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Molecular cloning, characterization and tissue expression of porcine creatine transporter

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ABSTRACT

The present study was to clone porcine creatine transporter from Landrace pig, and investigated its tissue distribution. The full-length cDNA of porcine skeletal muscle creatine transporter (CrT) was first cloned by using rapid amplification of cDNA end technology and the full sequence of porcine skeletal muscle CrT was 2,374bp, with a 1,908bp open reading frame encoding a 635-amino acids protein (a predicted molecular mass of 70.4 kDa). The RT-PCR assay showed that the porcine CrT was highly expressed in porcine skeletal muscle, followed by kidney, lung, heart, liver, brain and spleen. *In-situ* RT-PCR and enzyme-histochemistry technology were also used to detect the distribution of CrT mRNA in porcine *psoas major* muscle (PM), the result showed that the area of precipitation was significantly correlated with the area of muscle fibre and the area of type red muscle (ßR) fibre was significantly lower than that of intermediate muscle (aR) or white muscle (aW) types in porcine PM. This is the first systematic report of molecular cloning, characterization and tissue expression of porcine CrT for a better understanding of the creatine metabolism in pigs.

Key words: Creatine transporter, Histochemistry, Molecular cloning, Porcine

Creatine (Cr), an amino acid derivatives, is synthesized from arginine, glycine and adenosyl-methionine (Walker 2006, Bera *et al.* 2008). It is partially synthesized by kidneys, pancreas and liver, as well as ingested from food, and thereafter mainly transferred to the skeletal muscle, brain and testes (Wyss and Kaddurah-Daouk 2000, Salomons *et al.* 2003). Recent evidences suggested that the brain and testes may also synthesize Cr (Moore 2000, Braissant *et al.* 2001). A proportion of Cr entering the cell is phosphorylated to form Cr phosphate (PCr), which provides an immediate source of ATP for energy requiring process.

Cr plays an important role in rapid energy provision, its major stores in skeletal muscle. Interestingly, skeletal muscles, which contain the high concentration of PCr and total Cr, do not synthesize their own Cr (Braissant *et al.* 2001; Snow and Murphy 2001). Previous studies showed that most of the Cr is absorbed via a specific CrT from the plasma into the respective tissue (Walzel *et al.* 2002). CrT is an important component of the Cr kinase/PCr system, which is necessary for the Na^+/Cl^- dependent Cr uptake into the cell, and the process of Cr transport is saturable. So far, researchers have cloned the CrT from rat and cattle, but little is known about the porcine CrT. Considering the pivotal role of CrT in the energy metabolism, it is important that its biology is fully understood, the aim of this study was to clone the cDNA of CrT and investigate its tissue distribution.

MATERIALS AND METHODS

Animals and samples: In experiment1, 3 female Landrace pigs with a mean BW of 90kg were selected and killed, *Longissimus dorsi* muscle were sampled for skeletal muscle CrT clone; in experiment 2, male landrace pigs (5), 5-month-old, were used to investigate the expression pattern of CrT. Heart, lung, liver, all brain, kidney, spleen and *psoas major* muscle were collected, rapidly frozen in liquid nitrogen, and then stored at –80°C.

RNA extraction and cDNA synthesis: Total RNA was extracted from pig tissues using trizol reagent according to the manufacturer's instructions. The RNA extracts were treated with RNase-free Dnase 1 to remove contaminating genomic DNA. The concentration and quality of the RNA was assessed by spectrophotometry and checked by 1% agarose gel electrophoresis, stained with 10µg/mL ethidium

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bromide. The first strand cDNA was synthesized using reverse transcriptase with Oligo (dT) $_{20}$.

Partial CrT cDNA sequence amplification: Two homologous *Sus scrofa* CrT expressed sequence tags (ESTs) were obtained from GenBank, assembled into one EST contig and checked manually. One pair of primers (forward primer: 5' -CCA CGA AGA CTG TGC CAA TG-3'; reverse primer: 5' -GGC GAT GAA AGC CAG ACC-3') were designed based on the contig sequence and used for reverse transcription PCR amplification. The PCR products were extracted from 1% agarose gel and ligated to PGM-T vector transformed into *E. coli.* (DH5α). The recombinant clones were screened by bacterial colony PCR. The selected positive recombinant clones were sequenced by a biological engineering technology and services.

