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Tumour necrosis factor-related apoptosis-inducing ligand expression in patients with diabetic nephropathy

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Abstract

Objective: The objective of this study was to evaluate the expression profile of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in patients with diabetic nephropathy (DN).

Methods: A total of 126 Chinese subjects were enrolled in this study, including 42 patients with diabetes mellitus (DM), 42 patients with DN and 42 healthy controls. Real-time polymerase chain reaction was performed to analyze levels of *TRAIL* mRNA in peripheral blood mononuclear cells (PBMCs). Serum levels of soluble TRAIL (sTRAIL) and various cytokines were detected with a commercially available enzyme-linked immunosorbent assay kit.

Results: Compared with the control group, the levels of *TRAIL* mRNA in PBMCs and sTRAIL in sera were both significantly decreased in the DM and DN patients ($P < 0.05$). Conversely, levels of interleukin (IL)-1, IL-6, tumour necrosis factor-α and monocyte chemotactic protein-1 were higher in the DN group than in the control group. Serum levels of TRAIL positively correlated with *TRAIL* mRNA levels in all of the subjects examined ($P < 0.05$).

Conclusions: These results provide support and a theoretical basis for further research of TRAIL in regard to the pathogenesis of DN.

Keywords

Diabetic nephropathy, TRAIL, IL-1, TNF-α, IL-6

Introduction

As living standards and lifestyle changes have improved, the prevalence of diabetes mellitus (DM) has increased yearly worldwide.[1] It has been conservatively calculated that the number of DM cases will increase from 382 million in 2013 to 592 million in 2035.[2] In a large-scale epidemiological survey, the prevalence of DM was found to be 9.7% among 20-year-olds, type 2 diabetes (T2DM) accounted for approximately 90% of these T2DM cases, and 20–40% of T2DM patients experienced diabetic nephropathy (DN).[3] DN is a major chronic vascular complication of T2DM and it is also the most common single cause of end-stage renal disease worldwide. DN is also associated with increased cardiovascular mortality and represents a huge economic burden to society.[4] To date, the mechanism and development of DN have not been elucidated. However, there are a variety of mechanisms that have been found to contribute to the development of DN, including haemodynamic pathways, hyperglycaemia, inflammatory cytokines and genetic disposition.[5]

It has been demonstrated that inflammatory factors may play an important role in the pathogenesis of DN.[6–8] Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumour necrosis factor (TNF) ligand superfamily,[9] whose levels were reported to change in various inflammatory/autoimmune diseases, including systemic lupus erythematosus,[10] human immunodeficiency

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Table 1. Primer sequences of TRAIL and β-actin.

Name		Sequence	Amplicon size (bp)
TRAIL	Sense	5′-GAGAACTTCCCCATTGGACATC-3′	161
	Antisense	5′-GCATTTGATGTAGAACCCGCA-3′	
β-actin	Sense	5′-CACCCAGCACAATGAAGATCAAGAT-3′	317
	Antisense	5′-CCAGTTTTTAAATCCTGAGTCAAGC-3′	

TRAIL: tumour necrosis factor-related apoptosis-inducing ligand.

virus-1 viraemia[11] and rheumatoid arthritis.[12] In addition, experimental evidence has suggested that TRAIL could modulate the immune system, whereby it might potentially protect against the development and/or progression of DM.[13] In 2008, Lorz et al. reported that TRAIL expression was higher in the kidneys of diabetic patients than in the kidneys of healthy indiviuals.[14] In another study, serum levels of TRAIL (sTRAIL) in T2DM patients were found to be significantly lower compared to those of a healthy group.[15]

Despite these results, the relationship between TRAIL and DN remains unclear. What is more, previous studies did not clarify the relationship between the expression of TRAIL protein in peripheral blood mononuclear cells (PBMCs) and DN. Therefore, in our study, we analysed levels of *TRAIL* mRNA in PBMCs and sTRAIL in sera in healthy controls, DN patients and T2DM patients.

Materials and methods

Research subjects

Between August 2015 and June 2016, a total of 42 T2DM patients and 42 DN patients were treated at the First Affiliated Hospital of Wannan Medical College (Wuhu, China), and were enrolled in this study. In addition, 42 healthy individuals from the general population were enrolled to establish a control group. These controls were not related to the enrolled patients. Patients who had taken any type of anti-inflammatory drug and those with a past or present history of chronic illness such as tuberculosis, rheumatoid arthritis, other autoimmune diseases and type 1 diabetes (T1DM) were excluded.

