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RESEARCH ARTICLE

Novel mutation c.1210-3C>G in *cis* with a poly-T tract of 5T affects *CFTR* mRNA splicing in a Chinese patient with cystic fibrosis

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Abstract **Cystic fibrosis (CF) is a rare autosomal recessive disease with only one pathogenic gene cystic fibrosis transmembrane conductance regulator (*CFTR*). To identify the potential pathogenic mutations in a Chinese patient with CF, we conducted Sanger sequencing on the genomic DNA of the patient and his parents and detected all 27 coding exons of *CFTR* and their flanking intronic regions. The patient is a compound heterozygote of c.2909G>A, p.Gly970Asp in exon 18 and c.1210-3C>G in *cis* with a poly-T of 5T (T5) sequence, 3 bp upstream in intron 9. The splicing effect of c.1210-3C>G was verified via minigene assay *in vitro*, indicating that wild-type plasmid containing c.1210-3C together with T7 sequence produced a normal transcript and partial exon 10-skipping-transcript, whereas mutant plasmid containing c.1210-3G in *cis* with T5 sequence caused almost all mRNA to skip exon 10. Overall, c.1210-3C>G, the newly identified pathogenic mutation in our patient, in combination with T5 sequence in *cis*, affects the *CFTR* gene splicing and produces nearly no normal transcript *in vitro*. Moreover, this patient carries a p.Gly970Asp mutation, thus confirming the high-frequency of this mutation in Chinese patients with CF.**

Keywords cystic fibrosis; CFTR; splicing mutation; minigene

Introduction

Cystic fibrosis (CF) is a monogenic inherited disease, referring to the only pathogenic gene cystic fibrosis transmembrane conductance regulator (*CFTR*). *CFTR* is located on chromosome 7q31 and encodes cAMP- and ATP-dependent chloride channels that are abundant in the apical membrane of respiratory epithelial cells, submucosal glands, exocrine glands, liver, sweat ducts, and the reproductive tract. Consequently, CF affects multiple systems [1–3]. CF is the most common life-limiting autosomal recessive disorder in individuals of northern European background, with a 1:3200 incidence rate in live births of this population [4]. However, the CF prevalence rate in China is low. Although the exact rate of patients with CF in China has not been determined, the predictions span from 1:10 000 to 1:350 000 [5]. CF phenotypes in Chinese patients are relatively non-classic and may suffer from chronic airway infection, bronchiectasis, and a significant elevation in sweat chloride concentration but with pancreatic sufficiency, enhancing the trouble of diagnosis compared with Caucasians who have more frequent CF cases and more classic CF phenotypes [6].

Since *CFTR* has been identified to be responsible for CF, more than 2000 mutations in *CFTR* have been reported, and this number recorded by the Cystic Fibrosis Gene Analysis Consortium continues to grow. The most common pathogenic mutation, ΔF508, among Caucasians is rarely reported in CF individuals of Chinese origin. By contrast, mutations detected in Chinese patients with CF

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tend to be unusual among the Caucasian population [7,8]. The c.2909G>A in exon 18 (p.Gly970Asp or G970D) of *CFTR*, which is restricted to Chinese patients with CF and has a frequency of 9.8% (12 in 122 Chinese CF patients), becomes the most common variant [7,9]. Hence, the Chinese population may have a specific CF mutation spectrum.

Polymorphisms within *CFTR* can also lead to insufficiency, absence, or malfunction of CFTR protein and affect intrinsic chloride activity, such as TG repeats and poly-T tract referring to *CFTR* exon 10 splice acceptor site [10]. The mutual effects of various numbers of TG repeats associated with diverse lengths of poly-T tracts can exert different influences on the splicing process of exon 10 [10]. The complete-exon 10-deleted *CFTR* mRNA accounts for 25% of total *CFTR* transcripts in normal individuals with a 7T structure. By contrast, the 5T polymorphism may reduce its competitive capacity as a splice acceptor site recognized by splicing branch sites and produce 90% exon 10 lacking mRNA of total mRNA [10,11].

