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Clinical Value of Urinary Wilms' Tumour-1 and Mu-Glutathione S-Transferase Gene Expression in Kidney Injury in Type 2 Diabetes Mellitus

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Objective: To investigate the diagnostic value of urinary Wilms' tumour-1 (WT-1) and mu-glutathione S-transferase (Mu-GST) gene expression for detecting kidney injury in type 2 diabetes mellitus (T2DM).

Methods: Patients treated between October 2022 and June 2023 were divided into two groups: a diabetic nephropathy (DN) group (105 patients) and a diabetes mellitus (DM) group (100 patients). Additionally, 100 healthy individuals undergoing routine medical check-ups were selected as the control group. The urinary albumin/creatinine ratio (ACR), as well as urinary WT-1 and Mu-GST expression levels, were measured. The sensitivity and specificity of these markers for predicting renal injury were evaluated.

Results: The levels of ACR, WT-1 and Mu-GST in the DN group were significantly higher than those in the DM and control groups. The ACR in the DM group was also significantly higher than that in the control group ($P < 0.05$), and WT-1 and Mu-GST gene expression levels demonstrated a positive correlation with ACR (r = 0.391 and 0.342, respectively). The sensitivity and specificity of WT-1 were 74% and 95%, respectively, whereas those of Mu-GST were 70% and 96%, respectively. The combined detection of WT-1 and Mu-GST yielded a sensitivity of 82% and a specificity of 97%.

Conclusion: The levels of WT-1 and Mu-GST gene expression are closely related to T2DM kidney injury, helping to identify the location of kidney injury to some extent, which provides valuable information for the clinical diagnosis and treatment of kidney injury.

Keywords: type 2 diabetes mellitus kidney injury, urinary microalbumin/creatinine ratio, wilms' tumour-1, mu-glutathione S-transferase, diagnosis

Introduction

Diabetes mellitus (DM) is a metabolic disease caused by multiple factors and characterised by hyperglycaemia.[1,2] The incidence of type 2 DM (T2DM) continues to rise, and the incidence of kidney injury in patients with T2DM is also increasing.[3] The clinical symptoms of kidney injury in T2DM are relatively insidious, exhibiting slow and progressive development. If diagnosis and intervention are not prompt, the condition becomes irreversible after entering the stage of massive proteinuria, resulting in increased patient mortality.[4] Therefore, the timely and effective intervention and treatment of patients with DM and kidney injury are of great value in delaying the deterioration of the disease and improving patient quality of life.[5]

The primary biochemical indicators for diabetic kidney injury include blood creatinine, urea nitrogen, cystatin C and the urinary microalbumin/creatinine ratio (ACR). However, these methods are susceptible to extrarenal factors and have limitations, such as poor sensitivity and specificity, as well as difficulties in localising the site of kidney injury.[6–8] The Wilms' tumour-1 (WT-1) gene, the earliest identified tumour suppressor gene linked to tumour development, is notably expressed in the glomerular podocytes of adult kidneys.[9,10] The detachment of podocytes from the glomerular basement membrane is a key pathological mechanism contributing to proteinuria.[11] Recent

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studies indicate that WT-1 gene expression is present at varying levels in the urine of patients with diabetic nephropathy (DN), chronic kidney disease and rheumatic kidney damage, suggesting its potential for predicting glomerular damage and prognosis.[12] According to Dan Gao et al,[13] the detection of WT-1 gene expression may serve as an earlier indicator of diabetic kidney injury than traditional urine protein tests. The mu-glutathione S-transferase (Mu-GST) gene, specifically expressed in distal renal tubular epithelial cells, is a marker of distal renal tubular injury.[14] Mu-GST is an enzyme involved in detoxification and metabolism within cells. In the kidneys, its elevated expression in urine serves as an indicator of distal renal tubular damage. When the distal renal tubule is injured, the epithelial cells are shed, leading to increased Mu-GST expression in urine, which can serve as an early warning for injury. However, no studies have yet investigated the diagnostic utility of WT-1 and Mu-GST genes for diabetic kidney injury.

Therefore, to further confirm the clinical significance of WT-1 and Mu-GST gene expression levels in diagnosing diabetic kidney injury, this study examines WT-1 and Mu-GST gene expression in the urine-shed cells of healthy people, patients with DM and patients with diabetic kidney injury. It compares them with ACR, a commonly used renal function detection index.