Rapid amplification of cDNA end (RACE)-PCR: To obtain the full-length cDNA sequence of CrT, RACE technology was carried out by using the 5'/3'-RACE kit. Total RNA was prepared as described above. Two gene-specific primers (GSPs) for CrT gene cDNA were designed according to the obtained partial cDNA sequence (GSP1: 5' -AGC TTC GAC CAG TCG GGC TTG AGA TAG-3'; GSP2: 5' -CGC CTT GGA CGG CAT CAT CTA CTA TCT C-3') and synthesized. The RACE cDNA was prepared with an SMART RACE cDNA. Amplication kit according to the manufacturer's protocol. The resulting sequences were verified and subjected to cluster analysis.

Quantification of mRNA levels by real-time RT-PCR analysis: Three housekeeping genes (GAPDH, 18S, U6) frequently used as a reference in real-time PCR experiments, were tested for expression stability under the experimental conditions. Primers for housekeeping genes and target gene were designed with primer 5.0, the sequence of primers are shown in Table 1. Real-time RT-PCR was performed using SYBR Green PCR mix. 1μL cDNA template solution was added to 20μL containing 10μL SYBR Green mix, and 1μL each of forward and reverse primers. The real-time RT-PCR condition was as follows: 95°C for 1min; 40 cycles of 95°C for 15s, 50! for 15s and 72°C for 40s; 95°C for 1min, 50°C for 30s and 95°C for 30s. The identity of each product was confirmed by sequencing. Amplification and melt curve analysis were performed in Mx3005. We used $R=2^{-\Delta Ct}$ (ΔCt = Ct of the target gene-Ct of the housekeeping gene) to calculate the relative expression ratio (R). Real-time PCR efficiencies were evaluated by the amplification of dilution series of cDNA according to equation 10 (-1/slope) and were consistent between target mRNA and housekeeping gene mRNA. Negative controls were performed in which cDNA was substituted by deionized water.

In-situ RT-PCR: Tissue samples (10μm thick), mounted on capillary slides, were deparaffinized and rehydrated according to standard histologic methods. The slide-mounted tissue samples were digested with proteinase k at 37°C for 20 min and treated with RNase-free DNase 1 (50U per tissue sample) at 37°C for 3 h. Tissues samples were then rehydrated with ethanol 100%, 75% and 50% respectively. The two-steps of RT *in situ* PCR approach was modified from a previously described technique (Yong *et al.* 2009). N10 primers were used to synthesis the first-strand cDNA. 50μL of the following mixture was added to each slide: 41.5μL RNase-free water, 1μL dNTP mix (10mM each), 2μL CrT primers (10μM each), 0.5μL Ex TaqTM Polymerase (2.5U/μL), 5μL 10×PCR buffer. The slides were covered and placed in the thermal cycle. The amplification condition was as follows: 94°C for 5min; 30 cycles of 94°C for 1min, 58°C for 1min and 72°C for 1min; 72°C for 7min. After a series of stringent washes with phosphate buffer solution, the amplification samples were rehydrated. Then distribution droplets streptavidin marked by horse radish peroxidase (200: 1), incubated in the PCR instrument 37°C for 2 hours. The reaction was revealed using the chromogen diaminobenzidine for 20 min. After washing in double distilled water and rehydrating, slides were examined with a light microscope and images were captured.

Enzyme histochemistry: Ten-micrometer thick frozen PM sections were cut on *cryostat microtome* at –25°C and mounted on glass slides. Myosin ATPase and uccinodehydrogenase activities were detected after acid (pH

Table 1. Primers used in quantitative RT-PCR

Gene	Primer sequence	Products length	GenBank accession no.
CrT	F: 5'-GGACGGCATCATCTACTATCTC-3' R: 5'-CCACGAAGCCAGCAAAG-3'	218	NM_001177327
GAPDH	F: 5'-CACCATCTTCCAGGAGC-3' R: 5'-CTTCAAGTGAGCCCCAG-3'	118	NM_001206359
18s	F: 5'-AATTCCGATAACGAACGAGACT-3' R: 5'-GGACATCTAAGGGCATCACAG-3'	145	NR_002170
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'	95	NR_003027

The sample data obtained from real-time PCR analysis were subject to one-way analysis of variance (ANOVA). P values were calculated by student's t-test (n=5). P values less than 0.05 were considered statistically significant. Statistical analysis was performed using SPSS 18.0 for windows.

