Clinical and pathological significance of Orai1 channel expression in human diabetic nephropathy

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Background: Targeted therapies for diabetic nephropathy (DN) are lacking, partly due to their irreversible nature. The role of Orai1, a store-operated Ca\(^{2+}\) channel, in DN remains debated, with conflicting evidence on its effect on proteinuria in animal models. We aimed to elucidate the functional relevance of Orai1 expression for clinicopathological parameters in patients with DN.

Methods: In this study, we included 93 patients diagnosed with DN between 2009 and 2019. Immunohistochemical staining for Orai1 was performed on paraffin-embedded kidney sections. The significance of Orai1 expression in human DN was assessed by examining its correlation with DN’s pathological and clinical parameters using Pearson’s correlation coefficient and univariate logistic regression.

Results: Orai1 was significantly overexpressed in DN patients compared to control. A strong correlation was observed between increased Orai1 expression and higher Renal Pathology Society DN classification, enhanced interstitial fibrosis and tubular atrophy scores. Positive correlations with serum creatinine levels and prognosis of chronic kidney disease (CKD) by glomerular filtration rate (GFR) and albuminuria category were noted but the estimated GFR was inversely related to Orai1 expression. Orai1’s association with advanced CKD stages persisted even after adjusting for confounding variables in multivariate logistic regression analysis.

Conclusion: Orai1 expression is closely associated with histological and clinical severities of DN, suggesting its potential as a predictive biomarker for disease progression and prognosis. These findings provide new perspectives on therapeutic interventions targeting Orai1 in DN.

Keywords: Biomarkers, Chronic renal insufficiency, STIM1, Prognosis, Store-operated Ca\(^{2+}\) entry
Introduction

Diabetic nephropathy (DN), a main cause of chronic kidney disease (CKD) and a representative disease of end-stage kidney disease (ESKD) worldwide, occurs in 10%–30% of patients with diabetes mellitus (DM) [1]. Its hallmark features include early deposition of the extracellular matrix (ECM) and myofibroblast accumulation within the glomerulus, eventually leading to glomerulosclerosis and ESKD [2]. Renal fibrosis, characterized by α-smooth muscle actin–expressing myofibroblast leading to excessive ECM production, is a pivotal pathological prognostic factor of DN [2].

Dysregulation of intracellular Ca\(^{2+}\) signaling has been implicated in fibrogenesis in various tissues, including the kidneys [3]. Among the primary mechanisms of Ca\(^{2+}\) entry into non-excitable kidney epithelial cells are the class C transient receptor potential (TRPC6) and stored-operated Ca\(^{2+}\) (SOC) channels. Upregulation and gain of function of TRPC6 channels have been associated with the development of tubulointerstitial fibrosis and focal segmental glomerulosclerosis [4–6]. However, reports on the role of Orai1, a pore-forming subunit of the SOC channels, in renal fibrosis are contradictory [2]. While the overexpression of Orai1 in the proximal tubules aggravates fibrosis [2], its activation in mesangial cells ameliorates ECM deposition, indicating a protective role [7]. Moreover, recent observations have shed light on the distinct pathological roles of Orai1 in type I and II DN [8]. Orai1 expression is downregulated in the proximal tubules of type I DN, and its blockade worsens proteinuric pathologies [8]. Conversely, glomerular overexpression and overactivation of Orai1 promote proteinuric diseases in type II DN [8].

Despite the observation of Orai1 overexpression in human renal specimens with minimal change disease (MCD) [2], animal models have provided accumulating evidence supporting Orai1-mediated renal fibrogenesis. Notably, the pathological role of Orai1 is cell type-specific and context-dependent in DN pathologies, including fibrosis [8]. However, deficiency of evidence related to the clinicopathological significance of Orai1 in human DN persists.

In this study, we aimed to explore the Orai1 expression and investigate its correlation with the pathophysiological significance of Orai1 in human DN. Furthermore, we assessed the potential of Orai1 as a valuable biomarker for predicting CKD progression and DN prognosis.

