Associations between *INSR* and *MTOR* polymorphisms in type 2 diabetes mellitus and diabetic nephropathy in a Northeast Chinese Han population

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ABSTRACT. We explored the associations of *INSR* and *mTOR*, 2 key genes in the insulin signaling pathway, and the susceptibility to type 2 diabetes mellitus and diabetic nephropathy. Three single-nucleotide polymorphisms (SNPs) (rs1799817, rs1051690, and rs2059806) in *INSR* and 3 SNPs (rs7211818, rs7212142, and rs9674559) in *mTOR* were genotyped using the Sequenom MassARRAY iPLEX platform in 89 type 2 diabetes patients without diabetic nephropathy, 134 type 2 diabetes patients with diabetic nephropathy, and 120 healthy control subjects. Statistical analysis based on unconditional logistic regression was carried out to determine the odds ratio (OR) and 95% confidence interval (95%CI) for each SNP. Combination analyses between rs2059806 and rs7212142 were also performed using the χ^2 test and logistic regression. Among these 6 SNPs, 4 (rs1799817, rs1051690, rs7211818, and rs9674559) showed no

association with type 2 diabetes mellitus or diabetic nephropathy. However, rs2059806 in *INSR* was associated with both type 2 diabetes mellitus (P = 0.033) and type 2 diabetic nephropathy (P = 0.018). The rs7212142 polymorphism in *mTOR* was associated with type 2 diabetic nephropathy (P = 0.010, OR = 0.501, 95%CI = 0.288-0.871), but showed no relationship with type 2 diabetes mellitus. Combination analysis revealed that rs2059806 and rs7212142 had a combined effect on susceptibility to type 2 diabetes mellitus and diabetic nephropathy. Our results suggest that both *INSR* and *mTOR* play a role in the predisposition of the Han Chinese population to type 2 diabetic nephropathy, but the genetic predisposition may show some differences.

Key words: Diabetic nephropathy; *INSR*; *MTOR*; Polymorphisms; Type 2 diabetes mellitus

INTRODUCTION

Diabetes, particularly type 2 diabetes mellitus, continues to be a global health care problem. Kidney injury is a frequent and devastating complication of diabetes, particularly in chronic cases with poor nutritional control. Diabetic nephropathy is the major cause of chronic kidney disease and contributes significantly to increased morbidity and mortality among diabetic patients. It is also one of the most significant long-term complications and a leading cause of end-stage renal disease. The incidence and prevalence of end-stage renal failure in patients with type 2 diabetes has increased recently in both the Western world and Asia (Ritz, 1997). Investigations of candidate genes and genome-wide association studies have resulted in the identification of loci associated with genetic susceptibility to diabetic nephropathy (Tanaka and Babazono, 2005; Freedman et al., 2007) and type 2 diabetes mellitus (Wu et al., 2009; Liu et al., 2010; Ohshige et al., 2010). A genetic association study of type 2 diabetes mellitus and type 2 diabetic nephropathy will provide information regarding the pathogenesis of these 2 diseases, indicate those who are at risk, and provide new targets for therapy.

Genes involved in insulin signaling pathways are associated with susceptibility to type 2 diabetes mellitus (Laukkanen et al., 2004). Both *INSR* and *MTOR* play important roles in the insulin-signaling pathway (Avruch et al., 2005; Tremblay et al., 2005; Zick, 2005). The *INSR* gene encodes the insulin receptor, an important mediator between the extracellular and intracellular insulin signaling pathways, and is essential for the action of insulin. *INSR* is involved in both adipogenesis and beta-cell insulin secretion, but may also be involved in type 2 diabetes mellitus (Brunetti et al., 2011). Furthermore, *INSR* expression in the kidney changes during diabetes (Gatica et al., 2013), suggesting that it is a factor contributing to the development of type 2 diabetic nephropathy.

The metabolic actions of insulin are mediated by the insulin receptor/IRS/PI3-kinase signaling pathway (Ogawa and Kasuga, 2006). The protein mTOR belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family and regulates inhibition of PI3K, which generates peptide growth factors central to the diverse responses elicited by insulin (Rui et al., 2001; Harrington et al., 2005). mTOR serves as an intracellular sensor for energy metabolism,

nutrient availability, cellular and organism growth regulation, and metabolic adaptation to the environment. Furthermore, mTOR activation may play a pivotal role in the development of end-stage renal disease (Mori et al., 2009). Therefore, we suggest that mTOR signaling is a potential therapeutic target for type 2 diabetes mellitus and diabetic nephropathy (Sataranata-rajan et al., 2007).