This report describes an 18-year-old male from Peking Union Medical College Hospital (PUMCH) with disseminated bronchiectasis and a high level of chloride in sweat chloride. This patient carries a novel mutation c.1210-3C>G in *cis* with $TG_{12}T_5$, resulting in almost all *CFTR* mRNA transcripts lacking exon 10. This patient also carries the second pathogenic mutation, G970D, the most common mutation in Chinese patients with CF so far. Our study aimed to expand the CF mutation spectrum of the Chinese population and provide extra cases supporting the previous findings of G970D's high frequency among Chinese patients with CF.

Materials and methods

Patient and clinical evaluation

Our patient, with both parents unaffected, is an 18-year-old male suffering from continuous coughing for 10 years. The patient had symptoms of expectorating yellow-green purulent sputum and hemoptysis after eating biscuits, was sent to PUMCH, and diagnosed with CF three years ago. The forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were 40.4% and 56.3% of the predicted value, respectively, and the FEV_1/FVC ratio was 71.1%, suggesting impaired pulmonary function. An evaluated chloride level of 131 mmol/L in his sweat was detected (abnormal value: above 60 mmol/L). A chest computed tomography (CT) scan showed disseminated bronchiectasis in both lungs. Informed consent and agreements to participate in the study were obtained from the patient and his parents. All methods carried out in this study were approved by the Institutional Review Board committee at PUMCH.

Detection of mutations in the *CFTR* gene

Genomic DNA was extracted from the peripheral blood leukocytes of the patient and his parents by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) by standard methods described in the protocol. Sanger sequencing was subsequently performed on the amplified gDNA to detect mutations in all 27 coding exons and their flanking intronic regions of *CFTR*.

Minigene plasmid construction

In vitro minigene assays were conducted using *pCAS2* vectors with a multicloning site for insertion of the target sequence between two encoding exons (exons A and B) [12]. The target fragment ranges from 414 nucleotides (nt) at the 3′ end of intron 9 to 402 nt at the 5′ end of intron 10 encompassing the whole sequence of exon 10, with 15-bp extensions on both ends that are complementary to the ends of the BamH1 cloning site of the linearized minigene vector (Fig. 1). After amplification, the target sequence was cloned into a minigene vector by using the In-Fusion HD Cloning Kit (Takara, Japan). The wild-type minigene plasmid had a $(TG)_{12}T_7$ structure followed by a C 3 bp downstream, which is designated (7T + C). The 5T + G plasmid was also constructed in the same way, which had a $(TG)_{12}T_5$ structure followed by a G 3 bp downstream. Similarly, the 5T + C plasmid with the $TG_{12}T_5$ sequence and C and the 7T + G plasmid with the $TG_{12}T_7$ sequence and G were generated through site-directed mutagenesis. All the above mentioned plasmids were verified by Sanger sequencing.

Transfection of minigene plasmid and cDNA sequencing

HEK293T cells were seeded at 1×10^6 per well, and after 6 h of cell adherence, four types (7T + C, 5T + C, 7T + G, 5T + G) of plasmids were transfected into the cells by using Lipofectamine 3000 reagent (Life Technologies, CA, USA). Cells were cultured with Dulbecco's modified Eagle medium (Gibco, USA) for 48 h and then washed twice with PBS. Total RNA was isolated under the standard RNA extraction procedure by using TRIzol (Gibco-BRL, San Francisco, CA, USA) and chloroform. Extracted RNA was qualified using a NanoDrop 2000 spectrophotometer (Thermo, Lithuania) and then reverse-transcribed into cDNA with a gross amount of 2 μg by using PrimeScript RT Master Mix (Takara, Japan). To analyze the splicing consequences of minigene vectors, we amplified the resultant transcripts by using the primer pair pCAS2-RTPCR-F (5′-GACCCTGCTGACCCTCCT-3′) and pCAS2-RTPCR-R (5′-GACGTGGGTAAGGAGGC-TG-3′) located in exons A and B. RT-PCR products were electrophoresed in 2% agarose gel by using GelRed

