ourn l re proof

unveils novel ther peutic t rget for nephrolithi sis y modul ting nd stress

ng i oyu ng ozhen ng enqi u ing iu iuli u ing o

https doi or

Journal Pre-proof

CAV1 unveils a novel therapeutic target for nephrolithiasis by modulating CaSR and ER stress

Yang Li[1,3,†], Baoyu Yang[2,†], Haozhen Wang[2], Wenqi Hu[1,3], Ting Liu[2], Xiuli Lu[2,*] Bing Gao[1,3*]

1. Department of Cell biology and Genetics, Shenyang Medical College, 146 Huanghe North Street, Shenyang 110034, China
2. Department of Biochemistry and Cell Biology, School of Life Science, Liaoning University, Shenyang 110036, China
3. Key Laboratory of Renal Calcification Disease Prevention and Treatment, 146

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

CAAAGGGATGCTTGGATTAGGT-3’. Samples identified as GG and GA through Sanger sequencing were selected as positive controls. For each experiment, normalization settings and reference genotypes remained consistent, and each sample was tested independently at least three times.

2.7 Cell culture

HK-2 cells were obtained from the Cell Resource Center of Life Sciences, Chinese Academy of Sciences (Shanghai, China). Cell culture was undertaken using Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L glucose, supplemented with 10% (v/v) fetal bovine serum, 1% penicillin-streptomycin, and 0.45 mM L-glutamine. Cells were equilibrated at 37°C using 5% CO_2 and 95% O_2.

2.8 Measurement of intracellular Ca^{2+} level

The intracellular Ca^{2+} level was measured with a calcium ion fluorescent probe Fluo-4

Journal Pre-proof

transcribed RNA was undertaken using TB Green™ Premix Ex Taq™ (Takara RR820A), with a 20

Journal Pre-proof

Tokyo, Japan). Densitometry analysis was undertaken using the software ImageJ (http://imagej.net/ImageJ).

2.12 Immunocytochemical analysis

To investigate the effect of COM exposure on CAV1 expression, HK-2 cells were incubated with anti-caveolin-1 mouse monoclonal antibody for 60 minutes after fixation and blocking, followed by ABflo488-conjugated goat anti-mouse IgG (ABclonal, AS037). Images lsnj g, t ng

Journal Pre-proof

T:0 Ê sr # m m -etol xa

Journal Pre-proof

Journal Pre-proof

induced by COM (Figure 1a and b). Notably, pretreatment with the CaSR inhibitor NPS2390 significantly attenuated the COM-induced upregulation of intracellular Ca^{2+} level, suggesting the involvement of CaSR in the disturbance of intracellular Ca^{2+}. Furthermore, the expression levels of CaSR and ER stress-specific markers, including glucose-regulated protein 78/binding immunoglobulin protein (GRP78/Bip) and C/EBP homologous protein (CHOP), were evaluated under the same conditions. A marked increase in the expression of these three proteins was observed following COM exposure, which was reversed by NPS2390 (Figure 1c-f). Upon ER stress, three signaling pathways of the unfolded protein response (UPR) that are characterized respectively by the activation of ATF6, IRE1-XBP1, and PERK-eIF2 are activated to deal with the accumulation of unfolded or misfolded proteins in ER [26]. NPS2390 also inhibited the increase of ATF6, XBP-1 expression and the phosphorylation level of PERK induced by COM (Figure 1c and g-i). Taken together, these results suggest that CaSR-mediated intracellular Ca^{2+} overload is intricately linked to COM-induced ER stress in HK-2 cells.

3.2 The integrated analysis of genetic variants and differentially expressed gene networks revealed that CAV1 may play a key role in the development of kidney stones

The pathogenesis of kidney stones is a multifaceted process involving numerous genes and proteins, forming a complex interaction network. Network analysis is a tool to delve into the intricate networks linking genes and proteins to the molecular mechanisms underlying the disease etiology[18]. A workflow corresponding to our network analysis is shown in Figure 2a. Firstly, susceptibility SNPs associated with kidney stones were selected from four GWAS datasets and 261 candidate SNPs that mapped to 199 susceptibility genes were obtained after removing duplicates (Su$pwe lysis is sh

i Â

= 0.019). This suggests that these 38 genes may increase the risk of kidney stones by perturbing molecular networks involved in nephrolithiasis. The top 10% of nodes were designated as hub genes including *EGFR*, *AR*, *CAV1*, and *CDH1* (Table 1). KEGG pathway analysis revealed enrichment in pathways like the Rap1 signaling pathway, focal adhesion, and pathways in cancer (Figure 2c). This comprehensive workflow and network analysis provides insights into the genetic factors contributing to kidney stone formation.