Methods

This study was approved by the Institutional Review Board of Yonsei University Wonju Severance Christian Hospital (No. CR320183). The requirement for informed consent was waived. All the procedures adhered to the principles of the Declaration of Helsinki.

Patients and tissue samples

A total of 93 patients diagnosed with DN at Wonju Severance Christian Hospital from 2009 to 2019 were enrolled in this study. Biopsy tissues from 10 patients diagnosed with MCD, characterized by histologically normal kidneys and a favorable clinical prognosis, served as the control group. Both paraffin-embedded blocks and fresh frozen tissues were utilized for pathological diagnosis. Slides stained with Periodic acid-Schiff and methenamine silver, along with the accompanying pathological reports, were reviewed, reclassified, and quantified according to the Renal Pathology Society classification (RPS class) proposed in 2010 [9]. The RPS class was launched by the Research Committee of the Renal Pathology Society and has since been used to predict the renal prognosis for DN [9,10]. In addition, electronic medical records were reviewed to collect the clinical parameters.

Immunohistochemistry

A Ventana Benchmark Ultra automatic immunostaining machine (Roche Diagnostics) was used for immunohistochemistry (IHC). The 4-μm sections were deparaffinized in xylene, rehydrated in graded alcohols, and subjected to pretreatment with CC1 (formalin-fixed solution; Roche Diagnostics). The sections were washed with reaction buffer, followed by incubation with Orai1 antibody (Proteintech) at a 1:100 dilution. Bound antibodies were detected using the UltraView Universal DAB Detection Kit (Roche Diagnostics), and sections were counterstained with hematoxylin (Roche Diagnostics) according to the manufacturer’s instructions. The positive and negative control strains were also tested. The expression of Orai1 IHC was semi-quantified as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong).
Quantitative real-time polymerase chain reaction

Total RNA was extracted from the tissue samples using a RiboEx Total RNA Kit (GeneAll Biotechnology) and reverse-transcribed into complementary DNA (cDNA) using a cDNA Synthesis Kit (Toyobo) according to the manufacturer’s instructions. The abundance of messenger RNA (mRNA) was analyzed by quantitative real-time polymerase chain reaction (RT-PCR) with SYBR-green (Applied Biosystems) using sequence-specific human primers: Orai1, F-5’ TTGAGCCCGCAGCAAGCTTAAA 3’, R-5’ CATTGCCACCATGCGAAGC 3’; 18S, F-5’ CGGCCGTTATCCCCATGAC 3’, R-5’ GCCCTTCCGTCAATTCCT 3’. Experiments were performed in triplicates using a QuantStudio 6 Flex RT-PCR System (Applied Biosystems). The 2-ΔΔCT method was used to analyze the data with 18S (18S ribosomal RNA subunit) as the reference gene.

Pathological parameters

Until recently, there was no alternative to the RPS class system utilized in this study for DN. Pathological factors incorporated into the RPS class include glomerular basement membrane thickening, mesangial expansion, nodular sclerosis, and global sclerosis. Additionally, this classification incorporates scores for tubulointerstitial and vascular lesions such as interstitial fibrosis and tubular atrophy (IFTA), inflammatory interstitial infiltrates, arteriolar hyalinosis (AH), and arteriosclerosis (AS) scores.

Clinical parameters

Collected data included age, sex, body mass index, serum creatinine, estimated glomerular filtration rate (eGFR), hemoglobin A1c (HbA1c), total 24-hour urinary protein, microalbuminuria, hypertension, duration of DM, and renal replacement therapy (RRT) status. Patients were stratified based on CKD prognosis by glomerular filtration rate (GFR) and albuminuria (CKD prognosis) as low-risk, moderate-risk, high-risk, and very high-risk based on the KDIGO (Kidney Disease: Improving Global Outcomes) 2012 guidelines [11].

