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The Association Between MTHFR C677T Polymorphism and Homocysteine Levels as Risk Factors for Coronary Artery Disease: A Case-Control study

Saeideh Amani[1], Ebrahim Mirzajani[2,3*], Seyed Mehrdad Kassaee[1], Minoo Mahmoudi[1], Fardin Mirbolouk[4]

Abstract

Objectives: Coronary artery disease (CAD) is the leading cause of disability and global death. Homocysteine is a major risk factor for CAD. High plasma homocysteine levels are an independent risk factor for this disease. Methylenetetrahydrofolate reductase (MTHFR) is one of the essential enzymes of homocysteine metabolism. In this way, mutations of genes encoding enzyme reduce its activity, which can increase blood homocysteine levels and ultimately raise the risk of CAD. Accordingly, this study aimed to investigate the association between MTHFR C677T polymorphism and homocysteine with CAD disease.

Materials and Methods: The present investigation is a case-control study conducted on the patients of Heshmat hospital, Rasht, Iran. The demographic characteristics of 90 patients and 76 controls were obtained by questionnaires and blood samples were collected to evaluate homocysteine levels and gene polymorphism. MTHFR C677T polymorphism and blood homocysteine levels were assessed using amplification refractory mutation system polymerase chain reaction (ARMS-PCR) and ELISA, respectively, and a comparison was made between the two groups. The statistical analysis of the data was performed using SPSS software, version 21.

Results: The frequency of C677T polymorphism genotypes in the control and patient groups was not statistically significant (P = 0.384). The highest frequency of genotype in the control (46.1%) and patient (50%) groups was shown in CT. Plasma homocysteine levels were significantly higher in CAD patients than in the control group (P = 0.001).

Conclusion: The results of this study showed that the TT genotype of C677T polymorphism has a protective effect on CAD. Although the results obtained for C677T polymorphism are not statistically significant, this genotype had little effect on atherosclerosis. Consequently, the interaction of MTHFR gene polymorphism with CAD may be due to C677T genotype and homocysteine levels in the selected population of Guilan.

Keywords: Coronary artery disease, Homocysteine, Methylenetetrahydrofolate reductase (MTHFR), MTHFR C677T polymorphism

Introduction

Coronary artery disease (CAD) is the leading cause of global death, especially in developed countries and some developing countries (1). About half of all cardiovascular disease fatalities are attributed to CAD (2). CAD is a multifactorial disease controlled by genetics and environmental factors (3). Improvement in environmental factors is effective in reducing the incidence and mortality of CAD, while genetic factors always play an influential role in CAD (4). Despite much progress in identifying the risk factors and mechanisms of CAD, the disease is not completely preventable and many patients get it without any risk factors. Additionally, traditional therapies are only involved in the recovery of 30% to 40% of populations at risk, and no effective treatment is currently anticipated for these patients (5). Serious risk factors such as lipoprotein-A, fibrinogen, homocysteine, and inflammatory markers specifically C-reactive protein (CRP) have been suggested for the treatment of this disease, and also, several studies are being directed on these risk factors (6).

The endothelium is a single layer of cell that covers the blood vessels. It has a critical role in many physiological functions, including controlling of blood flow, blood vessels permeability, and hemostatic balance. Endothelial cells produce a wide range of substances in response to various physical and chemical stimuli comprising vasodilation and vasoconstriction agents (7). Endothelial dysfunction is the primary cause of cardiovascular disease. Furthermore, it plays a significant function in all stages of CAD, containing stable angina, unstable angina, and acute myocardial infarction (8, 9). Plasma homocysteine is one of the most important risk factors for CAD, which plays an influential role in the progression of atherosclerosis through its effect on thrombolysis, endothelium, and platelets. Homocysteine is a product of methionine metabolism and metabolized by two pathways (10). Conversion of 5-10 methyl tetrahydrofolate to

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[1]Department of Biology, Hamedan Branch, Islamic Azad University, Hamedan, Iran. [2]Department of Biochemistry, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran. [3]Cellular and Molecular Research Center, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran. [4]Cardiovascular Diseases Research Center, Department of Cardiology, Heshmat Hospital, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran.
***Corresponding Author:** Ebrahim Mirzajani, Tel:(+98)9111329896, Email: e_mirzajani@yahoo.com