To further investigate kidney stone-related genes with expression change, a gene expression profile (GSE73680) of kidney stone patients was obtained from the GEO database. In the comparison of the C group and N group, 2,156 genes were upregulated and 267 genes were downregulated with log2 fold change ($|log2FC| > 2$). A PPI network was also constructed, revealing 345 proteins in the direct network (Figure 2d). The permutation test indicated that there were more edges than would be expected by chance (p = 0.0009). Moreover, this network was enriched in several KEGG pathways, including the PI3K-AKT signaling pathway, neuroactive ligand-receptor interaction, pathways in cancer, and focal adhesion (Figure 2e).

To clarify the most consistent pathogenic factors underlying kidney stones, our analysis focused on examining the intersection of the genetic variant network and differentially expressed network. Six genes (*DMBT1*, *ZNF408*, *CAV1*, *LRP2*, *CAV2*, and *HSPG2*) and two signaling pathways (focal adhesion and proteoglycans in cancer) formed the intersection between the two networks (Figure 2f and g). CAV1 and EGFR are known to be critical members of the focal adhesion signaling pathway, which is important in cell proliferation, gene expression, and cell survival. Furthermore, the SNP rs6867 variant of the *CAV1* gene has been reported to be associated with the risk of kidney stones in the northeastern Thai population[3]. To investigate this association further, we conducted a genotyping experiment on SNP rs6867 in kidney stone patients from Northeastern China (Figure 2h-j), and utilized the genotypes of the East Asian population from the 1000 Genomes Project database as controls. The statistical analysis results (Table 2) showed that carriers with the GA genotype were more prevalent among

Journal Pre-proof

CAV1 expression levels in cells treated with COM alone, while

Journal Pre-proof

by repairing the EGFR-AKT survival signaling pathway.

3.5 CAV1 overexpression inhibited COM-induced ER stress

Previous researches have shown that CAV1 could regulate CaSR-mediated Ca^{2+} homeostasis imbalances in HUVECs and Saos-2cells [14, 15]. However, the effect of CAV1 on COM-induced intracellular Ca^{2+}

Journal Pre-proof

2+

demonstrated a remarkable increase from week 2 after EG treatment, while the phosphorylation levels of EGFR and AKT showed a marked decrease following EG treatment (Figure 7a-d). Our previous experiments found that ER stress occurred in the kidneys of EG-treated SD

Journal Pre-proof

the EGFR-AKT cell survival signaling pathway, ultimately causing HK-2 apoptosis. On the contrary, CAV1 overexpression restored the EGFR-AKT signaling pathway and inhibited the CaSR-mediated ER stress, thereby protecting HK-2 cells from COM-induced apoptosis (Figure 8). These´ s

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

collecting duct, which is a common initial site for CaOx crystal deposition. Research indicates that high concentrations of oxalate can induce morphological changes and damage to both proximal tubule and collecting duct cell lines, whereas lower concentrations do not produce such effects[51]. Injured cells exhibit an increased affinity for crystal attachment, promoting the retention of crystals within the renal collecting duct[52]. Therefore, hyperoxaluria-induced cellular damage is a critical factor in the formation of CaOx stones. Although rats are frequently used as model organisms for kidney stone disease research, their kidney structure differs from that of humans; for instance, rat kidneys are considerably smaller

Journal Pre-proof

our experimental findings, further substantiating the significant role of the CaSR-CAV1-EGFR/AKT signaling axis in the formation of kidney stones.

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

signaling in regulating transepithelial calcium transport, Experimental biology and medicine (Maywood, N.J.), 246 (2021) 2407-2419.

[11] G. Vezzoli, L. Macrina, G. Magni, T. Arcidiacono, Calcium-sensing receptor: evidence and

Journal Pre-proof

A large-scale analysis of tissue-specific pathology and gene expression of human disease genes and complexes, Proceedings of the National Academy of Sciences of the United States of America, 105 (2008) 20870-20875.

Journal Pre-proof

Signaling: The Link between Nephrolithiasis and Vascular Calcification, International journal of molecular sciences, 22 (2021).

[31] G. Vezzoli, A. Terranegra, F. Rainone, T. Arcidiacono, M. Cozzolino, A. Aloia, E. Dogliotti, D. Cusi, L. Soldati, Calcium-sensing receptor and calcium kidney stones, Journal of translational medicine, 9 (2011) 201.

[32] A. Balistrieri, A. Makino, J.X. Yuan, Pathophysiology and pathogenic mechanisms of pulmonary hypertension: role of membrane receptors, ion channels, and Ca(2+) signaling, Physiological reviews, 103 (2023) 1827-1897.

[33] F.C. Gombedza, S. Shin, Y.L. Kanaras, B.C. Bandyopadhyay, Abrogation of store-operated Ca(2+) entry protects against crystal-induced ER stress in human proximal tubular cells, Cell death discovery, 5 (2019) 124.