Statistical analysis

The significance of Orai1 expression in human DN was assessed by examining its correlation with DN’s pathological and clinical parameters. Pearson’s correlation coefficient analysis was conducted to determine the relationships between the expression of Orai1 and individual pathological and clinical parameters. Associations between the expression of Orai1 and RPS class, eGFR, RRT status, and CKD prognosis were analyzed using univariate logistic regression. Subsequently, factors showing significant relationships in the above analyses were re-evaluated according to the other clinical factors of each patient and included in the multivariate logistic regression analysis. The predictive accuracy of each model was determined using c-statistics, corresponding to the area under the receiver operating characteristic curve. All statistical analyses were performed using IBM SPSS version 28.0 (IBM Corp.). A p-value of <0.05 was considered statistically significant.

Results

Baseline characteristics

Table 1 illustrates the baseline characteristics of all the

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Data</th>
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<tr>
<td>No. of patients</td>
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<tr>
<td>Age (yr)</td>
<td>68.4 ± 8.79</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male</td>
<td>68 (73.1)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (26.9)</td>
</tr>
<tr>
<td>Type of DM</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (7.5)</td>
</tr>
<tr>
<td>II</td>
<td>79 (84.9)</td>
</tr>
<tr>
<td>Unknown</td>
<td>7 (7.5)</td>
</tr>
<tr>
<td>Diabetes duration (yr)</td>
<td>10.33 ± 6.69</td>
</tr>
<tr>
<td>RPS glomerular classification</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3.14 ± 0.75</td>
</tr>
<tr>
<td>II + IIb</td>
<td>5 (5.4)</td>
</tr>
<tr>
<td>III</td>
<td>23 (24.7)</td>
</tr>
<tr>
<td>IV</td>
<td>37 (39.8)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>28 (30.1)</td>
</tr>
<tr>
<td>Baseline eGFR (mL/min/1.73 m²)</td>
<td>8.2 ± 2.6</td>
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</table>

Data are expressed as number only, mean ± standard deviation, or number (%).

DN, diabetes mellitus; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; RPS, Renal Pathology Society.

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patients, including age, sex distribution, type of DM, DM duration, RPS class, and baseline eGFR. The mean age of patients was 68.4 ± 8.79 years, and there were 25 female patients (26.9%). Additionally, seven patients (7.5%) had type 1 DM, and the mean DM duration was 10.33 ± 6.67 years. The number of patients classified as RPS classes I, I (Ia + Ib), II, and III were 5 (5.4%), 23 (24.7%), 37 (39.8%), and 28 (30.1%), respectively; the mean RPS class was 3.14 ± 0.75. The mean baseline HbA1c was 8.2% ± 2.6%. The mean baseline eGFR was 42.28 ± 36.42 mL/min/1.73 m².

Expression of Orai1 in diabetic nephropathy

The role of Orai1 in DN-related features such as fibrosis and proteinuria has been debated in rodent DN models [7, 12]. Orai1 expression was elevated in DN patient samples. We observed a significant elevation in the relative mRNA levels of Orai1 in DN tissues compared to normal tissues (Fig. 1A). IHC staining demonstrated pronounced Orai1 expression in the cytoplasm of the tubular and interstitial stromal cells, unlike in the control group, where Orai1 expression was negligible in the tubulointerstitial area. Notably, Orai1 expression increased concomitantly with the RPS class in human DN specimens (Fig. 1B).

Correlation of Orai1 expression with pathologic and clinical parameters

Orai1 overexpression was evident in patients with DN. Using the Pearson correlation test, we investigated the association between Orai1 expression and DN classification. A positive correlation was found between Orai1 expression and several factors, including RPS class, IFTA, interstitial inflammation, AH, and AS scores. In particular, RPS class and IFTA scores were positively correlated with Orai1 expression (Table 2). Orai1 expression also correlated positively with serum creatinine levels and CKD prognosis. Conversely, an inverse relationship was observed with eGFR levels, 24-hour total urinary protein, and 24-hour urine microalbumin, reaching statistical significance, suggesting that higher Orai1 levels were associated with lower eGFR values (Table 3).

