.590±0.228
	ISKD III	6	97.63±14.21*	69.51±18.39	70.56±11.13	1.848±0.260*
t			5.37	1.88	0.67	9.30
P			<0.01	>0.05	>0.05	<0.01

* P<0.01, compared with the ISKD II group.

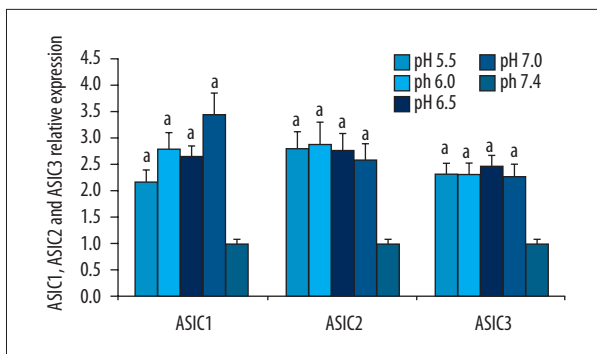


Figure 2. Effect of extracellular acidosis on ASICs expression in renal tubular epithelial cells (n=10). ASIC1, ASIC2 and ASIC3 mRNA expression in renal tubular epithelial cells under different pH concentrations was measured with gRT-PCR. The fold-changes in gene expression were calculated by using the delta-delta Ct method. The error bars represent the standard deviation of the mean (\pm SD). Symbol 'a' indicated statistically significant ($p < 0.01$) change, when compared to pH 7.4 group.

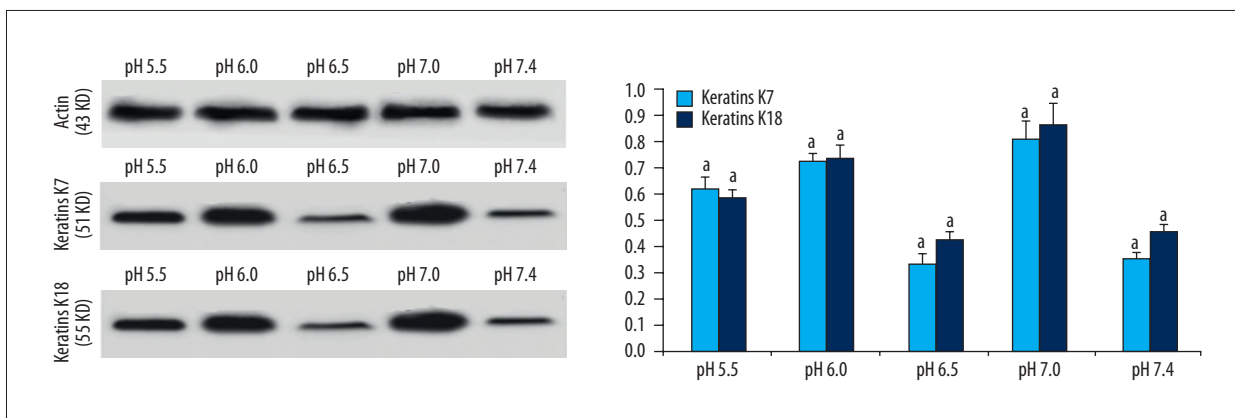


Figure 3. Keratins K7 and K18 protein expression in renal tubular epithelial cells under different pH concentrations (n=10). Keratins K7 and K18 protein expression in renal tubular epithelial cells under different pH concentration was measured with Western blotting method. Signal intensity of bands was measured (in triplicates) by Image J software and each value was normalized to β -actin signal intensity. The error bars represent the standard deviation of the mean (\pm SD). Symbol 'a' indicated statistically significant ($p < 0.01$) change, when compared to pH 7.4 group.