Materials and Methods

General Information

Between October 2022 and June 2023, patients with T2DM were retrospectively included in this study. Patients with a urinary albumin excretion rate (UAER) ranging between 30 and 300 mg/24 hours were included in the diabetic kidney injury group (DN) (105 patients).[15] Patients with UAER <30 mg/24 hours were included in the DM group (100 patients). Furthermore, age- and gender-matched healthy participants from routine physical examinations (UAER <30 mg/24 hours) were selected as the control group (100 patients). The inclusion criteria were as follows: (1) not using nephrotoxic drugs or immunosuppressants before enrolment; (2) meeting the diagnostic criteria of T2DM;[16,17] (3) having complete clinical data. The exclusion criteria were as follows: (1) acute diabetic complications (eg ketoacidosis); (2) the presence of primary or secondary kidney diseases (eg primary glomerulonephritis, nephrotic syndrome and lupus nephritis); (3) the presence of serious cardiovascular and cerebrovascular diseases or malignant tumours. This study was approved by the Ethics Committee of Kongjiang Hospital (LL-2023-KY-05), and all patients provided signed informed consent.

Instruments and Reagents

The TBA-120FR automated biochemical analyser (Toshiba, Japan), EX 3600 Plus automated nucleic acid extraction instrument (Shanghai Zhijiang Biotechnology, Shanghai, China), FQD-96A fluorescence quantitative polymerase chain reaction (qPCR) instrument (Hangzhou Bori, Hangzhou, China) and HYDRASYS automatic electrophoresis instrument (Sebia, Lisses, France) were used. The urine mAlb reagent (Shanghai Danli Biological, Shanghai, China), urine creatinine kit (Zhejiang Ningbo Quark Biological, Ningbo, China), nucleic acid extraction and purification kit (Shanghai Zhijiang Biological Technology), PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) Kit (Takara, Dalian, China), Probe qPCR Mix with UNG Kit (Takara) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) urine protein kit and acid purple dye (Sebia, Lisses, France) were also selected.

Urinary Albumin/Creatinine Ratio

A 5-mL urine sample was collected and centrifuged at 3000 revolutions per minute for 10 minutes. The supernatant was taken to determine UmAlb and urinary creatinine, and the value of ACR was calculated. Both the UmAlb and urinary creatinine were determined using the Toshiba TBA-120FR automated biochemical analyser. Subsequently, UmAlb was determined using immunoturbidimetry and urinary creatinine using the sarcosine oxidase method. The normal reference value for ACR is <30 mg/g, which is considered negative.[18]

WT-1 and Mu-GST Gene Detection

A 1.5-mL urine sample was collected, and nucleic acids were extracted using a nucleic acid extraction kit on the EX 3600 plus automated nucleic acid extraction instrument. Following extraction, the PrimeScript™ RT Reagent Kit with gDNA Eraser was used for reverse transcription. For qPCR amplification, the Probe qPCR Mix with UNG kit was employed. The amplification protocol consisted of 45 cycles, with the following steps: 95°C for 1 minute, 95°C for 5 seconds, 60°C for 30 seconds and 95°C for 5 seconds. The nucleotide sequences of the upstream primers, downstream primers and probes used for amplifying the WT-1 and Mu-GST genes are listed in Table 1. The relative expression of the target genes in the samples was determined using the 2-ΔΔCt method in RT-PCR for quantitative gene expression analysis.[19] In addition, GAPDH was used as the internal reference gene to normalise the expression levels of the detected genes. A relative expression level of WT-1 and Mu-GST genes (<1.5) is also regarded as negative.

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

The SDS-PAGE process is employed to differentiate types of proteinuria. In this study, 20 μL of SDS bromophenol blue diluent were combined with 80 μL of treated urine. A total of 5 μL of the mixture was carefully drawn and added through the gel sampling hole. The mixture was allowed to spread for 10 minutes before proceeding with the SDS-PAGE process. In this process, proteins with molecular weights greater than 70,000 are located below the negative pole, whereas proteins with molecular weights smaller than 70,000 are found above the positive pole. The region from the clear protein band to the negative pole contains large molecules (>70,000 molecular weight), whereas the region from the clear protein band to the positive pole contains small molecules (<70,000 molecular weight), demarcating the molecular boundaries. Large and small molecules flank the clear protein band on either side.[20]

