

Correlations Between the Genetic Variations in the *COL1A1*, *COL5A1*, *COL12A1*, and β -fibrinogen Genes and Anterior Cruciate Ligament Injury in Chinese Patients^a

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Context: A variety of factors have been linked to the occurrence of anterior cruciate ligament injury (ACLI), including sex, familial factors, and genetic variations.

Objective: To find the genetic loci associated with ACLI and explore the genetic mechanism of ACLI in order to provide a genetic basis for the diagnosis, prognosis, and treatment of patients with ACLI.

Design: Cross-sectional study.

Setting: Hospital.

Patients or Other Participants: Data from 101 Chinese Yunnan Han patients with ACLI and 110 Yunnan Han individuals without ACLI (control group) were collected.

Main Outcome Measure(s): The single nucleotide polymorphisms of *COL1A1* rs1800012, *COL5A1* rs12722 and rs13946, *COL12A1* rs970547 and rs240736 and the rs1800787, rs1800788, rs1800789, rs1800790, rs1800791, and rs2227389 in the β -fibrinogen (β -fib) promoter region were analyzed using restriction fragment length polymorphism and DNA sequencing detection, and their genetic associations with ACLI were assessed.

Results: Single nucleotide polymorphisms of *COL1A1* rs1800012, *COL5A1* rs12722 and rs13946, and the rs1800789

and rs1800791 in the β -fib promoter region showed no difference between patients with ACLI and control participants, but the changes of *COL12A1* rs970547 and rs240736 and the rs1800787, rs1800788, rs1800790, and rs2227389 genotypes in the β -fib promoter region were associated with ACLI. Furthermore, the rs970547 allele and genotype frequencies in male ACLI patients were different from the control group ($P < .05$): the frequencies of the rs970547 A and G alleles in the patients were 71.9% and 28.1%, respectively, and in the control group were 58.8% and 41.2%, respectively. The frequencies of AA, AG, and GG genotypes in the patients were 49.3%, 45.2%, and 5.5%, respectively, and in the control group were 27.5%, 62.7%, and 9.8%, respectively, suggesting that male carriers of rs970547 A and rs970547 AA were at high risk of ACLI.

Conclusions: Males with the rs970547 A allele and rs970547 AA genotype of *COL12A1* may be at high risk for ACLI. Low rs1800787 TT and high rs1800788 CT, rs1800790 AG, and rs2227389 CT frequencies as well as high TGA* of rs1800790, rs1800791, and rs2227389 in the β -fib promoter region may be genetic risk factors related to ACLI.

Key Words: polymorphisms, β -fib promoter, knee injury

Key Points

- Collagen susceptibility genes in Chinese Han participants with anterior cruciate ligament injury (ACLI) were different from those of other races.
- The rs970547 and rs240736 of *COL12A1* gene were correlated with ACLI in Chinese males. Male individuals carrying the rs970547 A allele of the *COL12A1* gene and the AA genotype might have an increased risk of ACLI.
- The promoter regions rs1800790, rs1800788, and rs1800787 of the β -fib gene and their haplotypes may be related to the occurrence of ACLI.

The anterior cruciate ligament (ACL) is an important stabilizing structure in the human knee joint. With the popularity and extensive development of national sports, ACL injury (ACLI) is a common injury. Although ACLI often results from a noncontact mechanism, the bending angle of the knee joint, the tension of the quadriceps muscle, and the ground reaction force on the leg

during exercise are closely related to the increase in knee valgus moment. The important role of the ACL in the knee joint is that, when the injury occurs, the anterior hypermobility of the tibia causes instability of the knee joint, which in turn damages the articular cartilage and menisci and further accelerates the degenerative changes of the knee joint and osteoarthritis.^{1,2} The incidence of ACLI in the US population is about 1/3000, which is significantly higher than that of the general population.³ The overall

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incidence rate of ACLI for active athletes in China is 0.43%.⁴ However, in recent years, the number of ACLIs in nonathletes increased to a level that was significantly higher than that of professional athletes.⁵

