

MALAT1 Gene Expression in Diabetic Patients with or without Nephropathy

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Abstract

Background: One of the main causes of end-stage renal disease worldwide is diabetic nephropathy, a catastrophic microvascular sequel of diabetes. The pathophysiology of DN must thus be urgently investigated in order to develop appropriate remedies. MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) is anticipated to be a novel target for the detection and therapy of diabetic nephropathy.

Objectives: This research primarily sought to verify the expression of the circulating lncRNA MALAT1 in peripheral blood mononuclear cells (PBMCs) of diabetic patients, diabetic nephropathy, and healthy controls and assess its relation to disease related criteria

Patients and methods: 50 diabetic volunteers in this trial sought medical attention at the Outpatient Clinic of Endocrinology & DM at Sohag University Hospital, between January 2022 and June 2022 and compare them with 25 apparently healthy persons. The expression of the lncRNA Malat1 was measured using quantitative real-time PCR (qPCR) in 25 people with diabetes, 25 people with DKD, and 25 healthy controls. The clinical relevance of the observations was then assessed.

Results: As compared to control, lncRNA MALAT1 expression in peripheral blood was substantially higher in the diabetics and DKD groups. Spearman correlation showing significant correlation between RQ and duration of DM as $P < 0.05$, also showing significant correlation between RQ and A/C ratio as $P < 0.05$, there was positive moderate correlation between RQ and HbA1C and showing significant negative correlation between RQ and eGFR as $P < 0.05$.

Conclusion: The best technique to identify diabetic nephropathy may be to combine the detection of urine ACR, serum creatinine, and eGFR with diabetes mellitus. lncRNA Malat1 is substantially expressed in DKD patients compared to diabetics and an apparently healthy group.

Keywords: DKD ; lnc RNA ; MALAT1 ; Gene expression.

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Introduction

A prevalent chronic metabolic condition that has a major negative impact on human health is diabetes mellitus (DM) (Galicia-Garcia et al., 2020). Diabetic nephropathy (DN), a severe microvascular consequence of diabetes, is one of the main causes of end-stage renal disease worldwide (Dagar et al., 2021). Unfortunately, DN cannot be stopped from progressing because of its complex pathogenic processes, which puts a tremendous financial burden on many families and society (Tang et al., 2021).

Long noncoding RNAs (lncRNAs) contribute to the pathophysiology and physiology of several disorders, including DN, and have more than 200 nucleotides. (Jiang and Ning, 2020). Lung cancer is where metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was initially discovered. (He et al., 2018). Several investigations have also revealed that MALAT1 has a function in transcription. (Arun et al., 2020).

As an illustration, in vivo cross-linking research has demonstrated MALAT1 binding to the chromatin of genes that are actively transcribed and controlling their expression at the transcriptional level. (West et al;2014). For instance, Zhang et al. (2019) research's work. In human renal tubular epithelial cells, hyperglycemia causes epithelial-to-mesenchymal transition and pyroptosis, which were mediated by MALAT1 (Liu et al., 2020). Diabetes-related microvascular problems such DKD and diabetic retinopathy are triggered by inflammation, which is modulated by the lncRNA MALAT1 (Thomas A et al;2016). Further researches are required to demonstrate the role of MALAT1 in DN as there are still few studies on the subject. This work mainly aims at identifying circulating expression of lncRNA MALAT1 in PBMCs of diabetic patients, diabetic nephropathy, and healthy controls and assess its relation to disease related criteria, analyse the function of

MALAT1 in DKD and its probable pathogenesis, which might be benefit for the identification of reliable biomarkers and novel treatment targets.

Patients and Methods

Subjects ; this case-control research included 75 Egyptian adult subjects (46 females and 29 males) who sought medical consultation at the Outpatient Clinic of Endocrinology & DM at Sohag University Hospital in Sohag , Egypt, between January 2022 and June 2022, full consent taken from participants ,the Sohag faculty of medicine's research ethics committee gave its approval to the study plan .The study's participants were split into three groups: Group I consists of 25 cases of (DM), with a mean age of **53.24±10.91** years (12 males and 13 females). Group II, there were 25 cases of DKD, with a mean age of **54.92±13.276** years (12 men and 13 females) . Group III: 25 healthy non-diabetic volunteers with a mean age of **31.24±13.206** years were enlisted as the control group (5 male and 20 female). By utilizing the Anova test, it was determined that there was a significant mean age difference between the study's groups (P value (0.05)).