4.3) pre-incubation (Dai *et al.* 2009). The muscle fibers were divided into types ßR, αR and αW (Ashmore and Doerr 1971). After staining, the slides were examined by an image processing system. Approximately 500 to 600 fibers were evaluated per sample, and fiber area was the average area of at least 50 fibers of each fiber type.

RESULTS AND DISCUSSION

Partial CrT cDNA sequence: Two homologous *Sus scrofa* CrT ESTs were obtained from GenBank (GenBank accession no.CV871555; CV866116), and assembled into one EST contig. One pair of primes was designed based on the EST contig for RT-PCR amplification. Finally, partial sequence of cDNA (607bp) was acquired. Sequence analysis showed that it shares a high homology (93.13%; 90.36%) with the corresponding sequences of mouse (GenBank accession no.AB077327) and cattle (GenBank accession no.AF027197).

Identification and characterization of porcine CrT cDNA: The entire cloned sequence (Fig.1) of the porcine CrT cDNA is 2374bp, including a 1908bp ORF coding 635 amino acids with an estimated mass of 70.4 kDa, similar in size to the CrT genes reported in other animals (Guimbal and Kilimann 1993, Sora *et al.* 1994). (Fig. 2) Porcine CrT protein exhibits an overall 90% amino acid identity to the mouse and cattle CrT protein. We further checked our RACE results by amplifying of open reading frame, the expected bands were gained by ORF PCR, and the sequencing results of ORF were also same as these of RACE (data not shown). Based on the above information, we can conclude that the cloned sequence is porcine CrT sequence. The full-length cDNA sequence of porcine CrT and its deduced AA sequence were deposited in the GenBank under accession number of GQ180109. Nucleic acid analysis showed that 3'-untranslated region (3'-UTR) contains a polyadenylation signal (AATAAA) 20 nucleotides upstream from the poly(A) tail, similar to the previous study (Simeone *et al.* 1986), so the sequence of CrT has an intact 3'-UTR. There are 12 putative membrane-spanning domains within porcine CrT, similar to other mammalian CrT (Guimbal and Kilimann 1993, Sora *et al.* 1994, Saltarelli *et al.* 1996).

Tissue distribution of porcine CrT mRNA: In this study, 18s rRNA was chosen as our housekeeping gene. The CrT mRNA expression in different tissues is shown in Fig. 3(A).