At present, our understanding of the pathogenesis of ACLI is not very clear. Many factors, including sex, family factors, and specific genetic variations have been shown to be related to ACLI.^{6,7} The incidence in women is reported to be higher than that of men.^{3,8,9} Further, the incidence of ACLI in first-degree relatives of ACLI patients is more than twice that of uninjured people,¹⁰ suggesting that genetic factors play a role in ACLI. Some collagen gene polymorphisms have been associated with ACLI. The *COL1A1* gene encodes a protein chain in type 1 collagen, a major structural component of ligaments. Anterior cruciate ligament-injured patients were 4 times more likely to have a family member with a ligamentous injury of any kind compared with control individuals.¹¹ Similarly, the CC genotype of a variant in the *COL5A1* has been found in female patients with ACLI. The *COL5A1* gene codes for a protein chain in type 5 collagen, found in ligaments and tendons.¹² Third, these investigators¹¹ found that the AA genotype of the *COL12A1* AluI polymorphism was overrepresented in female patients with ACLI. This gene encodes for protein chains in type 12 collagen, which is believed to regulate fibril diameter in ligaments.¹³ An association was reported between the chromosomal region 11q22 and the risk of ACLI.¹⁴ Several matrix metalloproteinase genes, including those that are physiological mediators of collagen cleavage and removal, are located on chromosome 11q22. Posthumus et al¹¹ confirmed that the *COL1A1* Sp1 binding site TT type was significantly less in ACLI patients in South Africa than in the control group, suggesting that this polymorphism may be a special genetic factor in the multivariate model of ACLI. It was speculated that the G-to-T substitution of the intron of the Sp1 binding site increased the affinity of the Sp1 transcription factor, thereby increasing the expression of the *COL1A1* gene.¹⁵ Analysis of the *COL5A1* gene polymorphism showed that the frequency of the *COL5A1* BstUI restriction fragment length polymorphism (RFLP) CC genotype of females with ACLI was higher than that of the control.¹² Female *COL12A1* AluI RFLP AA has also been associated with an increased risk for ACLI.¹⁶ In addition, polymorphisms of *β-fibrinogen* (*β-fib*) were involved in the rate-limiting steps of the formation of the *β*-chain and were closely related to elevation of the plasma fibrinogen level. Several studies^{17,18} have suggested that *β-fib* polymorphism was associated with an elevated plasma fibrinogen concentration and ischemic stroke. The correlation between these related gene polymorphisms and ACLI in the Chinese population has not been reported.

Our aim was to investigate the single nucleotide polymorphisms (SNPs) of the *COL1A1*, *COL5A1*, *COL12A1*, and *β-fib* gene promoter regions of ACLI patients in the Chinese population to identify their correlation with the pathogenesis of ACLI.

METHODS

General Information

The study involved 101 patients with ACLI (73 males and 28 females) and 110 healthy control participants without ACLI (69 males and 41 females). The average ages

Table 1. General Information of Patients With Anterior Cruciate Ligament Injuries and Control Participants

Characteristic	Group	
	Anterior Cruciate Ligament Injury (n = 101)	Control Individuals (n = 110)
Sex	No.	
Male	73	69
Female	28	41
Side of anterior cruciate ligament injury		
Left	41	0
Right	60	0
	Mean ± SD	
Age, y	33.62 ± 11.18	35.57 ± 12.78
Height, cm	167.51 ± 6.46	165.43 ± 7.82
Weight, kg	64.39 ± 8.07	63.57 ± 9.03

were 33.62 ± 11.18 years of the ACLI patients and 35.57 ± 12.78 years of the control participants. A total of 327 patients with signs and symptoms of knee injury underwent arthroscopic examination in our hospital; of these, 41 ACLI patients with left-sided injuries and 60 with right-sided injuries were selected for this study. Control individuals with normal knees were randomly selected from the general population, and magnetic resonance imaging confirmed they had no organic knee-joint injury such as ACLI, meniscal tear, or fracture. Control recruits were matched by age and sex to the ACLI participants. Demographic information is shown in Table 1. The trial was approved by our hospital and the Clinical Research Ethics Committee of First People's Hospital of Kunming University. All participants signed the consent form. Inclusion criteria were (1) clinical physical examination indicated knee instability; (2) magnetic resonance imaging examination confirmed fiber discontinuity by coronal and axial imaging, the empty notch sign on coronal imaging, thickening and edema of the ACL (increased signal intensity on T2 or intermediate-weighted sequences), the fibers being completely absorbed or the residual ACL stump adherent to the synovial envelope covering the posterior cruciate ligament; (3) arthroscopic examination of the ACLI; (4) nonmechanical damage. Exclusion criteria were (1) ACLI combined with ligament injury in another part of the knee joint; (2) patients with previous knee disease such as rheumatism or rheumatoid arthritis, septic arthritis, or knee-joint tuberculosis; or (3) patients with previous knee surgery.