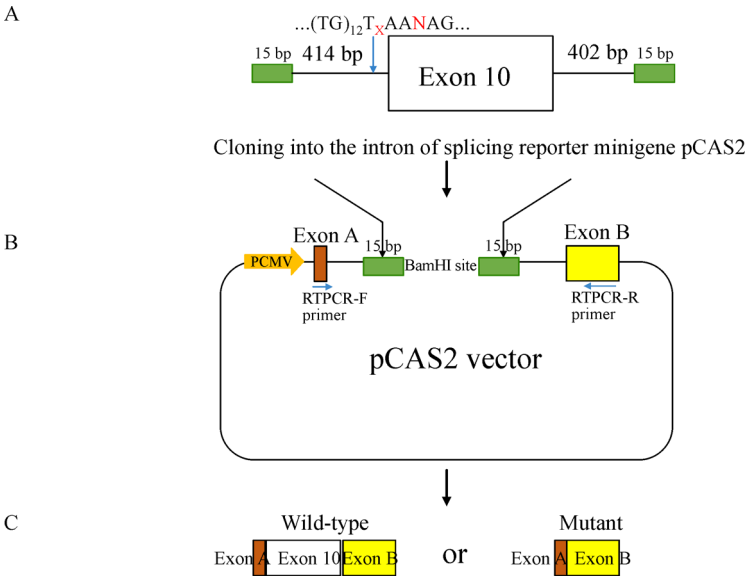

Fig. 1 Functional tests of splicing mutations via minigene assay. (A) A fragment of genomic DNA including exon 10, 414 nucleotide (nt) at the 3′ end of intron 9, and 402 nt at the 5′ end of intron 10 was amplified. Four types of target sequences encompassing two variants in intron 9 were designated as 7T + C (wild type), 5T + C, 7T + G, and 5T + G (carried by the patient). T_x stands for T_5 or T_7, and N stands for C or G nucleotide. (B) The amplicons were cloned into the *pCAS2* vector, which has a PCMV promoter (orange) and two exons (exons A and B, brown and yellow, respectively). Two green boxes on the *pCAS2* vector and target sequences represent two 15 bp fragments complementary to BamH1 ends, which help clone the target sequence into the *pCAS2* vector. (C) Predicted resultant transcripts of the minigene: wild-type transcript, including exon A + exon 10 + exon B, on the left; and mutant transcript, including exon A + exon B, skipping of exon 10, on the right.

staining (Biotium, Hayward, CA, USA) followed by UV imaging. In addition, TA cloning was conducted to distinguish the multiple transcripts from the RT-PCR products by using the pMD 18-T Vector Cloning Kit (Takara, Japan).

Results

Mutation detection in the patient

All 27 coding exons were amplified and sequenced for the patient and his parents, and the results showed that the patient carried two suspected pathogenic variants (Fig. 2A and 2B), c.2090G>A and c.1210-3C>G. The c.2909G>A in exon 18 (p.Gly970Asp) obtained from his mother is the most frequent *CFTR* mutation among Chinese patients with CF [9]. Interestingly, the other novel variant c.1210-3C>G in intron 9, inherited from his father, is just 3-bp upstream a poly-thymine (T) sequence of 5T on the same allele. Considering that the 5T allele causes a partially reduced level of normal CFTR mRNA *per se*, which is common even in the general population, the combination of 5T with G might contribute to the pathogenicity of the paternal allele [13]. Thus, a minigene assay was conducted to determine the effect of c.1210-3C>G in combination with the *cis* 5T sequence on *CFTR* mRNA splicing.

Functional testing results of the *CFTR* splicing mutation in the patient by using minigene assay

