

Single nucleotide polymorphism at –1087 locus of interleukin-10 gene promoter is associated with severe chronic periodontitis in nonsmoking patients

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ABSTRACT

Objective: Single nucleotide polymorphisms (SNPs) in the promoter region of interleukin (IL)-10 gene, which codes for the anti-inflammatory cytokine IL-10, have been associated with its level of production in chronic periodontitis. The prevalence of promoter SNP genotypes is known in other populations with chronic periodontitis, while its association in the Indian population is not known. Hence, the present study was designed to investigate the prevalence of IL-10 promoter polymorphism in a racially defined group of Indians with severe chronic periodontitis as the Indian population is known to be genetically diverse. **Materials and Methods:** Genomic deoxyribonucleic acid was extracted from 46 nonsmoking patients with severe chronic periodontitis and 45 subjects with healthy periodontium. A SNP locus at –1087 of IL-10 was chosen, as this locus has been frequently associated with chronic periodontitis in other population. Genotyping was carried out using allele-specific polymerase chain reaction (AS-PCR), and the frequencies of genotype were analyzed between the groups. **Results:** The distribution of genotype and allele frequencies showed significant differences between the study groups. The prevalence of genotype AA alleles at –1087 locus of IL-10 was significantly higher in severe chronic periodontitis patients compared to the healthy controls ($P = 0.05$). **Conclusion:** The study has identified a positive association between the occurrence of AA allele at –1087 locus of IL-10 gene and severe chronic periodontitis in nonsmoking patients.

Key words: Interleukin-10 alleles in periodontitis, interleukin-10 polymorphism in periodontitis, interleukin-10 polymorphism, promoter polymorphism in periodontitis, single nucleotide polymorphism in periodontitis

INTRODUCTION

Chronic periodontitis (CP) is an inflammatory disease of periodontium, which develops as a response to toxic factors secreted by periodontal pathogens. CP is a progressive condition that leads to gradual recession of periodontium accompanied by loss of supporting bone with tooth mobility as a consequence.^[1] Nearly 10% of the adult population are affected by severe forms of CP. The etiology is understood to be complex and multifactorial and are attributed to risk factors like plaque microflora, tobacco smoking,^[1] systemic diseases like diabetes^[1] and genetic factors.^[2] Deciphering the interplay between these factors is thus essential to understand the pathogenesis of the disease. Initiation and progression of severe CP begins

with toxic factors secreted by periodontal pathogens that promote the inflammatory process by triggering the production of inflammatory cytokines. Cytokines are usually produced transiently but in a regulated

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manner in varying concentrations by large number of cells such as T-cells, B-cells, macrophages, and monocytes that have different range of activity in order to effectively orchestrate the inflammatory response to injury.^[3] Hence, dysregulation in the production of cytokines may be expected to cause a subdued or accentuated response leading to untoward tissue destruction and disease progression.

Association of single nucleotide polymorphism (SNPs) with periodontal disease was first reported by Kornman *et al.* in 1997^[4] where the authors found association of SNPs in interleukin (IL)-1A (-889 in linkage with +4845), IL-1B (-511 in linkage with -31), IL-1B (+3954) and IL-1RN (variable number tandem repeat in linkage with +2018) with severe CP. Several genetic polymorphism studies on IL-1, tumor necrosis factor- α , IL-4, IL-6, IL-10, Fc γ RIIIa, Fc γ RIIIa, Fc γ RIIIb, Vitamin D receptor, CD14, TLR2, and TLR4 genes have since been reported in different populations with periodontal disease. Of the above genes, IL-10 synthesizes an inflammatory cytokine IL-10, which is produced by monocytes, Th2 cells, regulatory T-cells, and B-cells.^[5] The presence of IL-10 in periodontitis was first reported by Gemmel *et al.*^[6] and Yamazaki *et al.*,^[7] who investigated the level of IL-10 mRNA by real time-polymerase chain reaction and found higher levels of IL-10 mRNA in gingival biopsies of severe CP patients relative to peripheral blood mononuclear cells.