All subjects gave written informed consent. This study was approved by the Ethics Committee of Wannan Medical College.

Biochemical measures

Two venous blood samples were collected from each participant. One sample was collected into a plastic tube and centrifuged for 15 min at 2500 rpm. The serum from this sample was immediately stored at −80 °C. PBMCs were obtained from the other sample, which was collected in a tube containing ethylenediaminetetraacetic acid. PBMCs were isolated with Ficoll-Hypaque density gradient centrifugation (TBD, Tianjin) and stored at −80 °C.

Serum levels of TRAIL and various cytokines

Serum levels of TRAIL (Senxiong Biotech, Shanghai, China), interleukin (IL)-1 (Beijing 4A Biotech Co., Ltd), IL-6 (CUSABIO, Wuhan, China), TNF-α (CUSABIO), monocyte chemotactic protein-1 (MCP-1) (CUSABIO) and hypersensitive C-reactive protein (hs-CRP) (CUSABIO) were measured by using commercially available enzyme-linked immunosorbent assays, according to the manufacturers' instructions.

TRAIL mRNA in PBMCs

Total RNA was extracted from isolated PBMCs with an RNA extraction kit, according to the manufacturer's instructions. Purity and yield of the RNA samples were subsequently measured with a spectrophotometer at 260 nm and 280 nm. Moloney murine leukemia virus reverse transcriptase was used to synthesize cDNA from the RNA samples. Real-time quantitative reverse-transcriptase polymerase chain reaction assays were subsequently conducted in an ABI-Prism 9700 sequence detector (Applied Biosystems, Foster City, USA) according to the following cycling conditions: 90°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Sequences of the primers used are listed in Table 1. Data were individually normalized to the mean of the relative expression level of β-actin. The results were analyzed by Sequence Detection Software (Applied Biosystems) and were calculated using the $2^{-\Delta\Delta CT}$ method.[16]

Statistical analysis

SPSS 13 statistical software was used to perform statistical analyses. Data are reported as the mean ± SD for the quantitative variables (age, sTRAIL, body mass index (BMI) etc.) and as percentages for the categorical variables (gender, smoking status, etc.). The study population was divided into: (a) a healthy control group (CNT), (b) a DM group and (c) a DN group. One-way analysis of variance (least significant difference method) or the χ^2 test were used to compare differences in demographic and clinical

Table 2. Characteristics of the study population (*n* (%) / $\bar{x}$ ± s).

Variable		CNT (*n* = 42)	DM (*n* = 42)	DN (*n* = 42)	*F*/$\chi 2$	*P*-value
Gender	Male	20 (47.62)	21 (50)	20 (47.62)	0.640	0.969
	Female	22 (52.38)	21 (50)	22 (52.38)		
Smoking	Yes	5 (11.90)	9 (21.43)	7 (16.67)	1.371	0.504
	No	37 (88.10)	33 (78.57)	35 (83.33)		
Drinking	Yes	6 (14.29)	8 (19.05)	6 (14.29)	0.475	0.788
	No	36 (85.71)	34 (80.95)	36 (85.71)		
Age		51.29 ± 7.04	56.48 ± 13.02	60.29 ± 11.90	9.640	0.001
BMI (kg/m^2)		23.54 ± 2.42	23.65 ± 3.62	25.07 ± 4.40	1.880	0.160

BMI: body mass index; CNT: healthy control; DM: diabetes mellitus; DN: diabetic nephropathy.

factors among the three groups. A multinomial logistic regression analysis was performed to identify whether any factors were independently associated with DN. Pearson correlation analysis was used to determine the relationship between *TRAIL* mRNA expression and various variables. *P*-values < 0.05 were considered statistically significant.

Results

Patient characteristics

The characteristics of our study population are summarized in Table 2. This population included 42 healthy volunteers (CNT) and 84 patients with T2DM, of whom 42 had DN. T2DM was diagnosed according to the World Organization criteria (1999). DN was diagnosed based on albumin excretion rate > 30 mg/24 h.

There were no statistically significant differences in the distribution of gender, smoking status, drinking status and BMI among the three groups ($P > 0.05$). The mean ages of the three groups were 60.29, 56.48 and 51.29 years, respectively, and there existed a statistical difference in age level ($P < 0.05$).