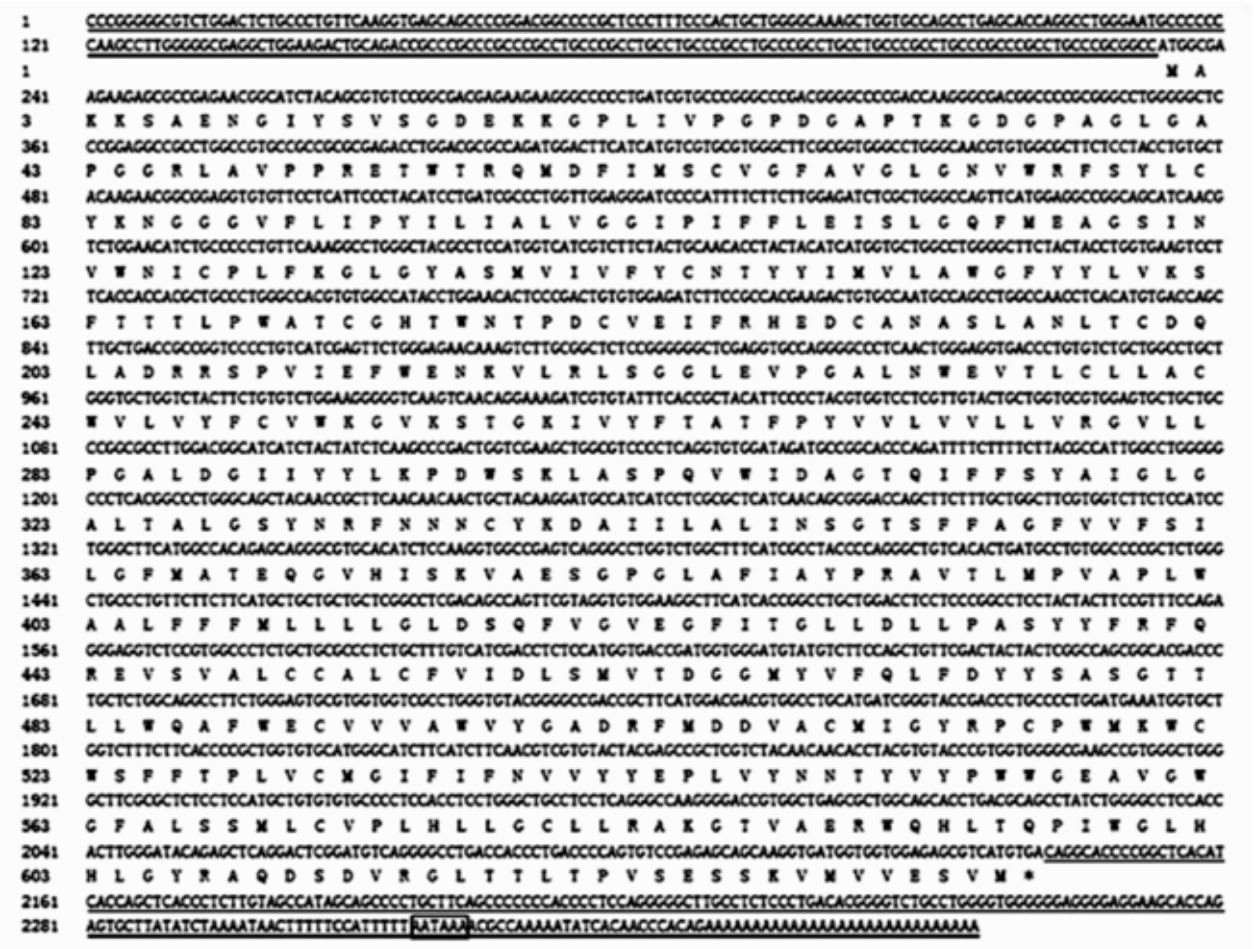

Fig.1. Nucleotide sequence of the porcine CrT and the amino acid sequence it is encoding for. The nucleotide and amino acid residue numbers are shown on the left. The sequence of 3'-UTR and 5'-UTR are underlined. Consensus polyadenylation signal AATAAA is boxed.