Single nucleotide polymorphisms in the promoter region of IL-10 gene have been identified at positions -1087, -592, -819 from the transcriptional start site, and have been associated with a number of inflammatory diseases like juvenile rheumatoid arthritis,^[8] systemic lupus erythematosus,^[9,10] inflammatory bowel disease,^[11] bronchial asthma,^[12] and CP. In case of CP, several independent studies have identified association of the SNPs with extent and/or severity of CP, albeit at varying frequencies in patients from diverse racial background [Table 1].

Although the genetic background of Indian population has been established to be unique from that of HapMap population,^[23] and that the genetic background is known to be diverse among the population in different geographical locations within India, the status of IL-10 promoter SNPs in Indian patients with CP remains yet to be investigated. In order to address this issue, the present study was designed as a pilot explorative study by including (a) Racially defined Tamil speaking Dravidians so as to eliminate confounding effect that may arise due to genetic diversity, and (b) IL-10 SNP

at -1087 alone as it has been shown to be consistently associated with chronic periodontitis in the European patients [Table 1].^[13,19,20]

MATERIALS AND METHODS

Subject selection

A total of 91 subjects were included in the study after obtaining informed consent from the patients. The study was approved by the Ethical Committee and Scientific Committee of the Institutional Review Board. The study group included 46 severe CP patients as the test group (Group A) and 45 periodontally healthy patients as the control (Group B). Patients from both genders in the age group 30-50 years were included when presented with clinical attachment loss ≥ 5 mm, probing pocket depth (PPD) ≥ 5 mm involving $\geq 30\%$ sites. Patients with above clinical presentation but with systemic diseases were excluded. Periodontally healthy subjects with no signs of periodontal disease and probing sulcus depth of > 3 mm were selected for the control group. Both the test group and control group of patients belonged to Tamil speaking Dravidian race.

Clinical evaluation of the subjects

The evaluation of each subject was done, based on their demographic details, ethnicity, mother tongue, and racial background. A brief medical, dental history as reported by the patient and clinical parameters were recorded. Diagnosis and classification of periodontal disease status were established based on clinical parameters which included PPD and clinical attachment level (CAL) measured at six sites around each tooth using a University of North Carolina probe. The probe was directed to the long axis of the tooth. The indices taken for the study included standard dental evaluation procedures namely, plaque index (PII), oral hygiene index-simplified (OHI-S) and modified sulcular bleeding index (mSBI). All clinical recordings were performed by a single examiner. Intraexaminer calibration was achieved by two examinations of ten patients, 24 h before recruiting the subjects into the study. Calibration was accepted if measurements at baseline and after 24 h were similar to 1 mm at the 95% level.

Sample collection and deoxyribonucleic acid extraction

One milliliter of venous blood was collected by venipuncture from each patient in ethylenediaminetetraacetic acid (EDTA) coated tubes and stored at -80°C . At the time of deoxyribonucleic acid (DNA) extraction, blood

Table 1: Incidence of association of -1087, -819, and -592 SNPs in promoter of IL-10 gene with CP

Study order (year wise)	Periodontitis condition	Study population	Sample size	SNP loci in IL-10 promoter	SNP association with CP/AgP	References
1	AgP	Japanese	34 AgP, 52 controls	-506 to -1140	No association	Yamazaki <i>et al.</i> , 2001 ^[7]
2	CP and AgP	Caucasian	23 CP, 18 AgP, 21 controls	-592 and -819	No association	Gonzales <i>et al.</i> , 2002 ^[5]
3	CP	Swedish	60 CP, 39 controls	-1087	Positive association	Berglundh <i>et al.</i> , 2003 ^[13]
4	CP	Brazilian	67 CP, 43 controls	-592, -819, -1087	Positive association for -819 and -592	Scarel-Caminaga <i>et al.</i> , 2004 ^[14]
5	CP	Caucasian	122 CP, 114 controls	-1087	No association	Babel <i>et al.</i> , 2006 ^[15]
6	CP	Turkish	75 CP, 73 controls	-582, -819	Positive association for -592	Sumer <i>et al.</i> , 2007 ^[16]
7	AgP	Iranian	52 AgP, 61 controls	-1087	No association	Mellati <i>et al.</i> , 2007 ^[17]
8	CP	Brazilian	116 CP, 173 controls	-592	Positive association	Claudino <i>et al.</i> , 2008 ^[18]
9	CP	Swedish	53 CP	-1087	Positive association	Donati <i>et al.</i> , 2008 ^[19]
10	CP and AgP	German	27 CP, 32 AgP, 34 controls	-592, -819, -1087	Positive association of all 3 SNPs for AgP	Reichert <i>et al.</i> , 2008 ^[20]
11	CP and AgP	Taiwanese	145 CP, 65 AgP, 126 controls	-592, -819, -1087	Positive association for -592 in CP	Hu <i>et al.</i> , 2009 ^[21]
12	CP and AgP	Meta-analysis of 14 studies	1438 periodontitis (both CP and AgP), 1303 controls	-592, -819, -1087	Positive association for -819 and -592	Zhong <i>et al.</i> , 2012 ^[22]