Sample Collection and DNA Extraction

A total of 5 mL of peripheral venous blood of all participants was collected and anticoagulated with ethylene diamine tetra-acetic acid (Tianjin Flourish Chemical Co, Ltd, Hebei District, Tian Jin, China). Genomic DNA was extracted from samples using the AxyPrep Blood Genomic DNA Mini Kit (Shanghai Haorui Biotechnology Co, Ltd, Shanghai, China).

Detection of Polymorphic Loci

The SNPs detected were *COL1A1* gene intron rs1800012, *COL5A1* gene 3'-UTR rs12722 and rs13946, *COL12A1*

Table 2. Primers Used for Polymerase Chain Reaction Restriction Fragment Length Polymorphism Analysis

Location	Chromosome	Gene	Primers (5'-3')	Allele	Amplicon (bp)
rs1800012	17 q21	<i>COL1A1</i>	F: 5'-GGAAGACCCGGT-TATTGCT-3' R: 5'-C GCTGAAGCCAAGTCAAATA-3'	G/T	598
rs12722	9 q34	<i>COL5A1</i>	F: 5'-GAAGACGGTTCTGGAGATCG-3' R: 5'-GAAGGCACCTGCAGAATGAC-3'	C/T	667
rs13946	9 q34	<i>COL5A1</i>	Same as above	C/T	—
rs970547	6q12-q13	<i>COL12A1</i>	F: 5'-GAGAATCCAGAACAGCTCCACCAG-3' R: 5'-CATGGCTAGTATGGGACAG-3'	A/G	615
rs240736	6q12-q13	<i>COL12A1</i>	F: 5'-ACTTTTGCCTGGGTATGT-3' R: 5'-CTCCAAAACAAGTAGTAGC-3'	C/T	480
rs1800787	4	<i>β-fibrinogen</i>	F: 5'-ACGTCATAAAATAAAATCCTGTCTA-3' R: 5'-GAAGCTCCAAGAAACCATCC-3'	C148T	1560
rs1800788	4	<i>β-fibrinogen</i>	Same as rs1800787	C249T	
rs1800790	4	<i>β-fibrinogen</i>	Same as rs1800787	G455A	
rs2227389	4	<i>β-fibrinogen</i>	Same as rs1800787	G933A	
rs1800791	4	<i>β-fibrinogen</i>	Same as rs1800787	G855A	
rs1800789	4	<i>β-fibrinogen</i>	Same as rs1800787	G1420A	

gene exons rs970547 and rs240736 and the rs1800787, rs1800788, rs1800789, rs1800790, rs1800791, and rs2227389 in the *β-fib* gene promoter region.

Polymerase Chain Reaction Restriction Fragment Length Polymorphism

Polymerase chain reaction (PCR) mixtures were prepared according to the instructions supplied with the AmpliTaq Gold kit (Applied Biosystems, Foster City, CA), which contained 200 μM of deoxynucleoside triphosphate, 2.5 μL of 10 × reaction buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl), 1.5 mM MgCl₂, a 0.1 μM concentration of the primers with 2.5 U of Taq DNA polymerase, and 5 ng of template DNA. The volume of this mix was adjusted to 25 μL with sterile water. The PCR reaction conditions were as follows: an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 120 seconds, and a final extension step at 72°C for 10 minutes. The *COL5A1* (rs12722, rs13946) and *COL12A1* (rs970547, rs240736) amplicons were subsequently digested using BstUI (NEB), DpnII (NEB), AluI (NEB), and BsrI (NEB) for RFLP analysis. Primers used for PCR RFLP in this study are shown in Table 2.