Key Messages

- The TT genotype of the C677T polymorphism has a protective effect on CAD, indicating its potential as a genetic marker for reduced risk.
- The interaction between the MTHFR gene polymorphism, specifically the C677T genotype, and elevated homocysteine levels may contribute to CAD.

folic acid by enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), which plays an important role in homocysteine metabolism, leads to the formation of methyl tetrahydrofolate (11). In the methionine cycle, it reacts with adenosine triphosphate to form S-adenosyl methionine (SAM). The methyl group is activated in SAM, which is called activated methionine. The active methyl group of SAM can be transported to target substrates (receptors) such as DNA, and SAM can be converted to S-adenosyl-L-homocysteine. After the elimination of adenosine from S-adenosine homocysteine, homocysteine is produced by S-adenosyl-L-homocysteine hydrolase. Eventually, homocysteine is converted to methionine by acquiring a methyl group of 5-methyltetrahydrofolate or betaine. Homocysteine is naturally present in the blood. Elevated homocysteine levels (hyperhomocysteinemia) increase the risk of atherosclerosis, heart attack, stroke, blood clots in the arteries, and CAD more than other risk factors (12). Homocysteine levels have been linked to a variety of factors, including high blood pressure, high cholesterol, and smoking. The mutations in methylenetetrahydrofolate reductase (MTHFR) genes along with environmental factors have been reported to increase homocysteine. The association between MTHFR gene polymorphism and disease severity in CAD patients showed that homocysteine levels were significantly higher in these patients than in normal individuals (13). C677T is a functional polymorphism of the MTHFR gene that significantly alters MTHFR enzyme activity. The conversion of cytosine to thymine is occurred because of a point mutation in exon 4 at nucleotide 677 of the MTHFR gene and leads to the replacement of valine with alanine in the amino acid number 222. This point mutation occurs in the generation of an unstable and heat-sensitive MTHFR enzyme with low activity. Serum homocysteine (plasma) increases due to the reduced enzyme activity of MTHFR mutation. MTHFR enzyme function is 30% in homozygous and 65% in heterozygous compared to normal individuals (14). The C677T gene is associated with reduced enzymatic activity, Low serum folate levels, plasma, and red blood cells, and increased plasma homocysteine (15). C677T polymorphism by transferring C to T reduces the activity of MTHFR enzyme and increases plasma homocysteine under folate deficiency conditions (16).

The impact of genetic variation on different races, geographic diversity, and lifestyles has an undeniable role in cardiovascular disease. According to the different lifestyles of the people of Guilan province in comparison with other provinces, as well as the genetic differences, this study was done in order to determine the mutation of MTHFR gene C677T and plasma homocysteine levels measurement in patients with CAD whose disease was confirmed by angiography at Heshmat hospital, Rasht, Iran.

Material and Methods

Study Population

This study was performed on patients admitted to Heshmat hospital, Rasht, Iran. The patients were placed in two control and patient groups after the angiography and the agreement of the cardiologist. The study was conducted on 90 patients and 76 normal individuals. After obtaining written consent, a special questionnaire was completed for all patients. The patients were allocated to either the control or patient group after undergoing angiography and with the agreement of the attending cardiologist. To minimize this potential for bias, we used a random number generator to select the participants. According to angiographic outcomes, the control group had no clinical symptoms of the disease and had normal arteries without congestion.

The inclusion criteria for this study include cardiologist-confirmed patients with heart disease who underwent angiography and coronary artery blockage. On the other hand, patients who have a history of heart surgery, renal failure or kidney transplantation, liver disease, smoking, malignancies, and epilepsy were excluded from this study.