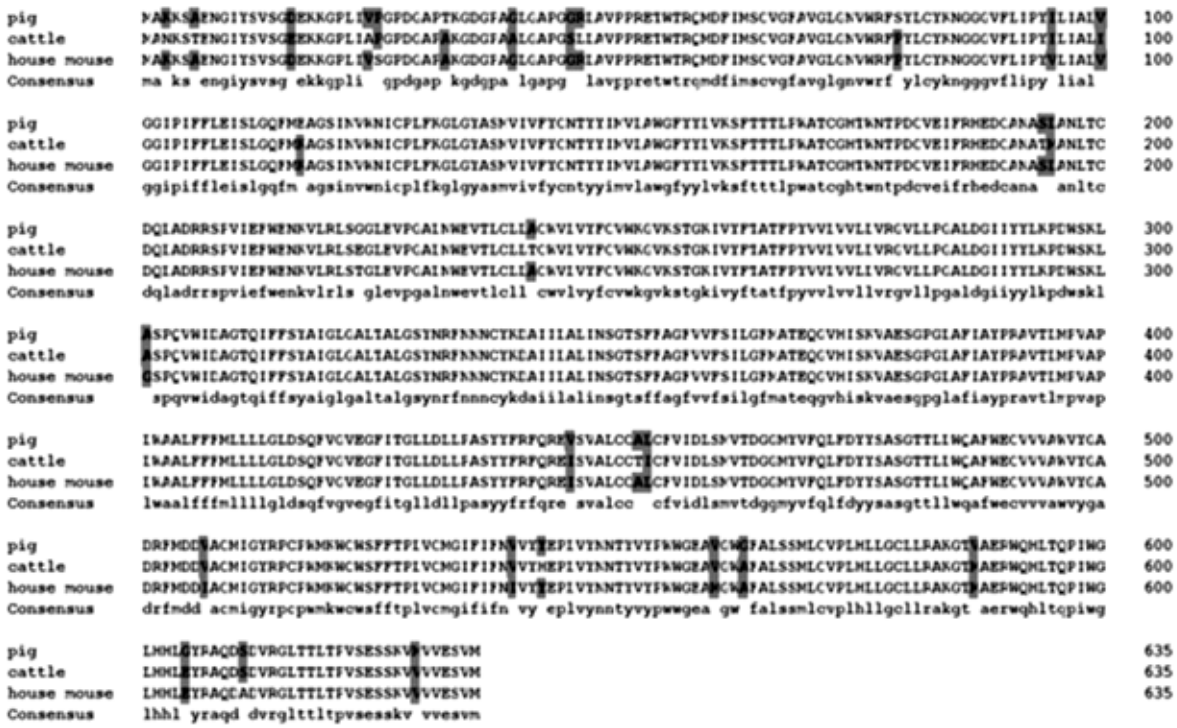

Fig.2. Multiple amino acid sequences alignments of CrT gene. Protein sequences were obtained from GenBank: ACS71529 (pig: *Sus scrofa*); AAB84029 (cattle: *Bos taurus*); BAC11857 (house mouse: *Mus musculus*).

The result showed that the skeletal muscle had the highest CrT mRNA abundance and the heart, liver, brain and spleen had the lowest CrT mRNA abundance. However, there is no significant difference among the heart, liver, brain, and spleen. Compared with skeletal muscle, lung and kidney had lower CrT mRNA abundance, and there is no significant changes between lung and kidney. Additionally, lung and kidney had higher CrT mRNA abundance than the heart, liver, brain and spleen ($P<0.05$). We conclude that the porcine CrT is widely expressed in pig tissues, which suggest that creatine metabolism is very important in pigs physiology. Interestingly, the skeletal muscle had the highest CrT mRNA abundance compared with other tissues, which is difference from the previous studies, it may be because of the different developmental stages of pig. When pig grows fast, the basal metabolic rate increases, the pig may need more CrT to satisfied the skeletal muscle for creatine metabolism. Most of the investigations revealed that the CrT mRNA were not expressed in the liver (Guimbal and Kilimann 1993, Sora *et al*. 1994), but in our study, we detected the CrT mRNA expression in the liver. The differences may be correlated with different methods and the source of the tissues.

Histochemistry and muscle fiber typing

In situ PCR is a histological technique that exploits the advantages of PCR for detection of mRNA directly in tissue section, It conjugates together, PCR and *in situ* hybridization (Lossi *et al.* 2011). Recently, *in situ* PCR has been used to detect low levels of mRNA within cells and tissue sections (Bagasra *et al*. 2005, Hishikawa *et al*. 2009). In this experiment, the *in situ* positive sign of CrT mRNA was mainly expressed within muscle fiber cells in porcine psoas major muscle (Fig. 3B). The muscle fiber with higher area had stronger signal intensity, and the area of precipitation was significantly correlated with the area of muscle fibre (Tables 2,3). Murphy (2001) found that the soleus and red gastrocnemius muscles contain about 2–3 fold greater transporter protein content than the white gastrocnemius muscle in rats (Murphy *et al.* 2001), and the soleus and the

Table 2. Area of diaminobenzidine precipitation and muscle fiber and their correlation (n=52)

Indexes	Area (μm^2)	Related coefficient
Diaminobenzidine precipitation	518.28±32.72	0.319
Muscle fiber	5159.53±207.76	

Table 3. Area of muscle fibers from porcine *Psosa* muscle (n=5)

Indexes	ßR	aR	aW
Area (μm^2)	3333.68 ±119.19[a]	4759.15 ±145.36[b]	6537.59 ±223.92[c]

Myofiber typing (αR: α-red fiber; ßR: ß-red fiber; αW: α-white fiber). Different characters mean that there is significant difference among different muscle fiber types ($P<0.05$).