According to American Diabetes Association (ADA) standards published in 2014 (fasting blood glucose level > 126 mg/dl or glycated haemoglobin (HbA1c) > 6.5%), DM was identified (Association, 2014) and without other serious illnesses including ;cancer, infection or pharmacological therapy that impact glycemia or albuminuria . Patients with DKD must fulfill the enrolment requirements for DKD diagnostic standards, which include persistent microalbuminuria , a high urine albumin creatinine ratio, and the exclusion of alternative causes of nephropathy such gout, systemic lupus, or individuals with cardiac conditions. By testing fasting blood glucose and HbA1c in the control group we rule out diabetes.

Anthropometrical and biochemical parameter measurements

Study participants were instructed to arrive fasting and without engaging in any physical activity. Early in the morning, peripheral blood (5 ml) and midstream urine samples were taken from all research participants. All trial participants provided general information that included age, type of DM, duration of DM, body height and body weight. Clinical examination was done to assess blood pressure and to exclude other causes of nephropathy, patients undergoes abdominal sonographic examination to assess kidney state, the method used to determine body mass index (BMI) was $\text{body weight}/(\text{body height})^2$ (kg/m^2). Three milliliters of venous blood used for laboratory detection of fasting blood glucose, serum creatinine (Cr) by Colorimetric reaction (Jaffe reaction), glycated hemoglobin (HbA1c) using the sandwich immunodetection technique by fluorescence immunoassay technology (Fineware, CAT NO.0350 from Texas) in the blood that had been treated with EDTA, glomerular filtration rate estimation: The MDRD (Modification of Diet in Renal Disease) recently proposed an equation that directly estimates GFR as follows: $\text{eGFR} (\text{ml}/\text{min}/1.73\text{m}^2) = 175 \times \text{standardized S}(\text{cr})^{-1.154} \times \text{age}^{-0.203} \times 1.212$ (if black) $\times 0.742$ (if female). Calculating microalbumin (UMA) urine creatinine, and albumin creatinine ratio: A sample of fasting midstream urine was taken, and the supernatant was obtained by centrifuging the mixture for 10 minutes at 3000 rpm. The measurement of microalbuminuria came next (MAU) using the One Step MAU Quick Quantitative Test (Fineware) catalog No.W206 and urine Creatinine chemically measured by using JAFEE LOT 42057, Biosystems.

The formula for calculating ACR was then used: $\text{ACR} = \text{urine albumin}/\text{creatinine}$. ACR readouts below 3 mg/g were deemed normal, whereas above 3 mg/g were deemed abnormal (high).

Quantitative real-time polymerase chain reaction (qRT-PCR) and gene expression assay

I-Total RNA Extraction

Two milliliters of venous blood used for RNA extraction, typical Ficoll-Hypaque density-gradient centrifugation procedure was used to separate the PBMCs (peripheral blood mononuclear cells). Then total RNA was extracted using the QIA amp RNA blood Mini Kit (QIAGEN, Catalog No. 52304 USA). According to the manufacturer's instructions. Nano Drop™ 8000 Spectrophotometer (Applied Biosystems, USA) was used to assess RNA concentration and purity, Extracted RNA is liable for degradation by RNAase so must be stored frozen in -80°C and to be converted to cDNA as early as possible.

II-Synthesis of cDNA from RNA by reverse transcription

Done by: RevertAid First Strand cDNA Synthesis Kit K1622 by Thermo Scientific, Lot 01007924. Reverse transcription was then performed in accordance with the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, The created cDNA was allowed to cool before being frozen and kept at -20°C until use.