[34] H.L. Ong, X. Liu, K. Tsaneva-Atanasova, B.B. Singh, B.C. Bandyopadhyay, W.D. Swaim, J.T. Russell, R.S. Hegde, A. Sherman, I.S. Ambudkar, Relocalization of STIM1 for activation of store-operated Ca(2+) entry is determined by the depletion of subplasma membrane endoplasmic reticulum Ca(2+) store, The Journal of biological chemistry, 282 (2007) 12176-12185.

Journal Pre-proof

Wieland, C. Kahn, Role of brain insulin receptor in control of body weight and reproduction, Science (New York, N.Y.), 289 (2000) 2122-2125.

[41] J. Park, H. Han, Caveolin-1 plays important role in EGF-induced migration and proliferation of mouse embryonic stem cells: involvement of PI3K/Akt and ERK, American journal of physiology. Cell physiology, 297 (2009) C935-944.

[42] J. Moreno-Càceres, D. Caballero-Díaz, Z. Nwosu, C. Meyer, J. López-Luque, A. Malfettone, R. Lastra, T. Serrano, E. Ramos, S. Dooley, I. Fabregat, The level of caveolin-1 expression determines response to TGF- as a tumour suppressor in hepatocellular carcinoma cells, Cell death & disease, 8 (2017) e3098.

[43] Y. Yang, S. Hong, Y. Lu, Q. Wang, S. Wang, Y. Xun, CAV1 alleviated CaOx stones formation via suppressing autophagy-dependent ferroptosis, PeerJ, 10 (2022) e14033.

[44] Y. Shu, S. Jin, Caveolin-1 in endothelial cells: A potential therapeutic target for atherosclerosis, Heliyon, 9 (2023) e18653.

[45] C.Y. Lee, T.Y. Lai, M.K. Tsai, Y.C. Chang, Y.H. Ho, I.S. Yu, T.W. Yeh, C.C. Chou, Y.S. Lin, T. Lawrence, L.C. Hsu, The ubiquitin ligase ZNRF1 promotes caveolin-1 ubiquitination and degradation to modulate inflammation, Nature communications, 8 (2017) 15502.

[46] A. Hayer, M. Stoeber, D. Ritz, S. Engel, H.H. Meyer, A. Helenius, Caveolin-1 is ubiquitinated and targeted to intralumenal vesicles in endolysosomes for degradation, The Journal of cell biology, 191 (2010) 615-629.

[47] V. Sathish, A.J. Abcejo, S.K. VanOosten, M.A. Thompson, Y.S. Prakash, C.M. Pabelick, Caveolin-1 in cytokine-induced enhancement of intracellular Ca(2+) in human airway smooth muscle, American journal of physiology. Lung cellular and molecular physiology, 301 (2011) L607-614.

Journal Pre-proof

[50] S. Robijn, B. Hoppe, B.A. Vervaet, P.C. D'Haese, A. Verhulst, Hyperoxaluria: a gut-kidney axis?,

Journal Pre-proof

Journal Pre-proof

Figures Legends

Figure 1. CaSR-mediated intracellular Ca^{2+} overload participated in the COM-induced ER stress in HK-2 cells. After pretreatment with 10 μM NPS2390 for 1 hour, HK-2 cells were

Journal Pre-proof

revealed complete consistency with the HRM results. The black arrows indicate SNP rs6867 (j).

Figure 3. COM-induced the post-translational degradation of CAV1 damage to EGFR/AKT cell survival signaling pathway. After exposing HK-2 cells to 100 µg/mL COM for 24 or 48 hours, apoptosis was detected using TUNEL assays (a). The percentage of TUNEL positive cells was expressed as the ratio of apoptotic cells to adherent cells (b). ** $p < 0.01$, *** $p < 0.001$ vs. 0h. CAV1 was observed using immunocytochemistry in HK-2

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Journal Pre-proof

Table 1. SNPs in the four hub genes from the GWAS PPI network

SNPs	Chromosome	Position	Alleles	Genes
rs7801956	7	55181937	A/G	*EGFR*
rs5031002	X	66859350	A/G	*AR*
rs6867	7	115987759	A/G	*CAV1*
rs12444784	16	67359924	A/G	*CDH1*

Table 2. The statistics of genotype and allele frequencies of SNP rs6867 (G>A) in patients with kidney stones

Gene	SNP rs6867	No. cases (%)	No. controls (%)	OR (95%CI)	Chi-square	*p* value
CAV1	Genotype					
	GG	306 (96.23)	1148 (98.12)	2.046(1.001 - 4.181)	4.014	0.0451*
	GA	12 (3.77)	22 (1.88)	—	—	—
	Allele					
	G	624(98.11)	2318(99.06)	2.026(0.997 - 4.117)	3.968	0.0464*
	A	12(1.89)	22(0.94)	—	—	—

*$p < 0.05$

Controls are from the East Asian population in the 1000 Genomes Project database

Journal Pre-proof

Highlights

- Calcium oxalate induces

Journal Pre-proof