CP: Chronic periodontitis, AgP: Aggressive periodontitis, SNPs: Single nucleotide polymorphisms, IL: Interleukin

samples were thawed and centrifuged at 500 rpm for 3 min at room temperature to pellet blood cells. Pelleted cells were washed thrice with red cell lysis buffer (containing 0.05% of saponin in 1X phosphate buffered saline (PBS), pH 7.5) to remove red blood cells from the whole blood. After the final wash, white blood cells appeared as a clear buffy coat to which 1 ml of cell lysis buffer (containing 0.1% sodium dodecyl sulfate, 25 mM EDTA, 75 µg/100 µl proteinase-K, and 200 mM Tris-Cl at pH 8 [Sigma-Aldrich, St. Louis, MO, USA]) was added and incubated at 57°C for 12 h with intermittent agitation. Cell lysates were then treated with 200 µl of 100 mM Ammonium Acetate, mixed and incubated at room temperature for 15 min. The samples were then centrifuged at 12,000 rpm for 15 min to precipitate protein fraction in cell lysate. Following this step, one-sixth volume of isopropanol was added to all the samples, mixed by vortexing and centrifuged at 12,500 rpm for 15 min at room temperature to precipitate the genomic DNA.

Allele specific polymerase chain reaction

The forward primer of allele-specific polymerase chain reaction (PCR), flanking -1087 region in IL-10 promoter was designed as described earlier,^[24] and is as follows:

- Interleukin-10F1: CTA AGG CTT CTT TGG GAA (-1087A specific)
- Interleukin-10F2: CTA AGG CTT CTT TGG GAG (-1087G specific).

Each of the forward primer specifically binds only to its respective allele.

The reverse primer was designed as a universal primer, the sequence of which is as follows:

Interleukin-10R: AGG CAC ATG TTT CCA CCT CTT CAG (common to both -1087A and -1087G alleles).

The primer sequences were synthesized at Genorime Facility, Chennai.

Polymerase chain reaction was performed under the following conditions: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, primer annealing at 60°C for 60 s, primer extension at 72°C for 60 s, with a final extension at 72°C for 5 min.

Statistical analysis

The data obtained were formulated and statistically analyzed using SPSS v17 software (IBM corporation). The basic characteristics of the study population like age and gender were analyzed using independent sample *t*-test. The mean of clinical parameters like oral hygiene index, PPD, CAL, PII, and mSBI were calculated and the intragroup and intergroup comparisons were derived using Independent sample *t*-test. The Chi-squared test was applied to examine the differences in genotype distribution, allele frequency between test and control groups. Deviation from Hardy-Weinberg equilibrium

was assessed by goodness-of-fit between the observed and expected numbers using Chi-square test with 1 degree of freedom. The comparison of clinical parameters with the genotype was performed within the groups and between the groups using one-way analysis of variance (ANOVA) analysis and the *P* value was obtained thereafter.

RESULTS

The study was undertaken to investigate the role of IL-10 at -1087 loci in patients with severe CP relative to age, gender and race-matched controls without periodontitis. Power calculations performed initially showed that the sample size required for ascertaining the significance of association of periodontal disease and genetic polymorphism with an alpha error of 1% and 90% power to be 41 for both severe CP and control groups. Based on this criterion, a total of 91 subjects, 46 with severe CP group, and 45 healthy controls were included in the study.