Single Nucleotide Polymorphism Sequencing

The amplicons of *COL1A1* (rs1800012) and *COL12A1* (rs970547, rs240736) and the rs1800787, rs1800788, rs1800789, rs1800790, rs1800791, and rs2227389 of the *β-fib* gene promoter region were sequenced to detect SNPs (Table 2). (Primers were provided by Shanghai Bioengineering Co, Ltd, Shanghai, China.) The haplotypes were generated using the 2 SNPs of rs12722 and rs13946 and 3 SNPs of rs1800790, rs1800791, and rs2227389 in the ACLI patients and control individuals.

Genetic Polymorphisms

The 351 and 316 bp DNA fragments generated by rs12722 BstUI RFLP suggested that they were homozygous for TT, and the 316-, 271-, and 80-bp fragments suggested a CC homozygote. The 418-, 194-, 40-, and 15-bp fragments produced by rs13946 DpnII RFLP were

homozygous for TT, and the 612-, 40-, and 15-bp fragments were homozygous for CC. The 460-, 139-, and 16-bp fragments produced by rs970547 AluI RFLP were defined as AA homozygotes, and the 599- and 16-bp fragments were homozygous for GG. The 480-bp fragment produced by rs240736 BsrI RFLP digestion was TT homozygous, and the 322- and 158-bp fragments represented CC homozygotes.

Statistical Analysis

The χ^2 test was used to analyze the frequency of allele, genotype, and haplotype distributions. Hardy-Weinberg equilibrium testing for genotypes was performed using the online calculator (<http://www.oege.org/software/hwe-mr-calc.shtml>). Haplotype analysis was carried out using <http://analysis2.bio-x.cn/myAnalysis.php>.¹⁴ Statistical significance was set at $P < .05$.

RESULTS

Clinical Results

Magnetic resonance imaging confirmed 101 patients with ACLI and 110 control participants without ACLI. Age, sex, height, and weight did not differ between the groups ($P > .05$; Table 1).

Allele and Genotype Frequencies

As shown in Table 3, the allele frequency was not statistically different between the patients and the control individuals ($P > .05$). The genotype frequency distribution was consistent with the Hardy-Weinberg equilibrium, except for rs1800791 ($P < .01$). In comparing genotype frequencies, we found that rs1800787 TT was less frequent in the patients than in the control group ($P < .05$). In contrast, rs1800788 CT, rs1800790 AG, and rs2227389 CT were higher in the patients than in the control group ($P < .05$). Other genotypes, such as rs1800012, rs12722, rs13946, rs970547, rs240736, and rs1800789, showed no difference between the groups ($P > .05$). We suggest that the changes of the rs1800787, rs1800788, rs1800790, and rs2227389 genotypes are associated with ACLI.

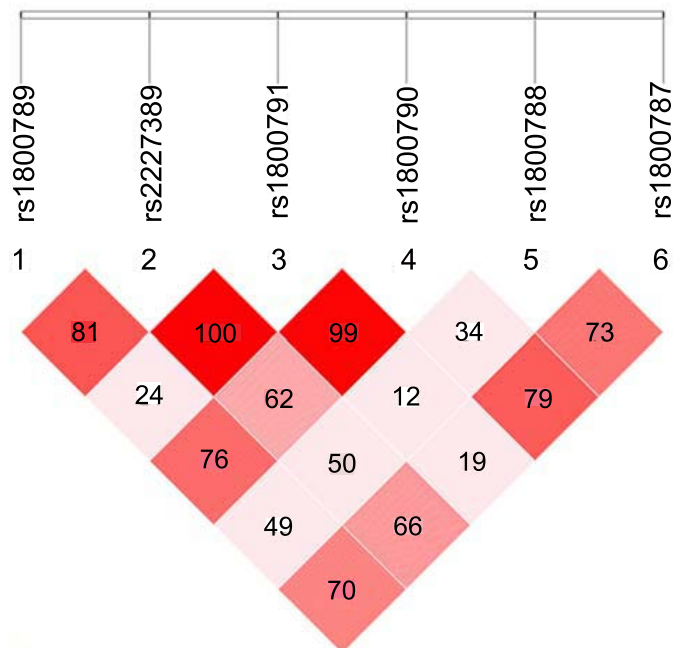


Figure 2. Linkage disequilibrium for the β -fibrinogen rs1800790, rs1800791, and rs2227389 polymorphisms in the patients with anterior cruciate ligament injury.