Investigation of Genotypes

After about 8 hours of fasting, blood was taken from each of the groups and collected in EDTA anticoagulant tubes. DNA samples were extracted using a High-Pure PCR Template Preparation kit (Roche). To ensure the purity of DNA, its quality was evaluated using gel electrophoresis. To determine the MTHFR genotype, the amplification refractory mutation system polymerase chain reaction (ARMS-PCR) technique was performed by the MTHFR SNP detection kit (Lytech, Moscow, Russia) along with specific primers (Table 1). Denaturing was carried out at 94 °C for 1 minute using Analytik Jena's thermal cycler. The PCR program was performed 30 cycles as follows: 93 °C for 10 seconds, 64 °C for 10 seconds, and 72 °C for 20 seconds. Following PCR, gel electrophoresis 1% was

Table 1. PCR Primer Sequences

Primer Name	Sequence 5' → 3'
MTHFR 677T (F)	GAA GGA GAA GGT GTC TGC GGT AGC
MTHFR 677T (R)	GAA GGA GAA GGT GTC TGC GGA AGT
MTHFR 677C (F)	GG ACG GTG CGG TGA GAGTG
MTHFR 677C (R)	CA AAG ACA CTT TCT TCACT

performed to ensure the amplification of the desired product.

Analysis of Plasma Homocysteine Levels

To prevent homocysteine from releasing red blood cells, blood samples were centrifuged for 30 minutes to evaluate homocysteine. Plasma samples were stored at -20 °C for subsequent studies. Next, the amount of plasma homocysteine levels was measured by the ELISA method using the AXIS-Shield kit and according to the manufacturer's instruction. Generally, the measurement was based on a competitive reaction between the S-adenosyl L-homocysteine (SAH) groups. In the sample, homocysteine was first converted to S-adenosyl L-homocysteine using adenosine, and hydrolase following the addition of the anti-SAH monoclonal antibody. The rabbit anti-mouse antibody conjugated with horseradish peroxidase (HRP) was added. The activity of the peroxidase enzyme was measured using an ELISA reader at a wavelength of 450 nm, and the absorbance was inversely related to the concentration of the Hcy in the sample.

Measurement of Lipid Profile

For the measurement of lipid profile, all participants were required to fast for a period of 12 hours before the test, this fasting period helped to ensure accurate and consistent lipid profile results. At our hospital laboratory, the concentrations of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using standard enzymatic methods. Commercially available kits by ELITech Company designed explicitly for lipid profile analysis were utilized for this purpose. The enzymatic methods employed in our laboratory are widely accepted and validated for lipid profile measurements. These methods involve enzymatic reactions that produce colorimetric changes proportional to the concentration of the lipid components being measured. The intensity of the color produced is then quantitatively measured using spectrophotometry. The normal ranges for serum TC, TG, HDL-C, and LDL-C levels in our study were determined based on established guidelines and previous research. The normal range for serum TC was 3.10-5.17 mmol/L, TG was 0.56-1.70 mmol/L, HDL-C was 0.91-1.81 mmol/L, and LDL-C was 1.70-3.20 mmol/L. By adhering to standardized procedures and using validated enzymatic methods, we aimed to ensure accurate and reliable measurement of the lipid profile in our study participants. These measurements provided valuable information about the participants lipid profile essential for assessing their cardiovascular health and risk of developing CAD.

Statistical Analysis

Statistical analysis was performed using SPSS software version 21. The chi-square test was used to evaluate the association between genotypes and CAD. Plasma homocysteine levels were measured and compared between the control and patient groups. The Mann-Whitney test was utilized to assess the significance of differences in homocysteine levels between the groups. To examine the association between MTHFR C677T polymorphism, homocysteine levels, and CAD, logistic regression analysis and Fisher exact test was performed. Adjustments for confounding variables and other relevant clinical parameters were made. Odds ratios (OR) with 95% confidence intervals (CIs) were calculated. Moreover, due to differences in the variable's frequency of studied genotypes compared to the natural frequency, the non-parametric tests, like Mann-Whitney and Kruskal-Wallis were used. *P* values less than 0.05 were considered statistically significant for all tests, while *P* values less than 0.25 was exerted for SNP.

Results

The study was accomplished on 90 patients with CAD and 76 normal healthy individuals without clinical symptoms of CAD, which included 54.2% of men and 45.8% of women. In this study, C677T mutations and their association with homocysteine levels were investigated. The frequency distribution of C677T genotypes in the patient and the control groups have been shown in Table 2. There was no significant difference between the genotypic distribution of the two groups ($P = 0.0384$). The CT genotype owns the highest percentage of frequency in the control (46.1%) and patient (50%) groups.