Baseline clinical and biochemical characteristics (Table 3)

After adjusting for age and gender, there was no significant difference in total cholesterol (T-Cho), low-density lipoprotein cholesterol and alanine transaminase among the three study groups ($P > 0.05$). In contrast, the levels of glucose (GLU), diastolic blood pressure, systolic blood pressure, triglycerides (TGs), serum creatinine (Cre) and uric acid in the DN group were higher than in the CNT group ($P < 0.05$). Conversely, the levels of high density lipoprotein cholesterol (HDL-C) and aspartate aminotransferase in the DN group were lower than in the CNT group ($P < 0.05$). Meanwhile, the levels of Cre and uric acid were higher in the DN group than in the DM group, and these differences were statistically significant ($P < 0.05$).

Lower levels of TRAIL *mRNA and sTRAIL in DN patients*

After adjusting for age and gender, significant differences in the levels of *TRAIL* mRNA in PBMCs and sTRAIL in sera were observed among the three study groups ($P < 0.001$). Further analysis with SNK-*q* tests revealed that *TRAIL* mRNA in PBMCs and sTRAIL in sera in the DN and DM patients were both lower compared with the CNT group, and that the differences were statistically significant ($P < 0.001$) (Table 4).

Levels of IL-1, IL-6, TNF-α, MCP-1 and hs-CRP were higher in DN patients

The serum concentrations of IL-1, IL-6, TNF-α and MCP-1 were found to be significantly elevated in the DN group compared to the CNT group ($P < 0.05$ for all of the cytokines examined). In contrast, the T2DM group exhibited significantly upregulated serum levels of IL-1 and no significant changes in the levels of IL-6, TNF-α or MCP-1 compared with the CNT group (Table 5).

Multinomial logistic regression

According to a multinomial logistic regression analysis, when age, sex, TRAIL mRNA and sTRAIL were taken as independent variables, patient age was found to positively correlate with DN, while the level of *TRAIL* mRNA in PBMCs and sTRAIL in sera negatively correlated with DN. However, after an additional adjustment for GLU, these relationships existed (Table 6).

Correlation between sTRAIL, TRAIL *mRNA and cytokine levels*

To examine the relationship between sTRAIL and other cytokines, correlation analyses were performed (Table 7). When these analyses were adjusted for GLU, sTRAIL was found to be positively associated with *TRAIL* mRNA levels

Table 3. Comparison of various parameters among the three groups ($\bar{x}$ ± s).

Parameter	CNT (*n* = 42)	DM (*n* = 42)	DN (*n* = 42)	*F*	*P*-value
GLU (mmol/L)	5.16 ± 1.10	9.94 ± 4.95[a]	10.37 ± 5.25[a]	19.027	< 0.001
SBP (mmHg)	120.20 ± 8.93	125.88 ± 16.25	137.90 ± 16.72[a,b]	10.624	< 0.001
DBP (mmHg)	75.43 ± 10.46	78.17 ± 10.54	81.90 ± 9.76[a]	4.271	0.016
T-Cho (mmol/L)	4.31 ± 1.06	4.63 ± 0.99	4.68 ± 1.42	0.440	0.645
TG (mmol/L)	1.13 ± 0.58	2.15 ± 1.78[a]	2.39 ± 1.48[a]	8.757	< 0.001
HDL-C (mmol/L)	1.40 ± 0.29	1.13 ± 0.22[a]	1.19 ± 0.30[a]	11.526	< 0.001
LDL-C (mmol/L)	2.36 ± 0.77	2.31 ± 0.89	2.31 ± 0.83	0.257	0.773
ALT (U/L)	19.62 ± 11.54	22.90 ± 13.10	22.26 ± 12.41	2.019	0.137
AST (U/L)	19.86 ± 7.83	14.31 ± 5.99[a]	16.69 ± 5.73[a]	7.355	0.001
Cre (μmol/L)	63.14 ± 15.31	60.37 ± 26.17	97.53 ± 58.66[a,b]	9.103	< 0.001
Uric acid (μmol/L)	291.73 ± 110.69	307.05 ± 91.65	362.91 ± 125.39[a,b]	3.075	0.050

ALT: alanine transaminase; AST: aspartate aminotransferase; Cre: serum creatinine; DPB: diastolic blood pressure; GLU: glucose; HDL-C: high-density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; SBP: systolic blood pressure; T-Cho: total cholesterol; TG: triglyceride.
[a]$P < 0.05$ versus CNT group.
[b]$P < 0.05$ versus DM group.

Table 4. Comparison of sTRAIL and TRAIL mRNA among three groups ($\bar{x}$±s).