Statistical Methods

For statistical processing, SPSS 25.0 software was used. For measurement data, if normal distribution was met, results were expressed as mean ± standard deviation (x ± s), and multiple group comparisons were performed using one-way ANOVA. If normal distribution was not met, data were presented as median and interquartile range (P25, P75). Count data were expressed as percentages (n [%]), and the $\chi 2$ test was used. The correlation between WT-1 and Mu-GST gene expression and ACR was assessed using Spearman's rank correlation coefficient. The sensitivity and specificity of each index (ACR and WT-1, Mu-GST gene expression) for detecting diabetic kidney injury were analysed using the receiver operating characteristic (ROC) curve. A value of $P < 0.05$ was considered statistically significant.

Results

General Information

In the DN group, 105 patients were included, 52 men and 53 women, with a mean age of 65.78 ± 12.02 years and a disease duration ranging from 5 to 20 years. In the DM group, 100 patients were included, 62 men and 38 women, with a mean age of 65.36 ± 11.14 years and a disease duration ranging from 3 to 18 years. Additionally, 100 healthy individuals were selected as the control group, 58 men and 42 women, with a mean age of 64.43 ± 10.67 years. Statistical analysis revealed no significant differences in age distribution or gender composition among the groups ($P > 0.05$),

Table 1 Primers and Probe Sequences Amplified by WT-1 and Mu-GST

Primer Number	Primer Name	Primer Sequence (5 "-3")	Base Number
SEQ ID NO:1	WT1 Forward Primer	ccatcacaacatgcatcagag	21
SEQ ID NO:2	WT1 Reverse Primer	ttgaccgcagttcacacactgt	22
SEQ ID NO:3	WT1 probe	cattctcaaactacagctggcg	22
SEQ ID NO:4	GSTM1 Forward Primer	gacttcattgtccctcttctc	21
SEQ ID NO:5	GSTM1 Reverse Primer	cactgccaggaaggaatgac	20
SEQ ID NO:6	Mu-GST probe	cagtagtgcagggaagagtaatga	24

Abbreviation: SEQ: sequence.

Table 2 Comparison of Characteristics of Enrolled Subjects

	DN Group (n=105)	DM Group (n=100)	Control Group (n=100)	χ^2/F	*P*
Age (years)	65.78±12.02	65.36±11.14	64.43±10.67	0.037	0.952
BMI (kg/m^2)	22.39±2.17	22.34±2.22	22.63±2.23	0.320	0.727
BUN (mmol/L)	35.76±6.25^{ab}	16.83±3.55^{a}	6.67±1.38	817.268	0.000
β2-MG (mg/L)	1.53±0.43^{ab}	0.71±0.21^{a}	0.16±0.02	428.306	0.000
UA (μmol/L)	462.36±132.25^{ab}	362.44±113.65^{a}	312.17±102.56	32.582	0.000
eGFR[mL/(min ·1.73m2)]	64.15±5.38^{ab}	87.32±4.73	95.26±4.12	101.162	0.000
ACR (mg/g)	59.13±9.98^{ab}	34.17±4.13^{a}	19.06±4.38	202.13	0.001

Notes: compared with control group, $^{a}P < 0.05$; compared with DM group, $^{b}P < 0.05$.
Abbreviation: ACR, ratio of urinary microalbumin to urinary creatinine; DM, diabetes mellitus; DN: diabetic nephropathy.

indicating their comparability. The ACR levels in both the DN and DM groups were significantly higher than those in the control group. Furthermore, ACR levels in the DN group were significantly higher than those in the DM group, with statistically significant differences observed in all group comparisons ($P < 0.05$) (see Table 2).

Expression results of WT-1 and Mu-GST Genes

The levels of WT-1 and Mu-GST gene expression in the DN group were higher than those in the DM and control groups, and the differences were statistically significant ($P < 0.05$). In the DM and control groups, the WT-1 and Mu-GST gene expression levels did not show statistically significant differences ($P > 0.05$). The results are presented in Table 3 and Figure 1.

Correlation Between WT-1 and Mu-GST Gene Expression and Albumin/Creatinine Ratio

The Spearman's rank correlation coefficient was calculated between WT-1 and Mu-GST gene expression and the ACR index. As indicated in Table 4, the gene expression levels of WT-1 (r = 0.391) and Mu-GST (r = 0.342) demonstrated a positive correlation with ACR indexes, and this correlation is statistically significant.