The effect of the C677T genotype on homocysteine levels was investigated in CAD patients and control groups using the Shapiro test.

Table 3 shows the homocysteine levels in the C677T

Table 2. The Frequency of the C677T Genotypes in the CAD and the Control Groups

Allele	Genotypes	Study Groups		χ2	Odds Ratio	95% CI	*P* Value
		CAD	Control				
C677T	CC	38 (42.2%)	30 (39.5%)	0.129	1.12	0.60 - 2.09	0.720
	CT	45 (50%)	35 (46.1%)	0.257	1.17	0.64 - 2.16	0.612
	TT	7 (7.8%)	11 (14.5%)	1.91	0.498	0.18 - 1.36	0.167
	T	32.8%	37.5%				
	C	67.2%	62.5%				

* The results are expressed as numbers (percentages).

Table 3. The Relation Between theC677T Genotypes and Plasma Homocysteine Concentrations in the CAD and the Control Groups

Genotypes	Homocysteine (μmol/L)		*P* Value[a]
	CAD	Control	
CC	17.07 ± 7.19	14.55 ± 8.01	0.032
CT	17.7 ± 11.21	12.42 ± 4.46	0.01
TT	19.5 ± 8.37	14.71 ± 9.25	0.151

The data are presented as the mean ± SD.
[a] Mann-Whitney test.

genotype in both CAD and control groups. As shown in Table 3, a significant increase in homocysteine levels was observed in patients with CC and CT genotypes compared to the control group ($P<0.05$).

The results of atherosclerosis in theC677T genotypes for both CAD and control groups (Table 4) showed that the highest and lowest frequency of vein thrombosis was observed in CT (50%) and TT (7.8%), respectively. Based on the chi-square test, no significant statistical difference was recognized between the number of clogged arteries in the C677T genotype of CAD and control groups ($P = 0.99$).

As shown in Table 5, the relation between the C677T genotypes and lipid profile was investigated. HDL was higher in the control group ($P = 0.027$). In other parameters of this study, no difference was observed ($P>0.05$).

The Pearson diagram shows the different variations in the measured elements of the gene. In this regard, there is a significant correlation between CC with TG, CT, and TT with fasting blood sugar (FBS) (Table 6).

Discussion

Cardiovascular disease refers to a group of multiple failures in which the heart, arteries, and vein lose their normal function (17). These diseases are the most common cause of disability and death in the world, including Iran (18). CAD is a multifactorial disease that has a higher risk in men, the elderly, smokers, and people with high blood pressure, lipid metabolism disorders, glucose metabolism disorders, obesity, inflammatory markers, increased homocysteine, and genetic factors. Homocysteine is a potential risk factor for CAD and MTHFR is associated with the risk of CAD (19,20). The MTHFR enzyme, as a methyl group transmitter, plays a vital role in homocysteine demethylation. The most important MTHFR gene polymorphism involved in homocysteine metabolism is

Table 4. Percentage of Coronary Artery Atherosclerosis in the C677T Genotypes of the CAD Patients

Genotype	Clogged Arteries			Total	*P* Value[a]
	1	2	3		
	No. (%)	No. (%)	No. (%)	No. (%)	
CC	8 (47.1)	12 (41.1)	18 (40.9)	38 (42.2)	0.99
CT	8 (47.1)	15 (51.7)	22 (50)	45 (100)	
TT	1 (5.9)	2 (6.9)	4 (9.1)	7 (100)	
Total	17 (100)	29 (100)	44 (100)	90 (100)	

Note: The results are expressed as numbers (percentages). [a] Fisher test.