Age and gender distribution between severe chronic periodontitis and control groups

The mean age was 41.89 years in severe CP and 43.13 years in healthy controls. Independent *t*-test with equal variance between the two groups revealed a *P* = 0.094 which was not statistically significant and hence was considered to depict age-matched individuals between the two groups. A total of 47 male subjects and 44 female subjects were present in both groups. Both Pearson Chi-square ($\chi^2 = 0.271$) and *P* = 0.378 values were not significant between the groups that showed gender match between both severe CP group and healthy controls [Table 2].

Clinical presentation in severe chronic periodontitis and healthy control groups

The mean of scores of OHI-S, PII, and mSBI were significantly higher in severe CP group relative to healthy controls group (*P* < 0.001). The mean of PPD and CAL were also several fold higher in subjects with severe CP than the healthy controls (*P* < 0.001) [Table 3].

Distribution of genotypes in severe chronic periodontitis and healthy control groups

The presence of genotype GG at -1087 locus of IL-10 gene produces significantly higher amount of IL-10 cytokine than IL-10 gene with AA genotype. Hence, in order to ascertain the occurrence of the genotype in the study samples, PCR was performed by combining either IL-10F1 and IL-10R to determine A allele or

IL-10F2 and IL-10R to determine G allele on DNA samples extracted from severe CP and control groups. A representative gel image of the samples along with positive controls is shown in Figure 1. Analysis of the distribution of genotypes among the study groups showed a higher percent occurrence of AA genotype in severe CP patients and GG genotype in healthy controls group subjects. The distribution of heterozygous AG genotype was comparable in both groups. Pearson $\chi^2 = 6.285$ (*P* = 0.043) and $\chi^2 = 5.8855$ (*P* = 0.0152) were obtained when either the three genotypes or alleles were compared among the CP and control groups [Table 4]. Hardy-Weinberg equilibrium analysis of the genotype distribution among the CP and control groups showed that the probability of differences between the observed and expected values due to chance to be very low ($\chi^2 = 9.011$, *P* < 0.0026 with 1 degree of freedom) [Table 5].

Distribution of allele frequency in severe chronic periodontitis group and healthy controls group

To enumerate G or A alleles that occur in the genotype at -1087 locus of IL-10 gene in the study groups, the data were analyzed by Pearson Chi-square test. Data analysis showed higher distribution of G allele in healthy controls (*n* = 26%, 66.66%) and A allele in severe CP (*n* = 79%, 55.24%), which indicated a clear association of low cytokine producing allele A with severe CP. Since

Table 2: Age and gender distribution between severe CP and healthy control groups

Variables	Severe CP (group A, n=46)	Healthy controls (group B, n=45)	Total (n=91)
Age in years (mean±SD)	41.89±5.322	43.13±4.320	
Independent <i>t</i> -test: <i>P</i> =0.094			
Gender (%)			
Male	25 (54.3)	22 (48.9)	47 (51.6)
Female	21 (45.7)	23 (51.1)	44 (48.4)
Total	46 (100)	45 (100)	91 (100)
Pearson $\chi^2=0.271$, <i>P</i> =0.378. SD: Standard deviation, CP: Chronic periodontitis			

Table 3: Comparison of mean values of the clinical parameters among the severe CP and healthy control groups

Variables	Severe CP group A, (n=46)	Healthy controls group B, (n=45)	<i>P</i>
OHI-S	3.281±1.144	2.122±0.809	<0.001
PII	1.779±0.357	1.3780±0.357	<0.001
mSBI	1.4888±0.314	0.4665±0.372	<0.001
PPD (mm)	6.42±0.697	1.923±0.404	<0.001
CAL (mm)	6.851±0.726	2.836±0.518	<0.001

CP: Chronic periodontitis, OHI-S: Oral hygiene index-simplified, PII: Plaque index, mSBI: Modified sulcular bleeding index, PPD: Probing pocket depth, CAL: Clinical attachment level

the A alleles occurred in at higher frequency in patients with severe CP, we next performed logistic regression analysis of the two genotypes - AG and AA with GG as reference, which showed a significance association of AA genotype with severe CP (odds ratio [OR] = 10.07%, 95% confidence interval [CI] = 1.1860-85.57). Similar analysis for A allele with G as reference showed significant association of A allele with severe CP (OR = 2.46%, 95% CI = 1.17-5.18) [Table 4].