Pathological prognostic and clinical factors associated with Orai1 expression in diabetic nephropathy

We compared the scores of each pathological and clinical factor to their mean values to overcome the limitations of the semi-quantitative IHC analysis. The Orai1 high expression group had significantly higher RPS class, IFTA scores,
interstitial inflammation levels, and serum creatinine levels. In contrast, eGFR levels were significantly lower in the high expression group compared to the low expression group (Table 4).

In univariate logistic regression analysis, both RPS class and CKD prognosis were associated with high odds ratios, indicating a strong positive correlation with Orai1 (Table 5). Subsequent multivariate regression analysis, adjusted for other relevant clinical factors, confirmed the robust association between CKD prognosis and Orai1 expression (Table 6). The c-index values for Orai1 expression, serum creatinine, eGFR, and 24-hour total urinary protein were 0.781, 0.905, 0.983, and 0.567, respectively, highlighting their predictive accuracy (Fig. 2).

**Discussion**

The pathological phenotypes of Orai1-mediated DN, including proteinuria and fibrosis, have been demonstrated...
to be cell-type-specific and context-dependent in rodent models [8]. Our study reveals that Orai1 is overexpressed in patients with DN compared to that in individuals with no DN. Orai1 expression was significantly correlated with representative pathological prognostic factors of DN, including the RP5 class (encompassing IFTA). Furthermore, Orai1 correlates with clinical parameters, such as serum creatinine levels, eGFR, and CKD prognosis.

Whether the upregulation of Orai1 exacerbates or mitigates renal fibrosis is still debated [8,13]. Upregulation of Orai1-mediated Ca\(^{2+}\) influx in the proximal tubule has been associated with the aggravation of transforming growth factor beta 1-induced epithelial-to-mesenchymal transition and fibrotic changes [2]. In contrast, Orai1 activation in mesangial cells appears to downregulate ECM protein expression, indicating its protective role in glomerular fibrosis in DM [7].

Our study provides evidence that the kidneys of patients with DN exhibit higher mRNA and protein expression of Orai1. IHC analysis further revealed prominent expression of Orai1 in the cytoplasm of tubules and interstitial stromal cells. Interstitial fibrosis is a characteristic feature of later stages of CKD. Furthermore, the Orai1 expression profile closely mirrored the clinicopathological features of DN and

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**Figure 2.** Receiver operating characteristic curve for predicting chronic kidney disease prognosis in the very high-risk group. (A) C-statistics for Orai1 expression (0.781), (B) creatinine (0.905), (C) estimated glomerular filtration rate (0.983), and (D) 24-hour total protein (0.567).
demonstrated a robust association with advanced CKD stages, as indicated by multivariate logistic regression analysis. In summary, our findings strongly suggest that Orai1 may play a significant pathophysiological role in the development of interstitial fibrosis in kidneys.

Whether Orai1 positively or negatively regulates DN features, including fibrosis and proteinuria, appears to depend on the type of DM [8,14]. Orai1-mediated SOC entry in mesangial cells has been associated with the negative regulation of ECM protein expression in a diabetic milieu [7,12]. In contrast, Orai1 overexpression has been observed in proximal tubular fibrosis induced by unilateral urethral obstruction or a high-fat diet and is positively correlated with interstitial fibrotic changes [2]. From a proteinuria perspective, Orai1 isoforms are highly expressed in the proximal tubules but are downregulated in type I DN. Functionally, the Orai1 blockade accelerates proteinuria in type I DN [14]. A recent study indicates that Orai1 is overexpressed in glomerular podocytes in a type II DN animal model. This study demonstrates that Orai1 inhibition ameliorates proteinuria and protects glomerular filter function [8]. In our study, Orai1 was upregulated, and its expression was positively associated with the clinicopathological characteristics and CKD in the kidneys of patients with type II DN. Accumulating evidence regarding the pathophysiological roles of Orai1 reveals bidirectional effects in rodent models of DN. Thus, our data from human patients with type II DN suggest that Orai1 may be a positive pathophysiological mediator of interstitial fibrosis in the kidney.