Efficacy of Molecular Indices and the Albumin/Creatinine Ratio in Evaluating Renal Injury

The results of the ROC analysis revealed that the ACR index had a sensitivity of 82% and a specificity of 88% in this experimental study. The WT-1 index had a sensitivity of 74% and a specificity of 95%, whereas the Mu-GST index had a sensitivity of 70% and a specificity of 96%. When combined, the detection sensitivity for WT-1 and Mu-GST reached 82%, with a specificity of 97%. Furthermore, the combined detection specificity for WT-1 and Mu-GST was significantly higher than that achieved by ACR alone (Table 5 and Figure 2).

Table 3 Comparison of WT-1 and Mu-GST Gene Relative Expression Levels of Subjects in Each Group (±s)

Groups	Number of Cases	WT-1	Mu-GST
Control group	100	0.97±0.05	0.98±0.04
DM group	100	1.02±0.23^{a}	1.01±0.25^{a}
DN group	105	2.32±0.58^{bc}	2.17±0.53^{bc}

Notes: Compared with control group, $^{a}P > 0.05$, $^{b}P < 0.05$; Compared with DM group, $^{c}P < 0.05$.
Abbreviations: DM, diabetes mellitus; DN, diabetic nephropathy.

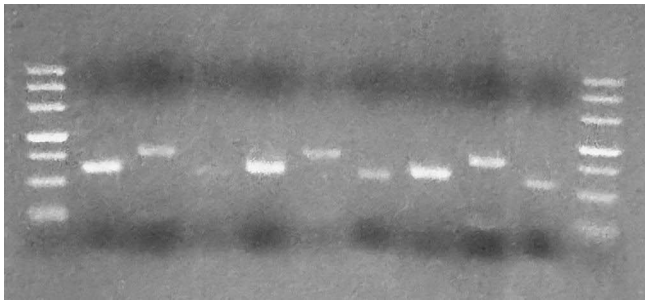

Figure 1 Electrophoresis of GAPDH, WT-1 and Mu-GST amplification products. DL1000 DNA Mark molecular weight range: 100 ~ 1000bp; 108bp was the amplification product of GAPDH mRNA. 112bp was the amplified product of WT-1 mRNA. 148bp was the amplified product of Mu-GST mRNA. Kim-1, a-GST and Clustr were the genes specifically expressed in proximal convoluted tubule injury. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Mu-GST: mu-glutathione s-transferase.

Urine Protein Electrophoresis Obtained from Patients Who Tested Positive for WT-1 and Mu-GST Gene Expression

Figure 3 presents the results of the urine protein SDS-PAGE process conducted on patients with positive WT-1 and Mu-GST gene expression, and Table 6 summarises the detected outcomes of various proteinuria types identified through the electrophoresis procedure. The SDS-PAGE process was performed on patients with positive WT-1 gene expression, including 58 patients with diabetic kidney injury and 3 patients with DM. Among the 58 patients with diabetic kidney injury, 52 exhibited glomerular proteinuria and 6 had mixed proteinuria. The three patients with DM had physiological proteinuria. Additionally, SDS-PAGE was performed on patients with positive Mu-GST gene expression, including 46 patients with diabetic kidney injury and 2 patients with DM. Of the 46 patients with diabetic kidney injury, 37 exhibited tubular proteinuria, 1 had glomerular proteinuria, 8 had mixed proteinuria and 2 had biological albuminuria. Furthermore, one patient with DM had physiological proteinuria. Figure 4 provides the semi-quantitative analysis of Mu-GST and WT-1 gene expression in patients with diabetic kidney injury.

Discussion

Renal function impairment in T2DM is closely related to glomerular, renal tubule and renal interstitial lesions. The selection of sensitive and ideal renal function impairment indicators is of great significance for the diagnosis and prognosis of T2DM.[20] Kidney function indicators widely used in clinical practice have obvious defects in sensitivity and specificity in a kidney injury diagnosis, as renal injury has progressed to a state of severe decompensation when they increase.[21,22] Urinary ACR is currently the most used index to judge kidney injury and diagnose DN.[23] Several studies[24,25] have also revealed that ACR has a stronger diagnostic effect than other indicators of kidney injury. However, the diagnostic sensitivity and specificity of ACR cannot fully meet the needs of clinical diagnosis or locate the site of kidney injury.