In the minigene assay, total RNA was isolated from minigene construct-transfected HEK293T cells, followed by RT-PCR to amplify transcripts of the 7T + C, 5T + C, 7T + G, and 5T + G plasmids. Sanger sequencing and electrophoresis were performed for RT-PCR products to distinguish the multiple transcripts, and the results are shown in Fig. 2C and 2D. Notably, the resultant transcripts of the 7T + C (wild type) plasmid had three bands. The top band corresponds to the wild-type transcript with a length of approximately 750 bp corresponding to the length of exon A from *pCAS2* plasmid + exon10 from inserted *CFTR* segment + exon B from plasmid. The bottom band is the mutant transcript with a length of nearly 500 bp, corresponding to exon A + exon B with exon 10 skipping. The middle band is the heterodimer of wild-type and mutant transcripts. To verify the heterodimer, we performed TA cloning assay, and the results showed that no transcript other than the wild-type or mutant transcript was inserted into the pMD 18-T vector (data not shown). Moreover, direct sequencing of the heterodimer could also confirm this finding, as the double peaks shown in the trace picture align perfectly with the combination of variant and wild-type transcript sequences. Electrophoresis results illustrated that in-frame exon 10 skipping *CFTR* mRNA

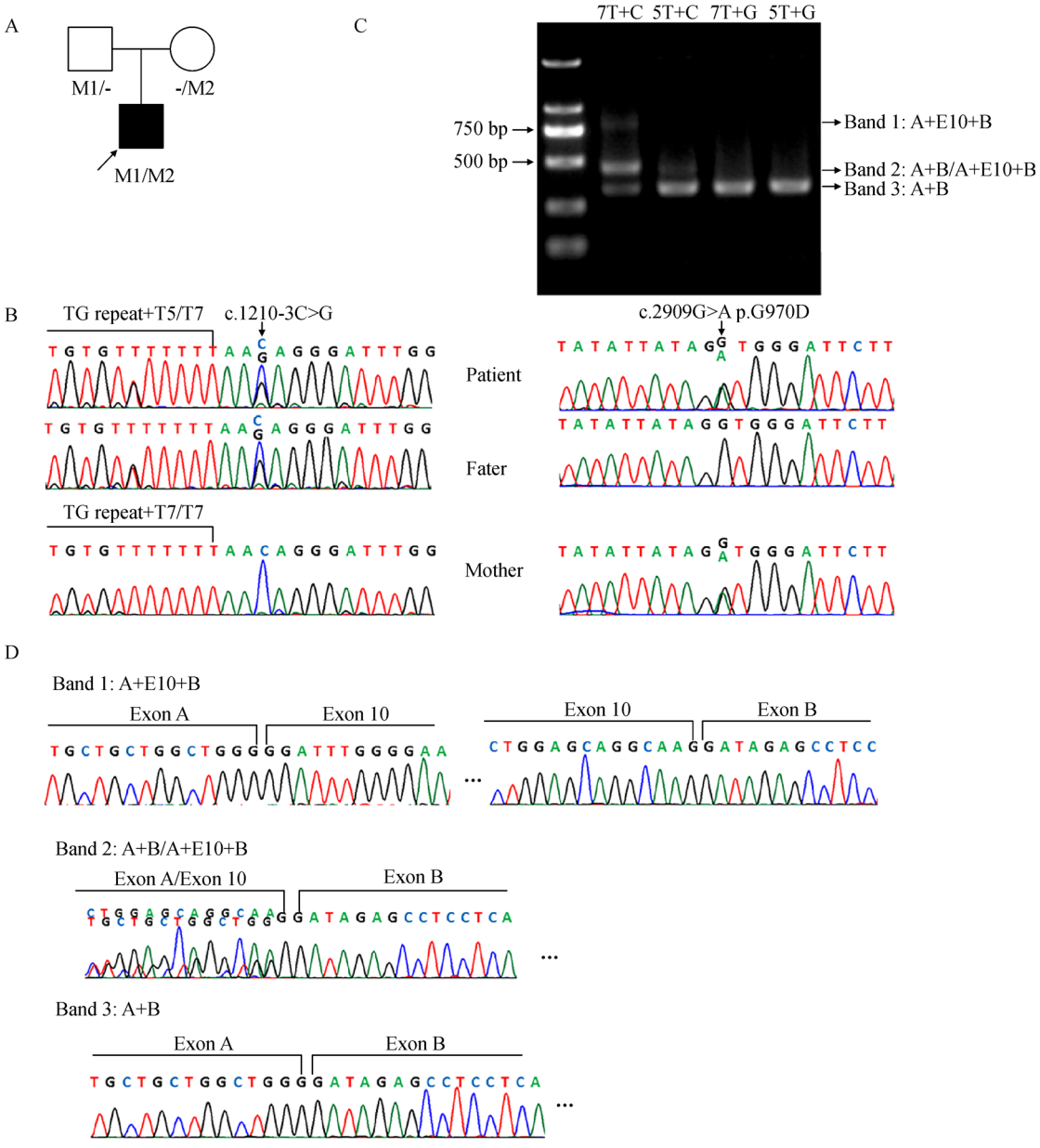

Fig. 2 *CFTR* mutations in the patient and his parents and their effect on splicing pattern. (A) Pedigree and genotype. Squares and circles symbolize males and females, respectively. No fill symbolizes unaffected individuals, and black fill symbolizes affected individuals. M1, mutation c.1210-3C>G; M2, mutation c.2909G>A (p.Gly970Asp); -, wild type. The arrow indicates the proband. (B) Sanger sequencing outcomes in the patient and his parents to verify their mutations. (C, D) Splicing patterns of plasmid: 7T + C, 5T + C, 7T + G, and 5T + G were shown by electrophoresis (C) and Sanger sequencing (D). Top band (band 1) is the wild-type transcript of exon A + exon 10 + exon B; middle band (band 2) is the heterodimer of wild-type transcript and mutant transcript; bottom band (band 3) is the mutant transcript of exon A + exon B possessing a deletion of exon 10.

was observed in individuals with all four different combinations but with different levels. 7T + C has an approximately equal number of transcripts with exon 10 and without exon 10. 5T + C has relatively more transcripts without exon 10 than with exon 10. 7T + G and 5T + G both have almost all transcripts without exon 10 and almost no transcripts with exon 10.

Discussion