Table 5. Lipid Profile and Blood Sugar Analysis of the C677T Genotypes

Allele	Lipids	Genotypes	Study groups			*P* Value[a]
			CAD	Control	Total	
C677T	Chol	CC	143.92 ± 45.08	151.90 ± 35.54	147.44 ± 41.05	0.121
		CT	145.76 ± 37.02	148.31 ± 33.82	146.88 ± 35.46	0.631
		TT	144.29 ± 38.65	134.18 ± 26.59	138.11 ± 31.13	0.727
	TG	CC	177.26 ± 122.76	155.53 ± 76.87	167.68 ± 104.87	0.767
		CT	139.02 ± 83.41	148.40 ± 74.23	143.13 ± 79.18	0.352
		TT	121.86 ± 52.15	135.91 ± 92.24	130.44 ± 77.55	0.999
	HDL	CC	32.28 ± 7.39	34.87 ± 6.44	33.42 ± 7.05	0.180
		CT	32.93 ± 6.58	32.87 ± 7.78	32.90 ± 7.08	0.899
		TT	28.14 ± 9.44	61.55 ± 80.54	48.56 ± 64.25	0.027
	LDL	CC	76.97 ± 29.38	84.53 ± 25.12	80.31 ± 27.64	0.205
		CT	79.24 ± 28.79	94.80 ± 89.98	86.05 ± 63.30	0.554
		TT	86.71 ± 24.99	75.73 ± 32.57	80.00 ± 29.58	0.328
	FBS	CC	135.78 ± 63.68	107.23 ± 26.52	122.80 ± 51.99	0.132
		CT	126.13 ± 40.20	136.60 ± 79.40	130.71 ± 60.34	0.942
		TT	121.83 ± 46.08	104.27 ± 36.43	110.47 ± 39.60	0.462

The data are presented as the mean ± SD. [a] Fisher test.

Table 6. Pearson Test Analysis of the Lipid Profile and Blood Sugar of Different C677T Genotypes

		CC	CT	TT
Chol	Chi-square	0.516	0.174	0.217
	df	1	1	1
	P	0.472	0.676	0.642
TG	Chi-square	3.830	0.909	2.443
	df	1	1	1
	P	**0.050**	0.340	0.118
HDL	Chi-square	3.373	2.741	0.060
	df	1	1	1
	P	0.066	0.098	0.807
LDL	Chi-square	2.816	0.689	1.742
	df	1	1	1
	P	0.093	0.406	0.187
FBS	Chi-square	2.255	8.130	4.872
	df	1	1	1
	P	0.133	**0.004**	**0.027**

called MTHFR C677T. Due to the MTHFR C677T gene mutation, cytosine replaces thymine, which causes valine to replace alanine in the enzyme structure. As a result, the enzyme becomes heat-sensitive, leading to a decrease in its activity (21). Declarations about the association of this polymorphism with cardiovascular disease, especially CAD are different. The results of this study showed that the C677T polymorphism of the MTHFR gene did not have a significant association between the CAD group and control ($P = 0.36$). The highest percentage of genotypes in the control and CAD groups were 46.1% and 50%, respectively. The meta-analysis study conducted by Azizi et al did not find a significant relationship between MTHFR gene polymorphism and metabolic syndrome (22).