To explore the relationship between polymorphisms in *INSR* and *mTOR* and the susceptibility to type 2 diabetes mellitus and type 2 diabetic nephropathy, 3 SNPs (rs1799817, rs1051690, and rs2059806) in *INSR* and 3 SNPs (rs7211818, rs7212142, and rs9674559) in *mTOR* were examined in a Han population from Northeast China using the MassARRAY iPLEX platform (Sequenom; San Diego, CA, USA).

MATERIAL AND METHODS

Subjects

A total of 134 diabetic nephropathy patients, 89 type 2 diabetes patients, and 120 healthy controls were enrolled between 2010 and September 2011 from Daqing Oil Field General Hospital in Northeast China. Type 2 diabetes patients were diagnosed according to the 2008 American Diabetes Association diagnostic criteria for diabetes and showed no evidence of renal dysfunction, with a disease course of more than 1 year. Type 2 diabetic nephropathy was confirmed in type 2 diabetes patients by renal biopsy in the absence of other causes of end-stage renal disease for more than 2 years. Control subjects were healthy, had no history of diabetes or other metabolic disorders, and were enrolled in the hospital during the same period. The study protocol was approved by the Clinical Research Ethics Committee of Nan-fang Hospital, Southern Medical University. All subjects provided informed consent before participating in the study.

DNA extraction

Peripheral blood samples were drawn from the participants at Daqing Oil Field General Hospital in Northeast China. The samples were delivered frozen by express mail to the School of Biotechnology, Southern Medical University, Guangzhou, Guangdong Province, China, and stored at -70°C. Genomic DNA was extracted from 200 μL peripheral blood using a Genomic DNA Purification kit (Tiangen Biotech; Beijing, China), according to the manufacturer instructions, and stored at -70°C until use.

Genotyping

In accordance with the primary literature and the Single Nucleotide Polymorphism Database, rs1799817, rs1051690, and rs2059806 in *INSR* and rs7211818, rs7212142, and rs9674559 in *MTOR* were selected as candidate SNPs. All SNPs were genotyped using the SEQUENOM MassARRAY matrix-assisted laser desorption/ionization time-of-flight mass spectrometry platform (Sequenom; San Diego, CA, USA). Primers were designed using a semi-automated method (Assay Design 3.1, Sequenom). Detailed information and primer sequences of the SNPs are summarized in Table 1.

Table 1. Information and primer sequences of SNPs examined.

rsID	Chr	Chr position	Gene	Location	SNP type	1st-PCRP[a]	2nd-PCRP	Extension primer
rs1799817	Chr 19	7125297	*INSR*	His1085His	C/T	ACGTTGGATGGTCATGAAGGGCTTCACCTG	ACGTTGGATGAAAGGGAAGGGTCAGGAAAG	ACCACCCCACTGGACTCACCAC
rs1051690	Chr 19	7116963	*INSR*	UTR-3	A/G	ACGTTGGATGAGGATTCTCACGACTCTACC	ACGTTGGATGTGGTAACCAAACGAGTCCAC	ACGATCTCTGAACTCCA
rs2059806	Chr 19	7166376	*INSR*	Ala550Ala	A/G	ACGTTGGATGGCCCTTATCAGAATGTGACG	ACGTTGGATGAATGTCTACCACCGTCCAAC	CAACTGTTGGAACCACA
rs7211818	Chr 17	78688903	*MTOR*	Intron	A/G	ACGTTGGATGGCTATGTTGTCTCTGTTGCC	ACGTTGGATGAACTCAAAGCAGAAGGAAAG	AGCGAAGGAAAGAAATAACAAAC
rs7212142	Chr 17	78623941	*MTOR*	Intron	A/G	ACGTTGGATGGAAAGAAAGGCATGGTTCTG	ACGTTGGATGGCTCTAATGGACTGAAACCC	TCTAGTCAACAGGGCTGCCATC
rs9674559	Chr 17	78715608	*MTOR*	Intron	A/G	ACGTTGGATGAATGGCTGAAGCCACGGGAT	ACGTTGGATGCTCTACACACCACCACCGAAC	AGCAGGCCTCGACTGGCCTCTCC

[a]PCR primer, 1st-PCRP means sense PCR primer, 2nd-PCRP means reverse PCR primer.