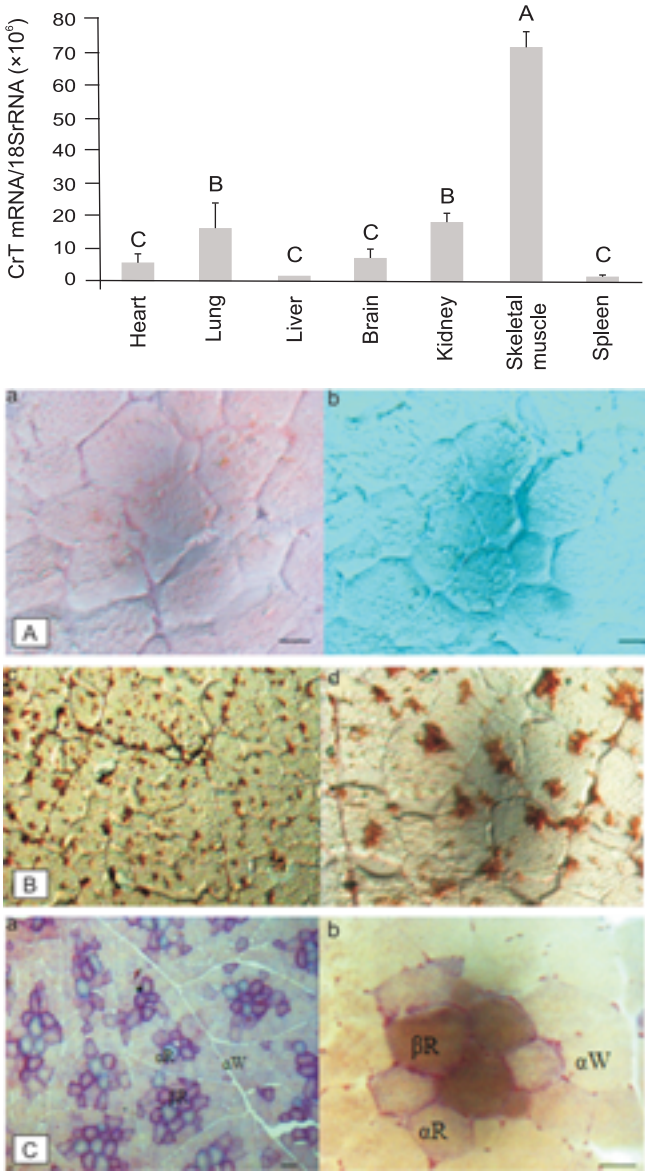

Fig. 3. Tissue distribution of porcine CrT mRNA and myofiber typing. (*A*) Tissue expression pattern of porcine CrT mRNA.18S rRNA gene was used as the internal control for qRT-PCR. Vertical bars represent the mean±SE (n=5). The different letter indicates that the values are significantly different (Pdþ0.05). (*B*) Determination of CrT mRNA in porcine *psoas* muscle by *in situ* RT-PCR. Positive signal was shown in aubergine. *a*: negative control without reverse transcription (10×40); *b*: negative control without PCR (10×40); *c*: treatment (10×10); d: treatment (10×40). (C) Histology of muscle fibers from porcine *psosa major* muscle. Myofiber typing (aR: a-red fibre; ßR: ß-red fibre; aW: a-white fibre). a: the magnification of microscope was 10×10. b: the magnification of microscope was 10×40.

red gastrocnemius contain predominantly type and I fibers, whereas the white gastrocnemius consists predominantly of IIb with IIx fibers (Delp and Duan 1996). Through the above information, we can conclude that the rat skeletal CrT protein was expressed in type I and IIa fibers, less in IIb fibers, and the distribution of CrT protein within skeletal muscle is fiber type-dependent. As shown in figure 3C and Table 3, the area of type ßR fiber was significantly lower than that of αR or αW types in porcine PM, indicated that the skeletal muscle CrT mRNA was mainly expressed in the fast muscle cell.

In conclusion, we have cloned and characterized the porcine CrT gene and investigated the CrT tissue expression in pigs. This is the first systematic report of molecular cloning, characterization and tissue expression of porcine CrT, which will be benefit for a better understanding of the creatine metabolism in pigs. The Cr will be especially relevant in the field of meat quality in which there is an increasing use of Cr as a source of additive for improvement of the meat quality.

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