Comparison of genotypes in severe chronic periodontitis and healthy control groups with clinical parameters by one-way analysis of variance

Since the allele frequency of A was higher in severe CP group, we next analyzed to understand the association of AA genotype with each of the clinical parameter. One-way ANOVA analysis showed highly significant association of AA genotype with all five clinical parameters namely, OHI-S, PII, mBS, mean probing pocket depth, and mean clinical attachment level ($P \leq 0.001$) [Table 6].

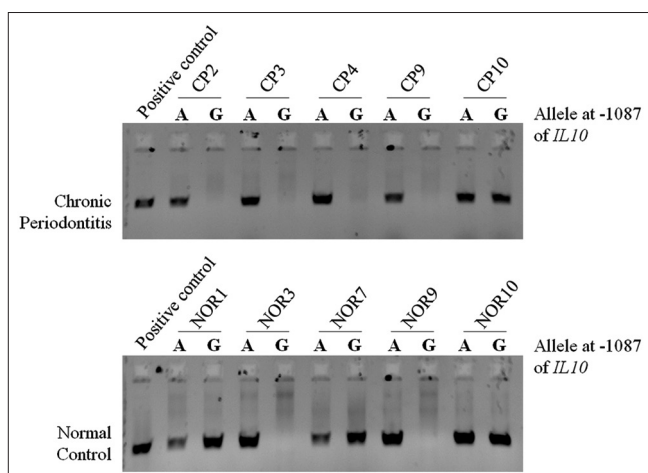


Figure 1: Representative gel image of allele-specific polymerase chain reaction of severe CP group and healthy control group samples

DISCUSSION

Genetically predetermined capability of an individual to respond to microbial and environmental factors has been shown to be associated with the susceptibility for CP. In particular, polymorphisms in genes encoding molecules of the host defense system, such as cytokines, have been targeted as potential genetic markers. IL-10 is one such anti-inflammatory cytokine, which plays a role in periodontitis by down-regulating the production of monocyte-derived pro-inflammatory cytokines and stimulating protective antibody production.^[14,15] Three SNPs at positions -1087, -819, and -592 from transcriptional start site of IL-10 have been associated with several inflammatory diseases including CP, and genotype at position -1087 has been associated with production of IL-10 cytokine in *in vitro* studies. In order to ascertain the prevalence of genotype at -1087, we analyzed 46 subjects with severe CP along with 45 subjects with healthy periodontium to understand the prevalence of SNPs in the promoter of IL-10. The subjects in both the groups were matched for age, gender and race. To eliminate possible confounding effects due to tobacco and other systemic diseases, only nonsmokers with no known systemic diseases were analyzed. The polymorphism of IL-10 at loci -1087 was assessed by allele-specific polymerase chain reaction technique (AS-PCR), which has been successfully used in earlier studies.^[24] AS-PCR on the study samples indicated a positive association of -1087(A/G) SNP with severe CP, which was determined based on: (1) Higher percent prevalence of lower IL-10 producing genotype AA in severe CP patients, and (2) higher percent occurrence of increased IL-10 producing genotype GG genotype in periodontally healthy individuals. A significant association of clinical symptoms such as increase in gingival inflammation, probing sulcus depth, and clinical attachment loss was

Table 4: Percentage distribution of genotypes and allele frequencies in severe CP and healthy controls groups, along with logistic regression analysis of IL-10 AG and AA genotypes with reference to GG, and A allele with reference to G

Genotype	n (%)			OR 95% CI	P
	Severe CP (group A, n=46)	Healthy controls (group B, n=45)	Total (n=91)		
GG	1 (2.2)	8 (17.8)	9 (9.9)	1.00	
AG	11 (23.9)	10 (22.2)	21 (23.1)	8.8 (0.929-3.3)	0.05
AA	34 (73.9)	27 (60.0)	61 (67.0)	10.07 (1.186-85.57)	0.0343
		Pearson $\chi^2=6.285$, $P=0.043$			
Allele					
G	13 (33.33)	26 (66.66)	39 (100)		
A	79 (55.24)	64 (44.75)	143 (100)	2.46 (1.17-5.18)	0.01
		Pearson $\chi^2=5.8855$, $P=0.0152$			

CI: Confidence interval, CP: Chronic periodontitis, OR: Odds ratio, IL: Interleukin