Group	*N*	sTRAIL (pg/ml)	*TRAIL* mRNA
CNT	42	180.65 ± 66.54	2.57 ± 1.83
DM	42	157.10 ± 68.37	0.96 ± 0.32
DN	42	118.63 ± 55.99[a,b]	0.90 ± 0.35[a]
F		10.022	27.453
P		< 0.001	< 0.001

Adjustment of age and sex.
CNT: healthy controls; DM: diabetes mellitus; DN: diabetic nephropathy; sTRAIL: soluble tumour necrosis factor-related apoptosis-inducing ligand; TRAIL: tumour necrosis factor-related apoptosis-inducing ligand.
[a]$P < 0.01$ versus CNT group.
[b]$P < 0.01$ versus DM group.

($r = 0.263$, $P = 0.003$), and to be negatively associated with IL-1 ($r = -0.181$, $P = 0.043$), IL-6 ($r = -0.187$, $P = 0.036$) and MCP-1 ($r = -0.247$, $P = 0.005$) levels in all of the study groups. However, the relationships between *TRAIL* mRNA levels and sTRAIL and various cytokines were not statistically significant in each of the study groups individually. These results may be due to the small sample size of our study.

Discussion

It has been estimated that the number of people that will develop DM worldwide will reach 592 million in 2035.[2] Both T1DM and T2DM increase cardiovascular and microvascular complications. Microvascular complications are the lead cause of end-stage renal disease (DN).[17] Accumulating evidence from both clinical and laboratory studies has shown that inflammation plays a significant role in the occurrence and development of DN and other microvascular complications.[6–8,18–22] TRAIL is a recently discovered member of the TNF superfamily, which has been linked to apoptosis, anti-inflammatory responses and cell proliferation. Moreover, in a proteomics study of kidney function decline, a receptor of TRAIL, TRAIL-R2, was identified as the strongest marker of kidney function decline. However, despite an increasing number of studies showing that TRAIL is associated with diabetes and kidney disease, the precise role of TRAIL in DN remains unclear.

In the apolipoprotein E-/- mouse model, TRAIL deficiency has been shown to aggravate renal disease and insulin resistance.[23] Two case-control studies conducted in Turkey and China further demonstrated that levels of sTRAIL were higher in healthy individuals than in individuals newly diagnosed with T2DM.[24–25] The latter results are consistent with our study.[24–25] In another study that was conducted in Turkey, levels of sTRAIL were found to be significantly lower in patients with DN with foot ulcers compared to healthy subjects.[15] In animal studies, an injection of SDR5 to block TRAIL accelerated autoimmune diabetes.[26] Taken together, the results of these studies indicate that TRAIL may play a protective role in DN and DM. The present case-control study compared serum levels of sTRAIL and *TRAIL* mRNA expression in patients with DN, T2DM and control subjects. To our knowledge, this is the first study to evaluate an association between *TRAIL* mRNA levels in PBMCs that were collected from DN and T2DM patients. The levels of *TRAIL* mRNA in PBMCs and sTRAIL in sera were both significantly lower in the DN and T2DM patients compared to the CNT group, but some research results are not consistent with our results. For example, expression of TRAIL was found to be significantly higher in the kidneys of diabetic patients than in the kidneys of healthy individuals.[14] Another study reported

Table 5. Comparison of cytokine levels among the three groups ($\bar{x} \pm s$).

Variable	CNT ($n = 42$)	DM ($n = 42$)	DN ($n = 42$)	*F*	*P*-value
IL-1 (pg/ml)	6.49 ± 0.92	7.34 ± 0.90[a]	8.65 ± 1.48[a,b]	38.762	< 0.001
IL-6 (pg/ml)	18.70 ± 5.61	21.19 ± 7.59	23.89 ± 10.90[a]	4.130	0.018
TNF-α (pg/ml)	7.75 ± 3.07	9.64 ± 4.70	13.82 ± 7.71[a,b]	12.138	< 0.001
MCP-1 (pg/ml)	17.52 ± 9.79	22.67 ± 10.32	58.89 ± 54.68[a,b]	21.213	< 0.001
hs-CRP (ng/ml)	6.14 ± 5.64	11.66 ± 6.49[a]	13.31 ± 8.47[a]	8.458	< 0.001

Adjustment of age and sex.
CNT: healthy control; DM: diabetes mellitus; DN: diabetic nephropathy; hs-CRP: hypersensitive C-reactive protein; IL: interleukin; MCP-1: monocyte chemotactic protein-1; TNF-α: tumour necrosis factor-α.
[a]$P < 0.05$ versus CNT group.
[b]$P < 0.05$ versus DM group.

Table 6. Multinomial logistic analysis between TRAIL mRNA, sTRAIL and DN.