Anderson et al (23) conducted a prospective study on CAD patients whose disease was confirmed by angiography. They found no statistically significant association between the frequency of C677T MTHFR gene polymorphism in the control and CAD groups. In the study of Nasiri et al (24) and Aleyasin et al (25), no significant relationship was observed in this case. A meta-analysis of 80 studies found that only a 14% risk of CAD was associated with the MTHFR genotype (26). Another analysis found that there was no association between this polymorphism and CAD in Europe, Australia, and North America, although their results in Asia found a link (27). Jain et al also showed a significant relationship that could be one of the reasons for this diversity in results, intake of folic(folate) in other countries (28). Evidence suggests that homocysteine is an independent risk factor for cardiovascular disease and elevated homocysteine levels are correlated with CAD (29). It also causes auto-oxidation through other thiols. Oxidative stress is produced by the reactivity of oxygen and leads to LDL oxidation, increased platelet aggregation, endothelial cell damage, and proliferation of vascular smooth muscle cells, and increases atherosclerosis (30). It means that increasing 5 µmol/L homocysteine is similar to 20 mg/dL and improves the risk of CAD. Besides a 3-4 µmol/L decrease in homocysteine, the risk of the disease is reduced by about 30-40% (31). Previous studies have shown that high plasma homocysteine levels may be a risk factor for CAD in Iranian patients (32). In the present study, the mean plasma homocysteine level in the CAD group was significantly higher than the control group and the result was consistent with the study of Ghazouani et al (33), Klerk et al (34), and Huh et al (35). Ma et al (36) examined plasma homocysteine levels in 100 control samples and 73 CAD patients. The results showed a remarkable increase in plasma homocysteine levels in the patient group compared to controls. Humphrey et al Observed that increasing 5 µmol/L of homocysteine increased the risk of CAD by approximately 20% (37). Another study confirmed that homocysteine may be a risk factor for the formation and progression of atherosclerosis in CAD. High concentrations of homocysteine also showed a significant association with CAD (36). In another meta-analysis study, there was a significant association between increased plasma homocysteine levels (normal levels of 5-15 µmol/L) and polymorphism of the MTHFR gene with ischemic heart disease, heart attack, venous thrombosis, and pulmonary embolism. An increased ratio of 5 µmol/L of homocysteine was reported to be 1.42 in 72 control subjects and 1.32 in 20 patients with CAD (38). Several studies in southern India have found no link between increased homocysteine and CAD. In another study, there was no significant difference in the homocysteine levels of the control and patient groups, but the heterogeneity of MTHFR gene mutations was shown in more than half of people with CAD with increased homocysteine. Hence, genetic differentiation in increasing homocysteine during folate deficiency is a risk factor for CAD (39), while other studies in the Indian population and a study in the UK found a significant association between increased homocysteine and CAD(11, 40, 41). Nevertheless, some research has shown that C677T polymorphism is not associated with CAD risk (42), due to homocysteine levels affected by a variety of factors such as genetic factors, eating habits, geographic and demographic differences, and lifestyle and lifestyle. Accordingly, differences in the results of studies can be due to several reasons mentioned above. Conspicuously, the quality and quantity of extracted nucleic acids both DNA and RNA is really of great importance and meanwhile, the investigation of epigenetics alteration particularly DNA methylation is recommended for CAD and may be DNA methylation will shed interesting information in this field (43-46).

Limitations of the study

The study's relatively small sample size may limit the generalizability of the findings to a larger population. A larger sample size would provide more robust and reliable results. Additionally, the study did not account for

certain confounding factors, such as lifestyle factors (diet, physical activity), medication use, and comorbidities that could influence the association between MTHFR C677T polymorphism, homocysteine levels, and CAD.

Future Directions for Research

For future studies, we suggest conducting a study with a larger sample size to investigate MTHFR polymorphisms so that the relationship between the study factors can be obtained more reliably. Additionally, it would be beneficial to consider the nutritional conditions of the people under investigation along with intervening factors such as smoking, diabetes, and the characteristics of the fat level of the people. Investigating the role of hyperhomocysteinemia in the mortality of patients is also suggested.

Conclusions

The prevalence of the C677T gene polymorphism was higher in CAD patients than in the control group. The TT genotype was found to be a protective genetic marker against CAD. Furthermore, homocysteine was identified as an independent risk factor for CAD pathogenesis. The elevated homocysteine levels observed in CAD patients compared to the control group may be attributed to mutations in the C677T gene.

Authors' Contribution

Conceptualization: Ebrahim Mirzajani.
Data curation: Saeideh Amani.
Formal analysis: Saeideh Amani.
Investigation: Saeideh Amani.
Methodology: Ebrahim Mirzajani, Seyed Mehrdad Kassaee, Minoo Mahmoudi, Fardin Mirbolouk.
Project administration: Ebrahim Mirzajani.
Resources: Saeideh Amani.
Supervision: Ebrahim Mirzajani.
Validation: Ebrahim Mirzajani, Seyed Mehrdad Kassaee, Minoo Mahmoudi, Fardin Mirbolouk.
Visualization: Ebrahim Mirzajani, Seyed Mehrdad Kassaee, Minoo Mahmoudi, Fardin Mirbolouk.
Writing–original draft: Saeideh Amani, Ebrahim Mirzajani, Seyed Mehrdad Kassaee, Minoo Mahmoudi, Fardin Mirbolouk.
Writing–review and editing: Saeideh Amani, Ebrahim Mirzajani, Seyed Mehrdad Kassaee, Minoo Mahmoudi, Fardin Mirbolouk.

Conflict of Interests

None.

Ethical Issues

This research was approved by the ethics committee of the University of Medical Sciences under the code IR.GUMS.REC.1396.64.

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