Cystic fibrosis (CF) is a rare autosomal recessive inherited disease associated with impaired pulmonary function and a defective digestive system. With symptoms affecting multiple organs and a relatively high incidence rate, CF has become one of the most frequent lethal diseases among

Caucasians [14]. Since *CFTR* was considered a CF-causative gene, more than 2000 mutations have been recognized, forming a defined *CFTR* mutation spectrum of Caucasian origin. By contrast, the morbidity of CF is low in China, considering that reports on CF among large populations in China is few. Atypical clinical syndromes and novel mutations unidentified previously in Caucasians also show an ethnicity-specific pattern in Chinese patients with CF [9]. In the present study, we aimed to identify potential pathogenic mutations in a CF patient of Chinese origin. By Sanger sequencing, we identified a low-frequency variant p.Gly970Asp (c.2909G>A, p.G970D) and a novel variant c.1210-3C>G in this patient.

p.Gly970Asp is located within the third cytoplasmic loop of the CFTR protein (Fig. 3), and its impairment of chloride conductance may lead to a potentiator-sensitive gating defect and a partial trafficking defect without RNA splicing alteration [7,15]. Amato found that the p.Gly970Asp mutant plasmid-transfected HEK293 cells show very low channel activity, demonstrating the mutation's pathogenicity [15]. In addition, with an allele frequency of 9.8%, p.Gly970Asp is the most commonly seen hotspot mutation in CF patients of Chinese origin [9]. This finding may encourage the eligibility of pharmacogene treatment focusing on p.Gly970Asp to correct the CF phenotype in Chinese patients.

The second mutation, c.1210-3C>G, in this patient is adjacent to the splice acceptor site of *CFTR* exon 10, in *cis* with a poly-T tract of 5T, 3 bp upstream. c.1210-3C>G is a novel mutation with no frequency data on GnomAD, and c.1210-3C shows a high degree of conservation among different species in UCSC database, indicating that c.1210-3C>G could be a pathogenic variant. Human splicing finder was chosen to predict the effect of c.1210-3C>G and the complicated outcome of c.1210-3C>G combined with the poly-T tract of 5T, both of which were predicted to most likely affect splicing pattern. This result suggests that c.1210-3C>G might contribute to the pathogenicity in this patient, which prompted us to test its effect on the splicing pattern. Unfortunately, the *CFTR* expression was very weak to be captured from peripheral blood leukocytes, and this study was restricted to a minigene assay *in vitro* because of the unavailability of the nasal epithelium from this patient.