Glomerular podocytes are terminally differentiated cells specifically arranged along the lateral basement membrane of the glomerulus. A decrease in the number of these cells is closely related to glomerulosclerosis. Genes such as WT-1 are specifically expressed in these cells, and detecting the expression of these genes can reflect the condition of podocytes.[26] Studies have reported[14] that detecting WT-1 gene expression may identify common diabetic kidney injury earlier than

Table 4 Spearman Correlation Analysis Results of WT-1, Mu-GST Gene Expression and ACR

Indicator Variable		ACR
WT-1	r	0.391
	P value	<0.05
Mu-GST	r	0.342
	P value	<0.05

Abbreviation: ACR, ratio of urinary microalbumin to urinary creatinine.

Table 5 Diagnostic Efficiency of ACR, WT-1 and Mu-GST Indexes

index	AUC	95% CI	Specificity	Sensitivity	P value
ACR	0.861	0.785~0.937	0.82	0.88	0.000
WT-1	0.820	0.720~0.920	0.74	0.95	0.000
Mu-GST	0.816	0.719~0.912	0.70	0.96	0.000
WT-1 association Mu-GST	0.882	0.799~0.966	0.82	0.97	0.000

Abbreviation: ACR, ratio of urinary microalbumin to urinary creatinine.

detecting urinary protein. It is believed that the initial pathological change in DM is a glomerular lesion. Recent studies have found that renal tubular injury in some patients with DN may precede glomerular injury. Urinary renal tubular injury markers are more valuable than microalbuminuria in predicting diabetic kidney injury.[27,28] Microalbuminuria is characterised by urinary albumin levels above the normal range but below detectable levels using a conventional dipstick. This corresponds to a UAER of 30–300 mg/24 hours, 30–300 μmol/L of creatinine or 20–200 μmol/L, based on results from two out of three urine collections. Recent studies on renal tubular biomarkers have mainly focused on proximal renal tubular markers (eg alpha 1-microglobulin and Kim-1), with few studies on distal renal tubular injury markers. Studies have reported[15] that the Mu-GST gene is specifically expressed in distal renal tubular epithelial cells. When the distal renal tubule is damaged, these epithelial cells are exfoliated, and the increased expression of the Mu-GST gene in urine can reflect distal renal tubular injury.

In this study, the ACR and the gene expression levels of WT-1 and Mu-GST in the DN group were significantly higher than those in the DM and control groups. The ACR in the DM group was also higher than that in the control group, with statistically significant differences. However, there were no significant differences in WT-1 and Mu-GST gene expression between the DM and control groups. Additionally, WT-1 and Mu-GST gene expression levels were positively correlated with ACR levels. These findings align with those of previous studies,[29,30] which reported undetectable WT-1 protein in urinary exosomes and WT-1 mRNA in urine samples from healthy controls. However, the elevated levels of WT-1 observed in patients with proteinuria, as compared to those without, suggest an association with renal injury.[31] Moreover, significantly higher urinary protein excretion, elevated serum creatinine and lower eGFR in patients who are WT-1 positive compared with those who are WT-1 negative, along with the strong association between