Statistical analyses

Hardy-Weinberg equilibrium for the 6 SNPs in the control group was assessed using the online tool SNPStats (http://bioinfo.iconcologia.net/SNPstats). Significant differences in genotype distributions between diabetic cases and controls were evaluated by χ^2 analysis. Associations between the polymorphisms and disease risk were assessed using odds ratios (ORs) and 95% confidence intervals (95%CIs) with binary logistic regression analyses, adjusted for age and gender. Statistical tests were implemented using the SPSS 13.0 software (SPSS, Inc.; Chicago, IL, USA). Linear regression analysis was used to assess the associations between genotype and clinical and biochemical characteristics. Combination analyses between rs2059806 and rs7212142 were carried out using both the χ^2 test and a logistic regression.

RESULTS

The characteristics of the 3 groups are shown in Table 2. A comparison among the 3 groups showed that age, body mass index, systolic blood pressure, diastolic blood pressure, and the fasting plasma glucose level were significantly different ($P < 0.05$). There were significant differences in the urine protein, urine glucose, urea, and creatinine levels between the diabetic nephropathy and type 2 diabetes groups ($P < 0.001$), while gender and CO_2 binding were very similar ($P = 0.520$, $P = 0.598$). Glycated hemoglobin was generally different ($P = 0.054$) between the 2 groups.

Table 2. Clinical and biochemical characteristics of the study subjects.

	Control	T2DM*	DN**
Male/Female	61/59	52/37	75/59
Age (years)	$52.7 \pm 6.44^\dagger$	56.80 ± 11.87	59.2 ± 12.35
BMI (kg/m^2)	$23.38 \pm 2.74^\dagger$	25.02 ± 3.40	25.48 ± 4.53
SBP (mmHg)	$117.67 \pm 11.63^\dagger$	132.96 ± 19.81	146.69 ± 29.14
DBP (mmHg)	$78.58 \pm 7.81^\dagger$	80.40 ± 11.27	82.67 ± 14.41
Fasting plasma glucose (mM)	$4.77 \pm 0.48^\dagger$	9.58 ± 3.78	10.75 ± 6.55
Urine protein			
-	118 (100.00%)	75 (88.23%)	17 (13.60%)
+	0 (0.00%)	8 (9.41%)	33 (26.40%)
++	0 (0.00%)	2 (2.35%)	23 (18.40%)
+++	0 (0.00%)	0 (0.00%)	52 (41.60%)
Unknown	2	4	9
Urine sugar			
-	118 (100.00%)	31 (36.47%)	45 (35.43%)
+	0 (0.00%)	7 (8.23%)	23 (18.11%)
++	0 (0.00%)	1 (1.18%)	13 (10.24%)
+++	0 (0.00%)	22 (25.88%)	35 (27.56%)
++++	0 (0.00%)	24 (28.24%)	11 (8.66%)
Unknown	2	4	7
Glycated hemoglobin	NA^a	8.48 ± 3.29	7.70 ± 2.08
Urea (mM)	NA	6.53 ± 2.15	11.75 ± 8.73
CO$_2$ binding (mM)	NA	25.29 ± 3.89	24.98 ± 4.49
Creatinine (μM)	NA	63.26 ± 20.15	198.63 ± 236.34

*Type 2 diabetes patients without diabetic nephropathy. **Type 2 diabetes patients with diabetic nephropathy. †Data are reported as means ± SD values. ^{a}NA means we did not collect the information.