RT-PCR analysis for gene expression assay

qRT-PCR was performed using the TaqMan® Gene Expression Assays for MALAT1 FAM™ dye-labeled MGB probe Part NO 4453320 were used, TaqMan® Endogenous Controls assay for GAPDH VIC dye-labeled MGB probe Part NO 4326317 was used as internal controls to normalize variations in total RNA levels. Quantitative PCR was performed with 2 μl of cDNA, 1 μl of each

assay (MALAT1 & GAPDH) and 10 µl of taqman gene expression assay Master Mix (Applied Biosystems - Lithuania) (LOT: 00989607) , 6 µl of nuclease free water. Real-Time PCR was employed by Step One/Step One Plus Real-Time PCR System from Applied Biosystems , every PCR run was carried out in triplicate. Relevant gene expressions were calculated using a comparative threshold cycle approach. Ct was the cycle threshold used by the $2^{-\Delta\Delta Ct}$ technique to determine relative RNA level as follow ; The

$2^{-\Delta\Delta Ct}$ value "RQ", which was determined using the formula: $\Delta\Delta Ct = ((Ct\ MALAT1 - Ct\ GAPDH)_{DM} - (Ct\ MALAT1 - Ct\ GAPDH)_{control})$, represented the degree of lncRNA MALAT1 expression.

The RT-PCR result presented in form of : amplification plots that showing MALAT1 expression to GAPDH as in (Fig.1), and gene expression plot that showing the different expression (RQ) between samples as showed in (Fig.2).

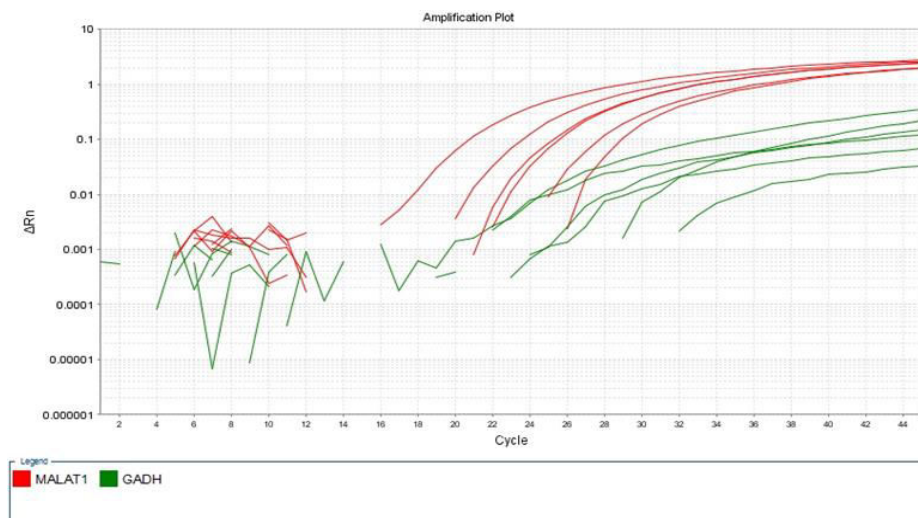


Fig.1. Amplification plot of a run of 6 samples, 5 case , 1 control showing MALAT1 expression to GAPDH



Fig.2. Gene expression plot showing the different expression (RQ) between samples

Statistical analysis

The acquired data was coded and checked prior to automated data entry. Statistical Package for the Social Science (SPSS) version 23 was used to statistically analyse the obtained data and present the results in tables and graphs. The Kolmogorov-Smirnov test was used to determine the data's normality. The graphs were produced using a chart maker. Student t test for parametric data and Mann Whitney for non-parametric data. The

correlation between parameters was determined using Spearman's rho test for non-parametric data. P 0.05 denoted statistical significance in each analysis.

Results

There is considerable distinction between the three groups in age (as diabetic nephropathy was detected mainly in elderly diabetics with very long duration of diabetes in our study) as shown in (Table.1)

Table.1. Difference in Age , Gender ,BMI ,Systolic and Diastolic blood pressure between study groups

Variables		Control (N=25)	Diabetic only (N= 25)	Diabetic nephropathy (N= 25)	p-value
Age by years	Mean \pm SD	31.24 \pm 13.206	53.24 \pm 10.91	54.92 \pm 13.276	
	RANGE	(15-64)	(35- 77)	(27-83)	<0.0003
Gender No. (%)	Female	20(80%)	13 (52%)	13 (52%)	0.064
	Male	5 (20.4%)	12 (48%)	12 (48%)	
BMI	Mean \pm SD	25.5 \pm 2.85	25.1 \pm 2.56	25.3 \pm 4.08	0.792
DBP	Mean \pm SD	117 \pm 14.8	119.8 \pm 18.6	126.8 \pm 19.7	0.68
SBP	Mean \pm SD	76.8 \pm 11.4	76.8 \pm 11.4	80.2 \pm 9.06	0.53