Linkage Disequilibrium Between SNPs

The linkage disequilibrium (LD) analysis of the SNPs of the collagen genes showed that rs12722 and rs13946 LD were higher in patients with ACLI than in control participants. The remaining sites rs1800012, rs970547, and rs240736 were less linked between the patients and control individuals (Figure 1). The β -fib gene promoter regions rs1800790, rs1800791, and rs2227389 had higher LD in the ACLI patients (Figure 2). The remaining regions rs1800787, rs1800788, and rs1800789 in the patient group were not associated with those in the control group.

Stratification Analysis

Further analysis showed that the frequencies of the rs970547 A and G alleles in males were different between the patient group and the control group ($P = .019$). The frequencies of A and G in the patients were 71.9% and 28.1%, respectively, and in the control participants were 58.8% and 41.2%, respectively. The frequencies of the rs970547 AA, AG, and GG genotypes in the patient group were 49.3%, 45.2%, and 5.5%, respectively, and in the control group were 27.5%, 62.7%, and 9.8%, respectively.

The rs970547 AA genotypes were greater in the ACLI patients ($P = .026$), indicating that male individuals carrying the rs970547 A allele and AA genotype had an increased risk of ACLI. No differences were found in the remaining SNPs. The analysis of SNPs in female patients did not show any statistical differences (Table 4).

Haplotype Analysis

According to the results of the LD analysis, the rs12722 and rs13946 haplotypes (Table 5) and rs1800790, rs1800791, and rs2227389 haplotypes (Table 6) were constructed. No statistical differences in rs12722 and rs13946 haplotype distributions were present between the groups (Table 5; $P > .05$). However, differences were noted between the haplotypes of TGG*, CGA*, and TGA* of rs1800790, rs1800791, and rs2227389. The detection rates of haplotypes TCG* and CGA* of rs1800790, rs1800791, and rs2227389 in patients were significantly lower than in control participants (Table 6; $P < .001$), whereas haplotype TGA* was higher in the patients than in the control group (Table 6; $P = .0228$), suggesting that this combination of SNPs was likely to be the genetic background of Chinese patients with ACLI.

DISCUSSION

Collagen Gene Variants and ACLI

At present, the genetic factors of ACLI are mostly focused on the polymorphism of collagen genes in different regions. In the Caucasian population of South Africa, Khoschnau et al¹⁹ observed that the polymorphism of the *COL1A1* Sp1 binding site was associated with ACLI in Caucasians. In 2009, Posthumus et al confirmed that the TT type of the Sp1 binding site was significantly less in South African patients with ACLI.¹¹ Population genetics showed that the frequency of the rs1800012 T allele was 20% and of the G allele was 80% in European populations. In the American population, T was 16% and G was 84%. In the Asian population, T was only 1% and G was 99%.¹² We did not observe an association between ACLI and the Sp1 binding sites in the Yunnan Han population, indicating that the polymorphism of rs1800012 in the Chinese population was insufficient and that it was not a site related to ACLI. Mokone et al²⁰ studied 111 cases of Achilles tendon injury in the Caucasian population of South Africa, 39 patients with spontaneous tendon ruptures, and 129 asymptomatic sports enthusiasts and found that only the rs12722-BstUI polymorphism of *COL5A1* gene was associated with chronic Achilles tendinitis. Among 85 Australian Caucasian patients with Achilles tendon injury, the rs12722-

Table 4. Comparison of Allele and Genotype Frequencies of rs970547 for Males in the 2 Groups

Single Nucleotide Polymorphisms	Allele Genotype	Patients With Anterior Cruciate Ligament Injury (n = 73), No. (%)	Control Individuals (n = 69), No. (%)	P Value	Odds Ratio
rs970547	A	105 (71.9)	81 (58.8)	.019	1.80
	G	41 (28.1)	57 (41.2)		
Allele rs970547	AA	36 (49.3)	19 (27.5)	.026	1.61
	AG	33 (45.2)	43 (62.7)		
	GG	4 (5.5)	7 (9.8)		

Table 5. Haplotypes of rs12722 and rs13946 in Patients With Anterior Cruciate Ligament Injury and Control Individuals