Table 5: Hardy-Weinberg equilibrium for genotypes

Genotype	Expected	Observed
GG	4.2	9
AG	30.6	21
AA	56.2	61

$\chi^2=9.011$, $P<0.0026$ with 1 degree of freedom

Table 6: Association of genotype AA with clinical parameters between the severe CP and healthy control groups

Genotype	Clinical parameter	Mean±SD		P
		Severe CP (n=34)	Healthy controls (n=27)	
AA	OHI-S	3.364±1.202	2.148±0.0837	<0.001
	PII	1.757±0.356	1.292±0.315	<0.001
	mBS	1.535±0.287	0.422±0.260	<0.001
	mPPD (mm)	6.466±0.726	1.935±0.433	<0.001
	mCAL (mm)	6.75±0.755	2.778±0.535	<0.001

OHI-S: Oral hygiene index-simplified, CP: Chronic periodontitis, PII: Plaque index, mPPD: Mean probing pocket depth, mCAL: Mean clinical attachment level, SD: Standard deviation, mBS: Mean bleeding score

observed in patients carrying AA genotype relative to other genotype (AG or GG) carriers. The finding may be considered as clinically significant as the sample analysis was done on a racially defined group, Tamil speaking Dravidians from the Southern region of India. Racial segregation of the samples was done to avoid confounding effects that may arise due to genetic diversity as the Indian population is known to be genetically diverse.^[23] Hardy-Weinberg equilibrium analysis confirmed the distribution of genotypes to be in harmony ($\chi^2 = 9.011$, $P < 0.002$) among the tested samples, which clearly suggested the occurrence of allele and genotype frequencies to be constant from generation to generation in an infinitely large interbreeding population. The higher occurrence of AA in severe CP patients and GG genotype in age and race-matched healthy individuals together advocates for a protective effect of GG allele in the analyzed samples, which is in agreement with earlier studies.^[6,24]

While the findings of the present study strongly support for the association of AA genotype at -1087 loci with CP, studies elsewhere excepting two on Swedish population have observed otherwise. Most of them have identified the positive association of SNPs at -819 and -592 loci with CP. Hence, we believe that the inclusion of -819 and -592 loci as well in the present analysis would have: (a) Provided a comparative prevalence pattern relative to other population, and (b) increased the significance of clinical association of IL-10 SNPs with severe CP.

It is noteworthy to mention that the genetic status of IL-10 cannot be considered as the sole determinant in the development of CP, as the etiology of CP is known to be multifactorial in nature. In our own study, we observed the low IL-10 producing genotype AA in 60% of subjects from the healthy control group as against 73.9% from severe CP group. Despite of having the AA genotype, the age-matched subjects in the control group did not develop periodontitis. This observation reiterates the genetic component as a risk factor, that is, in the presence of good oral hygiene measures the role of AA genotype remains insignificant. However, in the presence of precipitating factors the periodontium of AA genotype carriers suffer a dual insult, (1) Inflammatory reaction triggered by microbial toxins, and (2) lower production of IL-10 that may not be proportionate enough to extent of periodontitis, which accelerates the progression of periodontitis. It is also possible that the presence of polymorphisms in other ILs besides IL-10 SNP may also be essential for the progression of severe CP. For example, polymorphism in IL-1 and levels of IL-1, IL-8, and IL-17 in gingival crevicular fluid have been shown to be associated with severity of the periodontal condition.^[25-27]

To best of our knowledge, this is the first case-control study to have demonstrated a positive association between the -1087 IL-10 promoter polymorphism and severe CP in the Indian population. Although only a small representative population was investigated, the statistically significant association of -1087 SNP highlights for the requirement of a large scale case-controlled prevalence study by including age and gender-matched patients with severe CP from different races across India. The findings from these studies may then be effectively used to identify subjects with a higher risk of developing severe CP, so that appropriate preventive measures may be followed.

CONCLUSION

This is the first case-control study from India to investigate and identify a higher incidence of the decreased IL-10 producing genotype AA at -1087 loci in nonsmokers with severe CP, which indicated A allele at -1087 loci as a risk factor for the development of severe CP in subjects with poor oral hygiene.

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Conflicts of interest

There are no conflicts of interest.

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