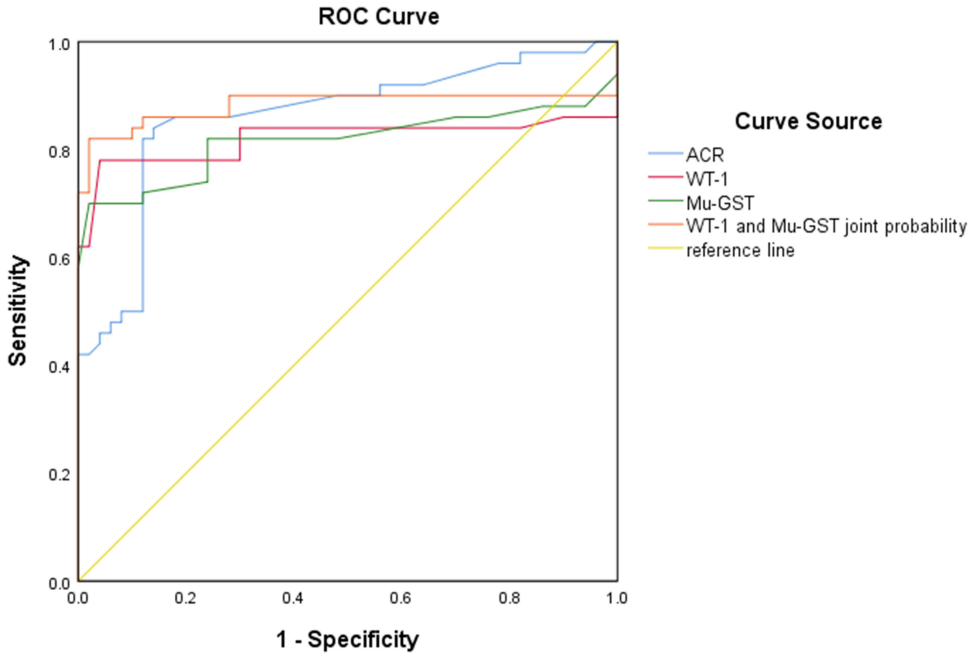

Figure 2 The ROC curve state variable for kidney injury.

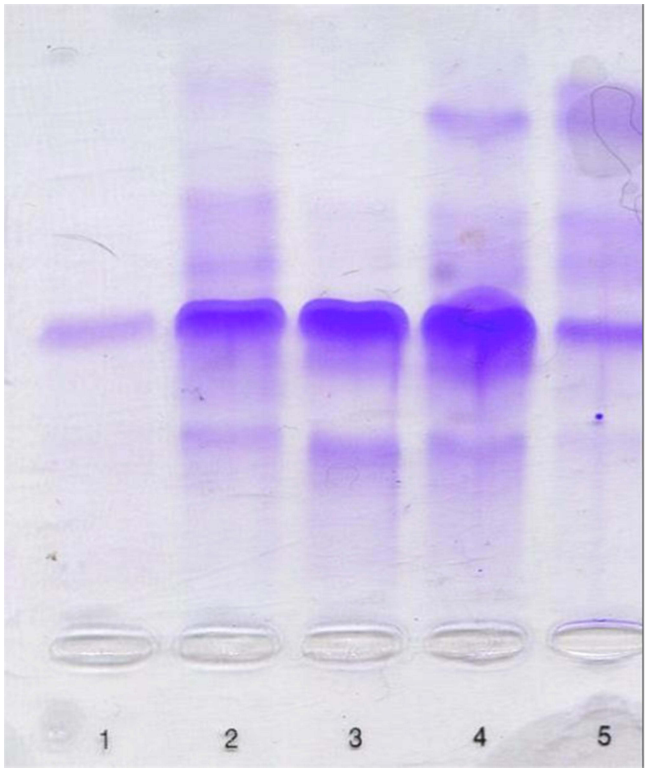

Figure 3 SDS-AGE electrophoretic map of urine protein. 1. Physiologic albuminuria; 2. Mixed albuminuria; 3. Glomerular albuminuria; 4. Mixed albuminuria; 5. Renal tubular albuminuria.

WT-1 levels and increased urinary protein excretion, highlight the potential of urinary exosomal WT-1 as a non-invasive biomarker for predicting early renal injury in patients with diabetes.

Our results indicate that the expression levels of WT-1 and Mu-GST in urine have a certain diagnostic value for DN. The sensitivity and specificity of the ACR index for DN diagnosis in this study were 82% and 88%, respectively, whereas the WT-1 gene index exhibited a sensitivity of 74% and a specificity of 95%. A similar study demonstrated that both ACR and WT-1 protein in urinary exosomes are equivalent in predicting a GFR <60 $mL.min^{-1}/1.73m^2$ in patients with DN (AUC: 0.92 for WT-1, 0.95 for ACR). Notably, WT-1 appears in a higher percentage of patients than proteinuria at earlier GFR cut-off values (eGFR <70/80/90 $mL.min^{-1}/1.73m^2$).[31] Additionally, the sensitivity and specificity of the Mu-GST gene index were 70% and 96%, respectively. The combined detection of WT-1 and Mu-GST yielded a sensitivity of 82% and a specificity of 97%, with the specificity significantly higher than that of ACR, though sensitivity was slightly lower. This may be attributed to the fact that WT-1 and Mu-GST primarily indicate glomerular and distal tubular injury, without capturing proximal tubular damage. However, the estimated sensitivity of WT-1 and Mu-GST was lower than their specificity, indicating that these markers are more effective in correctly identifying individuals without renal injury than in detecting all individuals with renal injury. This suggests that although the markers are highly specific, they may