The analysis of the 6 SNPs is shown in Table 3. The genotype distributions of the 4 SNPs rs1799817, rs1051690, rs205980, and rs7212142 were consistent with Hardy-Weinberg equilibrium in the control group ($P > 0.05$). Genotype frequencies for rs2059806 were sig-

nificantly different among the 3 groups (P = 0.043). The frequencies of genotypes GG and GA-AA of rs2059806 were 56.3 and 45.0% in controls, 71.1 and 28.9% in the type 2 diabetes group, and 54.9 and 45.1% in the diabetic nephropathy group, respectively. Compared with healthy controls, the GA-AA genotype of rs2059806 decreased the risk of type 2 diabetes mellitus, with an OR of 0.506 (95%CI = 0.274-0.935, P = 0.030) after adjusting for age and gender. However, the GA-AA genotype showed an increased risk of diabetic nephropathy (OR = 1.892, 95%CI = 1.047-3.418, P = 0.035). The SNPs rs1799817 and rs1051690 in *INSR* showed no differences among the 3 groups (Table 3).

For rs7212142,the distribution of GG-AA and GA differed among the 3 groups (P = 0.037). Of the control group, 57.1% of individuals carried genotype GG-AA and 42.9% had genotype GA, which was not significantly different from the type 2 diabetes group (46.6% GG-AA and 53.4% GA) or diabetic nephropathy group (64.1% GG-AA and 35.9% GA). However, compared with type 2 diabetes patients, the GA genotype of rs7212142 decreased the risk of diabetic nephropathy (OR = 0.501, 95%CI = 0.288-0.871, P = 0.014; Table 3).

To evaluate the combined effect of rs2059806 and rs7212142, the recombination of rs2059806 and rs7212142 was analyzed using an overall χ^2 test using logistic regression. There were significant differences between genotypes (Table 4); the combined genotype GA/AA-GG/AA showed greater protection against type 2 diabetes mellitus (OR = 0.289, 95%CI = 0.116-0.721, P = 0.008) and an increased risk of diabetic nephropathy (OR = 4.29, 95%CI = 1.17-10.63, P = 0.002) compared with GA-AA of rs2059806. Any such combined effect may be caused by their biological interaction and the *INSR* and *MTOR* genes may function within a single pathway.

DISCUSSION

Diabetic nephropathy is a progressive renal disease and represents a serious late complication of diabetes (Lehmann and Spinas, 1995); 20-50% of type 2 diabetic cases will develop into diabetic nephropathy. Multiple factors contribute to the pathogenesis of diabetic nephropathy resulting from metabolic abnormalities, hemodynamic alterations, and various growth and genetic factors (Parving et al., 1996). Insulin signaling occurs through the mitogen-activated protein kinase and PI3K pathways, via the insulin receptor in podocytes, and remodels podocyte cells (Welsh et al., 2010). Mutations in the insulin receptor may play roles in the pathogenesis of type 2 diabetes mellitus and diabetic nephropathy. The PI3K/AKT/*MTOR* pathway is important in diabetic nephropathy and *MTOR* signaling, and is activated in early diabetic nephropathy (Sakaguchi et al., 2006). mTOR is expressed primarily in renal podocytes, resulting in a number of deleterious effects such as hypertrophy of podocytes, which is critical for the development of diabetic nephropathy (Pagtalunan et al., 1997; Inoki et al., 2011).

In the present study, rs1799817 in exon 17 of *INSR* was not associated with disease. However, in South Indian individuals, this SNP is associated with type 2 diabetes mellitus (Bodhini et al., 2012). Within Mexican ethnic groups, rs2059806 showed no association with type 2 diabetes (Flores-Martinez et al., 2004). However, in our study, this SNP was not only associated with type 2 diabetes mellitus, but also with type 2 diabetic nephropathy. Furthermore, opposite associations were observed between this SNP and 2 other diseases. Compared with the AA genotype, the GA-AA genotype was associated with a 1.89-fold increased risk of diabetic nephropathy (P = 0.018, OR = 1.89, 95%CI = 1.05-3.42). The GA-AA genotype was a protective factor for type 2 diabetes mellitus (P = 0.033, OR = 0.51, 95%CI = 0.27-0.94).

Table 3. Analysis of all SNPs in *INSR* and *mTOR* among 3 groups.