Orai1 is intricately linked to key pathological prognostic factors of DN, especially the RPS class and IFTA scores, showing significant correlations. The RPS class standardizes the identification and scoring of DN lesions [9]. DN is characterized by various changes, including vascular lesions (AH and AS), tubulointerstitial damage marked by tubular basement membrane thickening and tubular cell hypertrophy, and progressive interstitial fibrosis in the later stages [15]. Early DN stages are characterized by mesangial matrix accumulation [9]. A notable positive correlation exists between Orai1 expression and serum creatinine levels and CKD prognosis. Conversely, negative correlations were observed with eGFR levels, 24-hour total urinary protein, and microalbumin, with statistical significance. Orai1 was useful for predicting the risk of progression of DN. These findings underscore the potential of Orai1 as a pathological and prognostic marker for DN and CKD.

Orai1 levels are inversely correlated with insulin secretion and kidney function, leading to potential improvements in blood glucose levels and an increased risk of hyperglycemia. Additionally, the activation of Orai1 channels is crucial for protein reabsorption in the kidneys [14]. Orai store-operated channels are present in human proximal tubules. Impairment of Orai channel expression or activity leads to decreased albumin absorption by the proximal tubular cells, resulting in proteinuria [14]. Therefore, the elevation of Orai1 may contribute to a decrease in proteinuria and albuminuria.

HbA1c, a reliable indicator of glycemic control, is associated with the occurrence and progression of diabetic complications [16,17]. Orai1 activation by insulin induces transient cytoskeletal remodeling in podocytes, resulting in glomerular filter disruption and albuminuria [8]. In a type II DN animal model, overexpression of Orai1 is particularly prominent in the early stages of DM, concurrent with hyperinsulinemia [8]. Furthermore, DN is characterized by microalbuminuria in its early stages, and glomerular hyperfiltration imposes shear stress that damages podocytes [18]. The confluence of podocyte damage, proteinuria, and increased Orai1 expression in diabetic mice suggests a correlation with HbA1c, which serves as an indicator of glycemic control [18].

In cases of severe DM, HbA1c levels are high but can decrease in situations where glomerular damage occurs or in advanced CKD cases. Despite no statistical significance, we observed a trend towards lower HbA1c levels with advancing CKD stages, potentially indicating better glycemic control or altered insulin metabolism in advanced DN. The inverse correlation between HbA1c and Orai1 might reflect this dynamic, highlighting the need for further investigation into Orai1’s role and expression concerning glomerular filter integrity and CKD progression in patients with type II DN.

This study’s limitations include focusing predominantly on patients with type II DM and a small sample size for RPS class I. The lack of fresh kidney tissue limited our ability to explore Orai1 gene variations across RPS classes. A significant constraint was the emphasis on Orai1 expression in the tubulointerstitium, excluding glomerular expression levels.

In conclusion, Orai1’s strong correlation with pathologi-
cal and clinical severity of DN underscores its potential as a biomarker and therapeutic target, offering new insights to understand the progression and prognosis of DN.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Data sharing statement

The data presented in this study are available from the corresponding author upon reasonable request.

Authors’ contributions

Conceptualization, Methodology: YK, SKC, ME
Formal analysis: JYL, JWY
Funding acquisition: ME, SKC
Investigation: YK, JYL, ME
Writing–original draft: YK, JYL, JSK, KHH, SKC, ME
Writing–review & editing: All authors
All authors read and approved the final manuscript.

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