*p values were derived by one-way ANOVA, Independent T test, or CHI Square test as appropriate. BMI : body mass index; DBP: diastolic blood pressure; SBP : systolic blood pressure

There is very highly significant difference P<0.001 of duration of DM and urine A/C ratio between 2 study groups

(diabetics and diabetic nephropathy patients) as showed in (Table.2) .

Table.2. Mann Whitney test of Duration of diabetes in years and urine Albumin creatinine ratio between the cases groups

Parameter		diabetic n=25	Diabetic nephropathy patients N=25	P value
Duration Of DM in years	Median(IQ)	3 (1-5)	12 (4-17)	0.000***
Urine A/C ratio	Median(IQ)	0.14 (0.12-0.2)	0.5 (0.4-0.6)	0.000***

Mann Whitney * P< 0.01 (very significant), **P< 0.05 (significant), and ***P< 0.001 (very highly significant) Non-significant, or p > 0.05

Comparison of other laboratory parameters among the studied population, by using the nonparametric Mann Whitney test, there is no significant difference in s. creatinine levels between the Control and solely diabetic groups. Using the non parametric test Mann Whitney, there was a significant difference in HBA1c, serum creatinine, eGFR, and RQ between controls and diabetic nephropathy (P 0.05). While there was no significant difference in eGFR as P > 0.05 between diabetic nephropathy patients and diabetic only patients, as shown in (Table.3)

Table 3. Comparison of HbA1C, creatinine, eGFR, and RQ between the studied groups

Parameters		Control (N=25)	diabetic (N=25)	Diabetic nephropathy patients (N=25)	P value (control vs. diabetic)	P value (control vs. diabetic nephropathy)	P value (diabetic vs. diabetic nephropathy)
HBA1C	Mean ±SD	5.06±1.08	7.7±2.02	9.6±1.6	0.000***	0.000***	0.001**
	Median(IQ)	4.5 (4.5-6.1)	7.1 (6.8-8.2)	9.95 (8.5-10.9)			
Serum Creatinine	Mean ±SD	1.26±1.4	1.0±0.2	1.2±0.317	0.74 NS	0.009**	0.006**
	Median(IQ)	1(0.9-1.0)	1.0 (0.9-1)	1.3 (1-1.4)			
eGFR	Mean ±SD	106.2±5.99	99.04±7.3	95.8±6.9	0.000***	0.000***	0.113NS
	Median(IQ)	105 (100-112)	99.3(96.2-100)	96.3 (89.0-100)			
RQ	Mean ±SD	0.3±0.33	1.69±0.99	6.5±2.91	0.000***	0.000***	0.000***
	Median(IQ)	0.09(0.08-0.55)	1.9(0.85-2.4)	6.04(4.15-9.05)			

Mann Whitney * P<0.01 (very significant), **P<0.05 (significant), and ***P< 0.001 (very highly significant) Non-significant, or p > 0.05

Results also showing that patients with diabetic nephropathy have higher MALAT1 expression than patients with diabetes alone and seemingly healthy individuals (control) as seen in (Table.3) and (Fig.3).