Variables	Model	B	OR	95% CI	*P*-value
Age	1	0.065	1.068	1.007–1.132	0.029
	2	0.120	1.127	1.035–1.228	0.006
TRAIL mRNA	1	−4.481	0.011	0.002–0.084	0.000
	2	−3.104	0.045	0.006–0.333	0.002
sTRAIL	1	−0.015	0.985	0.974–0.997	0.012
	2	−0.021	0.979	0.965–0.993	0.004
GLU	1	–	–	–	–
	2	1.104	3.015	1.516–5.996	0.002

Model 1: age, gender, *TRAIL* mRNA and sTRAIL; Model 2: Model 1 plus GLU.
CI: confidence interval; GLU: glucose; OR: odds ratio; sTRAIL: soluble tumour necrosis factor-related apoptosis-inducing ligand; TRAIL: tumour necrosis factor-related apoptosis-inducing ligand.

Table 7. Correlations between sTRAIL and cytokines.

Variable	*r*	*P*-value
IL-1	−0.181	0.043
IL-6	−0.187	0.036
TNF-α	0.068	0.454
MCP-1	−0.247	0.005
TRAIL mRNA	0.263	0.003

Adjustment of GLU.
IL: interleukin; MCP-1: monocyte chemotactic protein-1; TNF-α: tumour necrosis factor-α; TRAIL: tumour necrosis factor-related apoptosis-inducing ligand.

that patients with microalbuminuria or macroalbuminuria had significantly higher serum levels of TRAIL than individuals with normal albuminuria.[27] These findings suggest that TRAIL has the capacity to mediate both destructive and anti-inflammatory properties in DM and DN.

MCP-1 is an MCP that is directly involved in the inflammatory response of DN by activating the monocyte–macrophage system.[28] MCP-1 has been shown to stimulate the expression of downstream inflammatory factors (e.g., IL-1, IL-6, TNF-α, etc.) in renal tissue in a paracrine or autocrine manner, and this can aggravate both endothelial cell damage and the inflammatory response. In a study by Daniele et al.,[29] MCP-1 levels were found to positively correlate with the degree of inflammation, and the DM group received a higher inflammatory score than the control group. Serum levels of IL-6 are also considered to be an important marker of DN progression. When serum levels of IL-1 and IL-6 were examined in a group of DN patients from China, both factors were present at higher levels compared with healthy controls.[30] Similarly, Shikano et al.[31] found that levels of IL-6 in serum and urine were higher in their group of DN patients than in their control group. In the present study, serum concentrations of IL-1, IL-6, TNF-α and MCP-1 were significantly elevated in the DN patients compared to the healthy controls, and these data suggest that these four factors contribute to the promotion of inflammation in DN.

In early Newman epidemiological studies, DM patients were characterized by higher TG levels and lower HDL-C levels.[32] In the present study, the DM and DN groups had higher levels of TGs and lower levels of HDL-C compared to the CNT group. These observations may be the result of abnormal function by cell cholesterol transporter receptors. In an observational study conducted in Korea, cardiovascular events were shown to have a high prevalence in DM populations with a low HDL-C concentration.[33] In a retrospective cohort study conducted by Squillace et al.,[34] a high TG/HDL ratio was found to predict the risk of new-onset DM independent of other traditional risk factors. Taken together, these findings suggest that levels of HDL-C and TGs may represent important early markers of DN and DM.

In the present study, serum levels of TRAIL were found to weakly, yet positively, correlate with *TRAIL* mRNA levels in all of the patients examined. This result may be attributed to therapy. Moreover, it should be noted that the relationship between levels of *TRAIL* mRNA and sTRAIL among the DN and CNT groups did not reach statistical significance. This result may have been due to the small sample size of these groups in our study.

Additional limitations associated with this study include its cross-sectional design, which limited our ability to draw conclusions regarding the causality of the observed associations. Second, we did not collect glycated haemoglobin levels, glomerular filtration rate in the CNT group and patient medication data. Consequently, possible correlations between sTRAIL and the glycated haemoglobin level, glomerular filtration rate and the use of insulin, metformin or other glucose-lowering drugs could not be examined, and this represents the main limitation of our study. Thus, prospective studies, particularly those with more rigorous design, are needed to confirm the observations made in the present study.

In conclusion, our results support a role for TRAIL in DN and they provide a theoretical basis for further research of the pathogenesis of TRAIL in DN. However, due to the small size of our patient group, these results remain to be confirmed in a larger sample.

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