Gene	rsID	Model	Genotype	Normal (%)	T2DM (%)	DN (%)	Control *vs* T2DM			Control *vs* type 2 DN			T2DM *vs* type 2 DN		
							P value*	OR (95%CI)†	P value**	P value	OR (95%CI)	P value	P value	OR (95%CI)	P value
INSR	rs1799817	Codominant	CC	40 (33.3%)	33 (38.4%)	49 (36.6%)	0.173	1		0.558	1		0.616	1	
			CT	54 (45.0%)	43 (50.0%)	63 (47.0%)		0.91 (0.48-1.72)	0.776		0.91 (0.50-1.63)	0.738		0.97 (0.53-1.76)	0.919
			TT	26 (21.7%)	10 (11.6%)	22 (16.4%)		0.50 (0.21-1.21)	0.123		0.74 (0.35-1.57)	0.434		1.47 (0.61-3.55)	0.388
	rs1051690	Dominant	GG	113 (94.2%)	84 (94.4%)	126 (94.0%)	0.947	1		0.963	1		0.912	1	
			GA-AA	7 (5.8%)	5 (5.6%)	8 (6.0%)		0.91 (0.27-3.06)	0.873		1.37 (0.46-4.05)	0.570		1.15 (0.36-3.69)	0.818
	rs2059806	Dominant	GG	67 (56.3%)	59 (71.1%)	73 (54.9%)	**0.033**	1		**0.051**	1		**0.018**	1	
			GA-AA	52 (43.7%)	24 (28.9%)	60 (45.1%)		0.51 (0.27-0.94)	**0.030**		0.94 (0.56-1.60)	0.823		1.89 (1.05-3.42)	**0.035**
MTOR	rs7211818	Overdominant	AA-GG	14 (11.7%)	17 (19.5%)	14 (10.4%)	0.117	1		0.757	1		0.057	1	
			GA	106 (88.3%)	70 (80.5%)	120 (89.6%)		0.51 (0.23-1.13)	0.096		1.57 (0.67-3.66)	0.301		2.14 (0.99-4.63)	0.054
	rs7212142	Overdominant	GG-AA	68 (57.1%)	41 (46.6%)	84 (64.1%)	0.133	1		0.259	1		**0.010**	1	
			GA	51 (42.9%)	47 (53.4%)	47 (35.9%)		1.54 (0.87-2.72)	0.138		0.89 (0.52-1.54)	0.679		0.50 (0.29-0.87)	**0.014**
	rs9674559	Codominant	AA	75 (64.1%)	65 (76.5%)	90 (71.4%)	0.063	1		0.362	1		0.503	1	
			GA	21 (17.9%)	6 (7.1%)	15 (11.9%)		0.46 (0.15-1.47)	0.192		0.63 (0.24-1.62)	0.332		1.61 (0.50-5.19)	0.425
			GG	21 (35.7%)	14 (25.9%)	21 (38.4%)		0.76 (0.35-1.65)	0.483		0.92 (0.45-1.87)	0.819		1.14 (0.53-2.43)	0.737

†OR (95%CI) was adjusted for age, the bold values indicate P < 0.05. *P value was analyzed by χ^2 analysis between 2 groups. **P value was analyzed by logistic regression.

Table 4. Combined analysis of rs2059806 and rs7212142 among 3 groups.

Genotype	Control	T2DM	DN	P value*	Control *vs* T2DM			Control *vs* type 2 DN			T2DM *vs* type 2 DN		
					P value**	OR (95%CI)†	P value***	P value	OR(95%CI)	P value	P value	OR (95%CI)	P value
GG-GA	32 (27.11%)	29 (35.37%)	26 (20.00%)	0.038	0.036	1		0.573	1		0.008	1	
GA/AA-GG/AA	33 (27.97%)	9 (10.98%)	37 (28.46%)			0.29 (0.12-0.72)	0.008		1.05 (0.50-2.22)	0.899		4.29 (1.17-10.63)	0.002
GA/AA-GA	19 (16.10%)	15 (18.29%)	21 (16.15%)			0.73 (0.30-1.74)	0.473		1.25 (0.53-2.92)	0.615		1.49 (0.63-3.50)	0.362
GG-GG/AA	34 (28.81%)	29 (35.36%)	46 (35.0%)			0.84 (0.41-1.74)	0.642		1.41 (0.69-2.91)	0.346		1.74 (0.85-3.50)	0.132

†OR (95%CI) was adjusted for age, the bold values indicate $P < 0.05$. *P value was analyzed by R x C. **P value was analyzed by χ^2 analysis between two groups. ***P value was analyzed by logistic regression.