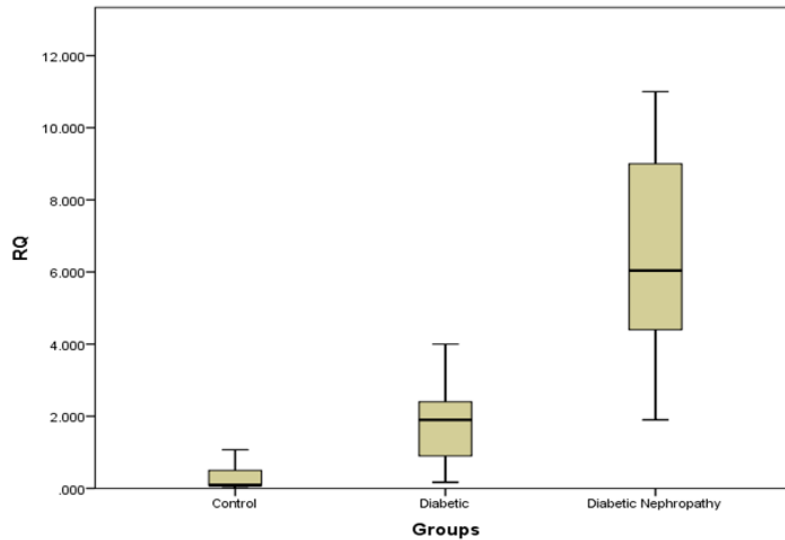


Fig.3. Box plot of RQ (MALAT1 gene expression) median difference among study groups.

Correlation between RQ and other study parameter (duration of DM , A/C ratio , HBA1C ,S.creatinine and eGFR) respectively as shown in (Table.4) , Spearman correlation coefficient showing significant" positive strong correlation between RQ and duration of DM as P<0.05, showing significant " positive strong "correlation between RQ and urine A/C

ratio as P<0.05, showing significant positive moderate correlation between RQ and HBA1C as P<0.05, showing significant positive mild correlation between RQ and serum creatinine as P<0.05 , while there was significant negative moderate correlation between RQ and eGFR .

Table 4. Spearman correlation coefficient between RQ with BMI , urine A/C ratio, HBA1C, s.creatinine, Duration of diabetes and eGFR respectively in all study participants:

Correlation between RQ and other parameters	R value	P value
BMI	-0.176	P=0.131
Urine albumin/creatinine ratio	0.657**	P=0.000
HBA1C	0.551**	P=0.000
Serum creatinine	0.300**	P=0.000
Duration of diabetes	0.692**	P=0.000
eGFR	-0.555**	P=0.000

**Correlation is significant at the 0.01 level (2-tailed)

Discussion

One of the main causes of end-stage renal disease worldwide is diabetic nephropathy , a significant microvascular consequence of diabetes (Dagar et al., 2021).DN cannot be stopped from progressing because of its

complex pathogenic processes, which puts a tremendous financial burden on many families and society (Tang et al., 2021). Prior research has shown that the primary pathogenic mechanisms of DN are apoptosis and inflammation, which play critical roles

in speeding DN development and worsening (Yang et al., 2022). The pathophysiology of DN must thus be urgently investigated in order to develop appropriate treatments.

Long noncoding RNAs (lncRNAs) contribute to the pathophysiology and physiology of several disorders, including DN, and have more than 200 nucleotides (Jiang and Ning, 2020). Lung cancer is where metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was initially discovered (He et al., 2018). Further research is required to demonstrate the role of MALAT1 in DN tubular epithelial cell apoptosis and inflammation as there are still few studies on the subject.

75 participants (25 with diabetes, 25 with diabetic nephropathy and 25 controls) were included in this case-control study. All participants had a thorough medical history, physical examination, age was substantially greater in the diabetic with nephropathy patients and merely diabetic patients in the current study compared to the control group (P value 0.001), but there was no significant difference in the distribution of sex between the three groups.

In accordance with our results Ashjari et al. (2022) in patients with DN and type 2 diabetes mellitus (T2DM) cases without neuropathy, transcript levels of MALAT1, microRNA (miR)-1-3p, and C-X-C motif chemokine receptor 4 (CXCR4) were examined, twenty DN patients and twenty T2DM participants without neuropathy were enrolled (as the control group), peripheral blood mononuclear cells (PBMCs) from each subject were collected to get total RNA, Real-time PCR was used to assess the target gene expression levels. Age and sex were similar between the two groups, but diabetic neuropathy's duration of the disease was much longer than that of type 2 diabetes mellitus alone (Ashjari et al., 2022).

SBP and DBP were comparable throughout the analysed groups in the current study.

Similar to what we found, Fawzy et al. (2018) shown that there was no discernible

difference in hypertension between diabetics and diabetics with ESRD..

In the current investigation, diabetic patients with nephropathy had substantially longer DM duration in years and a higher A/C ratio than diabetic patients alone (P value 0.001). Our findings are consistent with (Mariye et al., 2020) who examined the factors that contribute to diabetic nephropathy in individuals with diabetes in Ethiopia 168 cases and 672 controls with a mean age of 45.18 and 62.12, respectively, participated in the unmatched case-control research design. They claimed that the predictor of diabetic nephropathy was the length of time with diabetes mellitus since diagnosis.