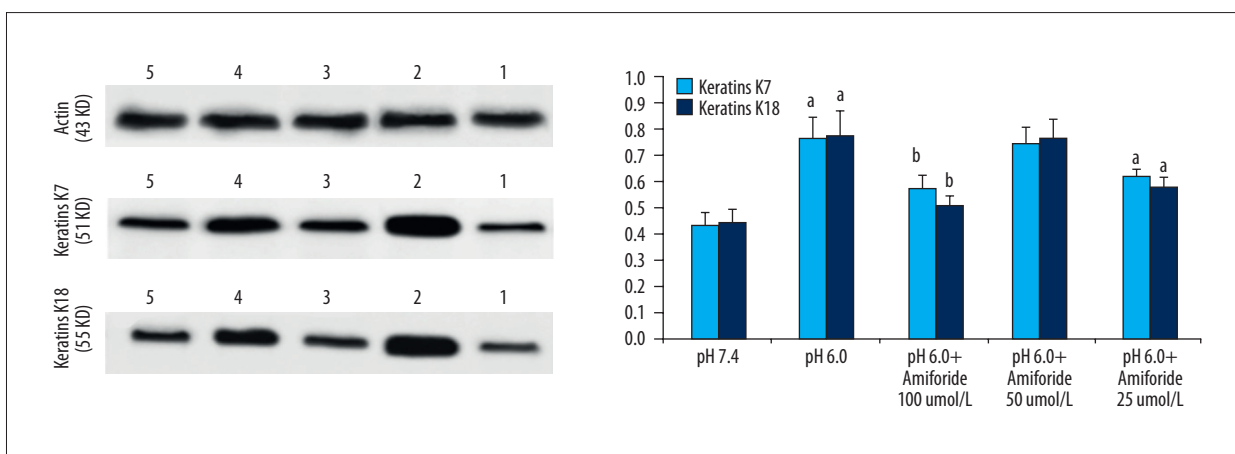


Figure 4. Acid-induced keratins K7 and K18 expression in renal tubular epithelial cells by Amiloride blocking ASICs (n=10). Signal intensity of bands was measured (in triplicates) by Image J software and each value was normalized to β -actin signal intensity. The error bars represent the standard deviation of the mean (\pm SD). Symbol 'a' indicated statistically significant ($p < 0.01$) change, when compared to pH 7.4 group. Symbol 'b' indicated statistically significant ($p < 0.01$) change, when compared to pH 6.0 group. 1 – pH 7.4; 2 – pH 6.0; 3 – pH 6.0+Amiloride 100 μ mol/L; 4 – pH 6.0+Amiloride 50 μ mol/L; 5 – pH 6.0+Amiloride 25 μ mol/L.

expression was closely related with ASIC1 expression ($r=0.53$, $P<0.01$; $r=0.56$, $P<0.01$, respectively).

Effect of Amiloride on acid-induced keratins expression in renal tubular epithelial cells

To determine if ASICs are involved in renal tubular injury, we observed the effect of the ASICs blocker, Amiloride, on acid-induced renal tubular epithelial cell injury. Figure 4 shows that keratins 7 and keratins 19 protein expression was significantly increased in pH 6.0 solution compared with that in pH 7.4 solution ($P<0.01$). However, Amiloride obviously inhibits keratins 7 and keratins 19 protein expression induced by extracellular acidosis.

Discussion

Henoch-Schönlein purpura nephritis (HSPN), the most serious long-term complication of HSP, is one of the most common renal diseases in children and adults. Renal involvement often determines the prognosis of HSP patients [3,4]. For this reason, it is important to identify the underlying mechanisms of the development of HSPN. The present study is the first to show higher ASIC1, ASIC2, and ASIC3 expression in kidney tissue, predominately in renal tubular cells. We also observed that the more severe renal pathological lesions are associated with higher ASIC1 expression in renal tubular cells was and higher urine protein level. These findings demonstrate that ASIC1 may be correlated with the development of HSPN.

Acid-sensing ion channels (ASICs) are known as proton-gated cation channels, which are activated in tissue acidosis conditions [14]. Inflammation in HSP is often accompanied by acidosis due to the release of lactate in the small vessels or glomerular capillaries, which may lead to activation of ASICs. This study also demonstrated that ASIC1, ASIC2, and ASIC3 expression was upregulated in cultured renal tubular epithelial cells in a low pH environment. Moreover, we found that extracellular pH fluctuates in a limited range (dropping from 7.4 to 7.0) and obviously influenced ASIC1 expression of renal tubular epithelial cells.

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Metabolic acidosis is one of the most common complications of kidney diseases, and it is mostly caused by renal tubular acidosis (RTA). In patients with renal transplantation, RTA was attributed to ischemic tubular injury in the early post-transplant period [15]. Acidification disorder due to tubular ischemia in HSPN patients appears to cause renal tubular injury. The extent of tubular involvement in HSP is a key factor in determining the development and prognosis of HSPN.

To investigate the relationship between ASICs expression and renal tubular injury, we measured renal tubular epithelial cell injury by identification of keratins expression. Keratins, the intermediate filaments of the renal tubular epithelial cell cytoskeleton, are novel sensitive markers of renal tubular cell injury [16]. Reports show that they can be upregulated and post-translationally modified in kidney diseases. The upregulation became significant early after disease induction and increased with disease progression [16]. In this study, we found that extracellular acidosis stimulated the expression of Keratins 7 and Keratins 18 proteins. Furthermore, K7 and K18 expression was closely related to ASIC1 expression, and blocking ASICs by Amiloride reduced keratins protein expression. Our data suggest that ASICs are involved in renal tubular cell injury induced by acidosis, and inhibiting ASICs had a protective effect on renal tubular cell injury.

Conclusions

This study is the first to show that ASIC1, ASIC2, and ASIC3 are expressed in kidney tissue from HSPN patients, predominately in the renal tubular cells. ASICs might be related to acid-induced renal tubular epithelial cell injury. However, the role of ASICs activation in the development of HSPN has not been fully elucidated, which will be done in the future.

Conflict of interest

None.

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