Haplotype	Patients, No. (%)	Control Individuals, No. (%)	χ^2	P Value	Odds Ratio (95% Confidence Interval)
CC*	75.38 (0.373)	89.00 (0.436)	1.512	.2189	0.779 (0.523, 1.160)
CT*	74.62 (0.369)	71.00 (0.348)	0.260	.6105	1.111 (0.740, 1.668)
TC	1.62 (0.008)	0.00 (0.000)			
TT*	50.38 (0.249)	44.00 (0.216)	0.722	.3955	1.221 (0.770, 1.938)

Table 6. Haplotypes of rs1800790, rs1800791, and rs2227389 in Patients With Anterior Cruciate Ligament Injury and Control Individuals

Haplotype	Patients, No. (%)	Control Individuals, No. (%)	χ^2	P Value	Odds Ratio (95% Confidence Interval)
CAA	0.00 (0.000)	0.38 (0.001)			
CAG	11.00 (0.053)	8.62 (0.032)	1.226	.2682	
CGA	0.00 (0.000)	13.55 (0.051)	10.920	.0010	1.665 (0.670, 4.139)
CGG	157.00 (0.755)	194.45 (0.731)	0.315	.5744	
TGA	40.00 (0.192)	31.07 (0.117)	5.189	.0228	1.127 (0.743, 1.709)
TGG	0.00 (0.000)	17.93 (0.067)	14.592	.0001	1.797 (1.080, 2.989)
TAA	0.00 (0.000)	0.00 (0.000)			

BstUI and rs13946-DpnII polymorphisms were associated with Achilles tendon injury.²¹ The frequency of the rs12722 BstUI CC genotype in female patients with ACLI was higher than in the control group.¹² This investigation demonstrated that the frequencies of rs12722 C and T alleles in the Yunnan Han population were consistent with the allelic frequency distribution previously reported in Asian populations, but they were not genetic risk factors in the Chinese population. Additionally, the findings for the *COL12A1* gene were different. Posthumus et al¹³ reported that, in the Caucasian population, the risk of ACLI was increased in females with rs970547 AluI RFLP AA. However, September et al²² did not find that the *COL12A1* gene was associated with Achilles tendon injury. We observed no difference in the rs970547 A and rs240736 C and T alleles compared with the patient group. Further analysis showed that male patients with ACLI carried more rs970547 A alleles and AA genotypes, but statistical differences were present in female patients.

***β* -fibrinogen Gene Variants and Anterior Cruciate Ligament Injury**

At the time of ACLI and after surgery, fibrinogen levels often increase significantly.^{23–25} The *β -fib* gene determines the synthesis of the fibrin β polypeptide chain, which in turn affects most of the changes in plasma fibrinogen levels. Deficient fibrinogen in anterior cruciate ligament wound sites has been demonstrated.²⁰ In this study, rs1800787 TT was lower in the patient group than in the control group. Also, rs1800788 CT, rs1800790 AG, and rs2227389 CT were higher in the patients than in the control participants, suggesting that changes in rs1800787, rs1800788, rs1800790, and rs2227389 genotypes were associated with ACLI. Here, rs1800787 TT seemed to confer protection against ACLI. The detection rate of haplotypes of TGG* and CGA* of rs1800790, rs1800791, and rs2227389 in the patients with ACLI was lower than that in the control group, whereas the TGA* was higher in the patients, indicating that this combination of changes is likely to be the genetic background of the ACLI pathogenesis in Chinese individuals.

CONCLUSIONS

The *COL1A1* gene Sp1 binding site rs1800012, as well as the rs12722 and rs13946 sites, were not susceptible sites for ACLI in the Chinese Han population. The *COL12A1* gene rs970547 and rs240736 had a correlation with ACLI in Chinese men. Male individuals carrying the *COL12A1* gene rs970547 A allele and AA genotype may have an increased risk of ACLI. The sites of the promoter region of the *β -fib* gene rs1800788 CT, rs1800790 AG, and rs2227389 CT and haplotypes of TGG*, CGA*, and TGA* of rs1800790, rs1800791, and rs2227389 may be associated with the genetic background of ACLI. The rs1800787 TT polymorphism may offer genetic protection against ACLI among Chinese individuals.

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