In the current study, HBA1C and RQ were considerably greater in patients with diabetic nephropathy than in patients with diabetes alone and controls, and they were also significantly higher in patients with diabetes alone than in controls (P value 0.001).

Our findings are consistent with Ashjari et al. (2022) who revealed that compared to type 2 diabetes mellitus, diabetic neuropathy had substantially higher HBA1C and MALAT1 levels. Our findings are consistent with Sahu et al., (2021) who studied 205 diabetic patients with and without microalbuminuria in a cross-sectional comparison research with 180 healthy controls (group I) and those with microalbuminuria in group III. They found that group I's HbA1c was considerably lower than those of groups II and III ($p < 0.01$).

In addition, Real-time quantitative polymerase chain reaction ($n = 90$ for each) was used on diabetics with and without ESRD, according to their findings, diabetic ESRD patients had blood MALAT1 levels that were higher than those of the study control group, with a median (quartile) value of 10.5 (1.41-126.7) ($p .001$). Just 20% of patient samples showed down-regulation with a relative expression ratio < 1.0 , compared to four fifths of patients who were up-regulated (Fawzy et al., 2020)

. In the current investigation, patients with diabetic nephropathy had considerably higher serum creatinine levels than controls ($P = 0.009$) and only diabetic patients ($P = 0.006$), but there was no statistically significant difference in serum creatinine levels between the two groups of patients. Our findings are consistent with **Sahu et al. (2021)** They found that healthy controls had the lowest levels of blood creatinine compared to other groups DM without micro albuminuria and those with micro albuminuria ($p < 0.01$) (**Sahu et al., 2021**). Also, **Fawzy et al. (2018)** found that ESRD diabetic patients' creatinine levels (Imol/l) were considerably higher than those of just diabetic patients (812.7 ± 273 vs. 86.4 ± 18.8 ; $p < 0.001$). Yet in contrast to what we found, **Asmamaw et al.(2020)** carried out a cross-sectional research to examine the usefulness of blood creatinine levels for the early diagnosis of renal disease in individuals with type 2 diabetes mellitus, they signed up 120 people (60 T2DM patients and 60 healthy controls), according to their findings, individuals with type 2 diabetes mellitus had considerably higher blood creatinine levels than healthy controls (**Asmamaw et al., 2020**). In the current investigation, eGFR was not substantially different between diabetics with nephropathy and just diabetic patients, although it was considerably lower in both groups when compared to controls (P value < 0.001). In contrast to what we found, **Fawzy et al. (2018)** compared to individuals with diabetes alone, patients with ESRD had considerably lower levels of eGFR ($p < .001$). This variation may be explained by a different study's sample size, patient characteristics, or the fact that it included ESRD patients receiving dialysis.

In the current study, there was a substantial negative link between RQ and eGFR, an insignificant association between RQ and BMI, and a significant positive correlation between RQ and other parameters (Albumin/creatinine ratio, HbA1C, s.creatinine, and duration of diabetes), our findings concurred with

Ashjari et al., (2022) who discovered a substantial favourable relationship between MALAT1, HbA1C, and the duration of diabetes.

Conclusion

Our findings showed a significant positive correlation between MALAT1 and urine Albumin/creatinine ratio, HbA1C, serum creatinine, and duration of diabetes and a significant negative correlation between MALAT1 and eGFR. LncRNA Malat1 is highly expressed in DKD patients compared to diabetics and an apparently healthy group, suggesting that MALAT1 is involved in the pathogenesis of DN.

Abbreviations

MALAT1	Metastasis associated lung adenocarcinoma transcript 1
DM	Diabetes mellitus
DN	Diabetic nephropathy
ESRD	End stage renal disease
DKD	Diabetic kidney disease
lncRNA	Long non coding RNA
eGFR	estimated Gomerular Filtration Rate
ACR	Albumin creatinine ratio
HbA1C	Heamoglobin A1C (glycated)
GAPDH	glyceraldehyde-3- phosphate dehydrogenase

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