These results suggest that the GA-AA genotype plays opposing roles in the development of type 2 diabetes mellitus and diabetic nephropathy; in the development of diabetes, it is a protective factor, while this genotype increases the risk of diabetic nephropathy development. No other study has reported a susceptibility effect of rs2059806 polymorphism on type 2 diabetes mellitus or diabetic nephropathy. These opposite results may be because of different pathogenic mechanisms of type 2 diabetes mellitus and diabetic nephropathy, possibly an innocent bystander of hyperinsulinemic states (Feliers et al., 2001) and hyperglycemia (Coward and Saleem, 2011). Multiple observations, such as elevated plasma and uric acid levels in type 2 diabetes, the reabsorption of which is correlated with the plasma insulin level, suggest that the kidney may not be insulin-resistant. As an index of insulin sensitivity, the insulin signaling pathway was activated in the renal cortex in type 2 diabetic mice (Feliers et al., 2001). Hyperinsulinism can lead to abnormal vasoreactivity, induce glomerular hyperfiltration, and contribute to albuminuria (Groop et al., 2005). Hyperglycemia can drive glucotoxicity of the kidney by increasing polyol and hexosamine levels, generating advanced glycation end products, activating signaling molecules, such as PKC and transforming growth factor-β and plays a critical role in hyperglycemia-induced kidney damage (Schrijvers et al., 2004; Dronavalli et al., 2008). High extracellular glucose initiates podocyte apoptosis, a key feature of diabetic glomerular damage *in vitro* and *in vivo* by inducing intracellular reactive oxygen species (Susztak et al., 2006). Glycemic control is crucial for diabetic nephropathy prevention and treatment (Sakaguchi et al., 2006; Welsh et al., 2010). Therefore, strong cases can be made for hyperinsulinism and glycemia as the central pathogenic insults to the kidney. To highlight this, variation in insulin receptors is key to insulin action and *INSR* may be related to type 2 diabetic nephropathy.

mTOR plays an important role in many renal diseases, serving as an important modulator of renal repair and recovery (Lieberthal and Levine, 2009). In the present study, *MTOR* showed a relationship with only type 2 diabetic nephropathy. In an overdominant model, the GA genotype of rs7212142 was significantly protective against diabetic nephropathy, with an OR of 0.501 (95%CI = 0.288-0.871, P = 0.014), compared with the type 2 diabetes mellitus group. There was no significant difference between controls and the type 2 diabetes mellitus group or between controls and the diabetic nephropathy group. Activation of mTOR signaling caused renal hypertrophy at an early stage of diabetes (Sakaguchi et al., 2006), and high mTORC1 activation led to mislocalization of nephrin, which is a podocyte cell-surface protein important for the function of the renal filtration barrier (Lu et al., 2011). In diabetic rats, treatment with a low-dose sirolimus inhibitor of *MTOR* had a beneficial effect on diabetic kidneys, but did not modify symptoms induced by diabetes (Lloberas et al., 2006). Overall, *MTOR* plays an important role in the pathogenesis of diabetic nephropathy.

MTOR is important for podocytes and may be regulated by an insulin receptor by activating PI3K signaling. The combined effects of the 2 SNPs, rs2059806 and rs7212142, were analyzed. The effect of the combined genotype GA/AA-GG/AA was different from GA-AA of rs2059806 and GG-AA of rs7212142, respectively, but was similar to the GA-AA genotype of rs2059806. The genotype GA/AA-GG/AA showed opposite effects on type 2 diabetes mellitus (OR = 0.289, 95%CI = 0.116-0.721) and diabetic nephropathy (OR = 4.285, 95%CI = 1.172-10.631), which was not simply an additive or multiplicative effect; this may have arisen from the interaction between *INSR* and *MTOR*. This suggests that *INSR*, along with *MTOR*, contribute to type 2 diabetes mellitus and diabetic nephropathy.

In conclusion, rs2059806 of *INSR* was associated with both type 2 diabetes mellitus and type 2 diabetic nephropathy,while rs7212142 of *mTOR* was associated with type 2 diabetic nephropathy but not type 2 diabetes mellitus. Combination analysis revealed that rs2059806 and rs7212142 have a cumulative effect on the susceptibility to type 2 diabetes mellitus and diabetic nephropathy. Whether the combined effects are caused by allele-allele interactions requires further investigation.

Conflicts of interest

The authors declare no conflict of interest.

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