CFTR exon 10 in-frame skipping mRNA is observed in normal individuals with the poly-T tract of 7T, and among the 5T, 7T, and 9T forms of the poly-T tract, referring to the exon 10 splice acceptor site, the shorter the poly-T tract, the higher the amount of aberrant *CFTR* mRNA transcripts in the respiratory epithelium, as described in Chin-Shyan's article [10]. However, the $TG_{12}T_5$ sequence may not be strong enough to be a disease-causing variant *per se* (data shown on the CFTR2 website). The level of human exon 10+ transcripts in the bronchial epithelium of non-CF individuals can account for as low as 8% of total *CFTR* transcripts [11]. This finding could be explained by the residual normal mRNA being abundant enough to exert *CFTR* function, considering that CF is an autosomal recessive disease but not a haploinsufficiency. By contrast, c.1210-3C>G, a possibly pathogenic mutation carried by this patient, seems to arouse the CF phenotype alone, considering that the normal transcript can hardly be seen in the 7T + G and 5T + G plasmids, according to the results of the minigene assay. The 5T variant alone is not a definitive CF-causing variant, but it may act synergistically with c.1210-3C>G in *cis* to produce a null allele [16,17]. An antecedent case was reported in which the combined effect of $TG_{12}T_5$ and p.Arg117His in the same chromosome as p.Phe508del in *trans* contributed to the CF phenotype, which also hypothesized the combined effect of two mutations [17].

CFTR exon 10 (regarded as exon 9 in reference article for legacy name) encodes the first 21% of NBF1, a domain that is critical to CFTR function [18]. The defective protein encoded by a transcript without exon 10 cannot exit from the endoplasmic reticulum and traffic through the Golgi

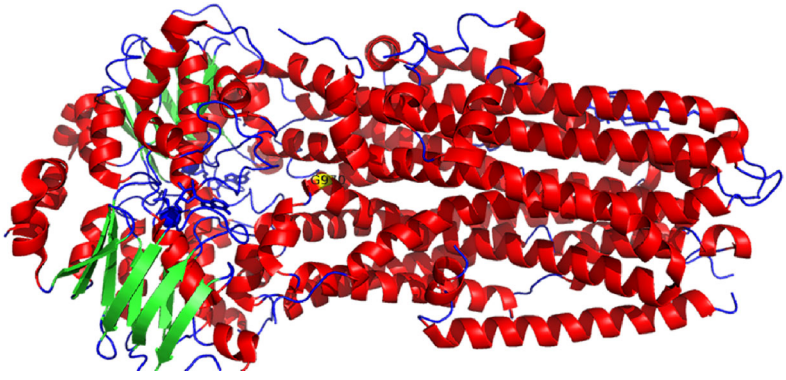

Fig. 3 Dephosphorylated, ATP-bound human CFTR protein structure, with G970 colored yellow in the center. This view shows that G970 is a critical residue for chloride ion channel function.

complex to the cell membrane. The resultant extremely decreased normal CFTR Cl^- channel in the cell membrane and may lead to the CF phenotype in our patient [11,19].

The present study reports a patient who carries p.Gly970Asp, a relatively high-frequency mutation among Chinese patients with CF, and a novel c.1210-3C>G mutation, which possibly acts synergistically with $TG_{12}T_5$ in intron 9 to cause the CF phenotype. To some extent, this study expands the mutation spectrum of the *CFTR* gene in patients with CF of Chinese origin and explains the potential pathogenicity of a novel splicing mutation.

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Compliance with ethics guidelines

Xinyue Zhao, Keqiang Liu, Wenshuai Xu, Meng Xiao, Qianli Zhang, Jiaxing Song, Keqi Chen, Yaping Liu, Xinlun Tian, Kai-Feng Xu, and Xue Zhang declare that they have no conflict of interest. All procedures followed were in accordance with the ethical standards of the *Helsinki Declaration* of 1975, as revised in 2000 and were approved by the Institutional Review Board committee at PUMCH. Informed consent was obtained from the patient for being included in the study.

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