Table 6 Results of SDS-AGE Urine Protein Electrophoresis (Example)

Types of Proteinuria	WT-1 gene Positive Expression	Mu-GST gene Positive Expression
Renal tubular albuminuria	0	37
Glomerular albuminuria	52	1
Mixed proteinuria	6	8
Physiologic proteinuria	3	2
Total	61	48

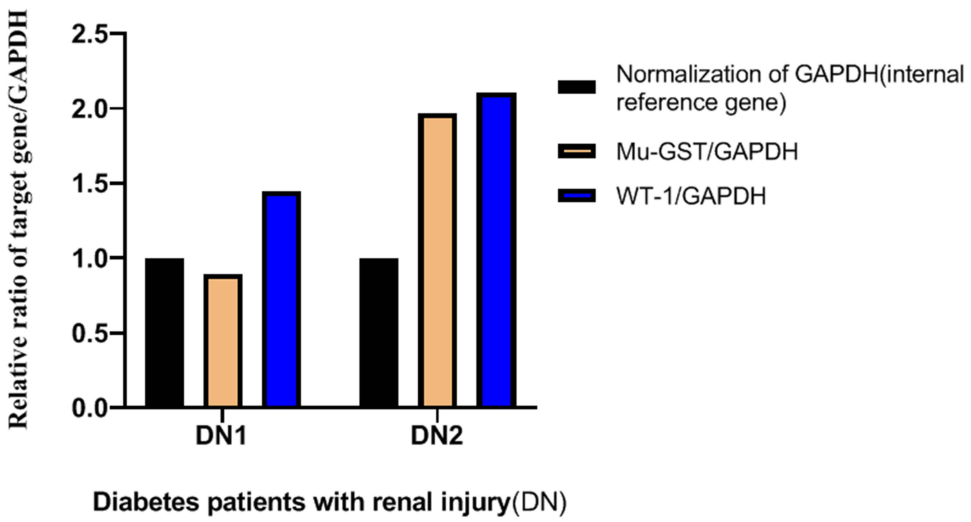

Figure 4 The semi-quantitative analysis of target genes Mu-GST and WT-1 in patients with diabetic kidney injury (DN1 and DN2).

miss some early or mild cases of DN, making them less ideal for early screening but valuable for confirming more advanced stages of the disease.

To investigate whether the urine protein types of patients positive for WT-1 and Mu-GST genes were consistent with the kidney injury sites indicated by these genes, urine protein SDS-PAGE was performed. The results revealed that all patients with renal injury and positive WT-1 gene expression exhibited glomerular proteinuria, suggesting that WT-1 gene expression indicates glomerular injury. Among the patients with DM, two individuals showed positive expression of the WT-1 gene, accompanied by the exclusive presence of physiological proteinuria. These findings imply that the detection of WT-1 gene expression may potentially precede urine protein detection in identifying typical diabetic kidney damage, consistent with the research findings of Gao et al.[13] Patients with renal injury who express the Mu-GST gene exhibit three types of proteinuria: tubular, glomerular and mixed. All of these are considered non-specific. Notably, one patient with DM had positive Mu-GST expression but only physiological proteinuria. These findings suggest that Mu-GST gene detection may indicate distal renal tubule damage before proteinuria becomes clinically apparent. However, further monitoring of the patient's condition is necessary to draw definitive conclusions.

This study has some limitations. The small sample size, constrained by time and funds, may introduce bias into the results. Additionally, the diagnostic ability of the proposed markers is less than that of the existing ACR marker. The study provides no evidence demonstrating the improved ability of the proposed markers in kidney disease. Further multi-centre research with a large sample size, including longitudinal studies tracking these biomarkers over the course of disease progression, would be valuable in clarifying their relative merits and limitations as diagnostic tools for DN.

Conclusion

In summary, the WT-1 and Mu-GST biomarkers are closely associated with diabetic kidney injury, and their combined detection is crucial for assessing the extent of kidney damage in patients with DM. Analysing the changes in WT-1 and Mu-GST gene expression in urinary cells can help clinicians localise kidney injury sites early. This non-invasive approach minimises patient harm.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Kongjiang Hospital (Approval No.LL-2023-KY-05). And all patients signed informed consent.

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Disclosure

All of the authors had no any personal, financial, commercial, or academic conflicts of interest separately.

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