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Cloning and Tissue Expression of *Clusterin* in *Bufo gargarizans*

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Abstract: *Clusterin (CLU) has become an important target in cancer-therapy and new anti-tumor drug development. In current study, cloning of CLU open reading frame (ORF) from Chinese toad by polymerase chain reaction (PCR) and CLU tissue distribution by real-time fluorescent quantitative PCR (qPCR) were carried out. As the results, 1 332 bp ORF (GenBank accession number: KX640823) was cloned encoding a protein consisting of 443 amino acid residues with 98% homology with Japanese toad CLU, which indicates the successful cloning of Chinese toad CLU ORF. qPCR indicated higher CLU expression in testis, and lower in other organs relative to the expression level in skin, while no expression was detected from spleen.*

Keywords: *Bufo gargarizans; Clusterin; Tissue distribution*

1. INTRODUCTION

Amphibian skin and skin secretions are rich in bioactive ingredients [1,2]. *Bufo gargarizans* (Chinese toad) is such an amphibian species providing "ChanSu" (toad skin secretions), "ChanYi" (toad cortex) and "ChanPi" (toad skin), all are the valuable materials of Traditional Chinese Medicine used in many clinical prescriptions [3-5]. Cinobufacini injection is water extract of dried toad skin of Chinese toad and its antitumor effect has been demonstrated [6]. The polypeptide mixture isolated from Cinobufacini injection showed the same antitumor activity as injection itself in vitro indicating polypeptides as the main antitumor ingredients [7]. Recent years, the authors screened skin cDNA plasmid library of Japanese toad (*B. japonicus formosus*), and some cDNAs with great potential for new-drug development have been cloned, such as *MCL1* [8], *SCP2* [9], *TAGLN2* [10], *PPDFP* [11] and *CLU* [12].

CLU, also known as SGP-2 (sulphated glycoproterin-2), TRPM-2 (testosterone repressed message-2) or APOJ (apolipoprotein J) [13], is divided into nuclear type (nCLU) and secretory type (sCLU) according to their locations in the cells [14]. sCLU is a highly glycosylated multifunctional protein, which is widely expressed in a variety of mammalian tissues and participates in many physiological processes, such as cell cycle regulation, DNA repair, and angiogenesis [15]. Recent studies have shown over-expression of *CLU* in several cancers, whose blocking enhances tumour cell sensitivity to chemotherapy and radiotherapy, therefore, *CLU* has become an important therapeutic target for cancer treatment [14,16].

Due to the lack of *CLU* data of Chinese toad, cloning of *CLU* open reading frame (ORF) by DNA polymerase chain reaction (PCR) and its tissue distribution by real-time fluorescent quantitative PCR (qPCR) were carried out in current study.

2. MATERIALS AND METHODS

2.1. Materials

Chinese toad was captured on the East lake Campus of Zhejiang A&F University, and ice anaesthetized before dissection. Their organs were removed and chopped into liquid nitrogen, which were kept at -80°C for RNA extraction later. The RNA Extraction Kit was purchased from Bocai Biotechnology Company (Shanghai, China); Real Master Mix (SYBR Green) for qPCR from Tiangen Biotechnology Limited Company (Beijing, China); PrimeScript™ II 1st Strand cDNA Synthesis Kit and *Taq* PCR Kit from TaKaRa (Dalian, China). Primer synthesis, TA cloning of PCR products and DNA sequencing were done by GENEWIZ (Suzhou, China).

2.2. Methods

RNA extraction and the first strand cDNA preparation RNA extraction and cDNA preparation of each organ of Chinese toad were followed Manufactures' Instructions as reported previously [9,11].

CLU ORF cloning for cloning of Chinese toad *CLU* ORF, primers (F1/R1) were designed based on the full length *CLU* cDNA of Japanese toad (GenBank accession number: JX035891) (Table 1). The reaction system of PCR was given in Table 2 and PCR was performed at 94°C for 40 secs, 54°C for 40 secs and 72°C for 90 secs, 30 cycles.

Sequence Analysis DNAstar/EditSeq was used to find ORF and deduce the amino acid sequence of encoded protein. Eleven CLU sequences of other animals were downloaded (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which were aligned and the phylogenetic tree was constructed with DNAstar/MegAlign.

Table 1. *Primers used in current study*

Primer	Sequence (5'-3')	Primer	Sequence(5'-3')
F1	GGACTCCAGAGCCGGA	R1	CAAAGCCCATTGTGTCC
F2	GGT GGTCATTCTGTTCAG	R2	GCCCTCCTTGTGCCTC
F3	TTGAGACCTTCAACACC	R3	CTTGATGTCACGCACAA

Table 2. *PCR constitution*

Components	*Taq* PCR	qPCR
10×*Taq* buffer	2.0 μl	0.0 μl
10 mM dNTPs	0.8 μl	0.0 μl
Taq (5U/ μl)	0.2 μl	0.0 μl
2×qPCR Master Mix	0.0 μl	5.0 μl
Primer F (2 pmol/ μl)	1.0 μl	0.5 μl
Primer R (2 pmol/ μl)	1.0 μl	0.5 μl
First strand cDNA	1.0 μl	0.5 μl
H_2O	14.0 μl	3.5 μl

CLU Tissue distribution analysis Primers both for *CLU* qPCR (F2/R2) (designed based on Chinese toad *CLU* ORF) and for reference gene of β-actin (F3/R3) [9,11] were given in Table 1. qPCR reaction system was given in Table 2, which was performed at 95°C for 3 secs, 52°C for 30 secs, 72°C for 20 secs, 40 cycles. For each organ, cDNA from 3 individuals were used and the expression level of *CLU* was relative to that of skin.

3. RESULTS

CLU cloning and homology analysis Sequencing analysis showed 1 332 bp *CLU* ORF of *B. gargarizans* (GenBank accession number: KX640823), which is the same as that of Japanese toad [12], while 23 bases are different between them (Fig. 1). Homology analysis at amino acid level indicated 98% between Chinese toad and Japanese toad, 65% with *Xenopus laevis* (NP_001080775.1) and *X. tropicalis* (AAH75303.1), and 42%~49% with *Homo sapiens* (NP_001822.3), *Pan troglodyte* (XP_001164647.1), *Gallus gallus* (NP_990231.1), *Numida meleagris* (AW21812.1), *Chelonia mydas* (XP_007071391.1), *Alligator sinensis* (XP_006033945.1), *Latimeria chalumnae* (P_006004784.1) and *Danio rerio* (AAQ56181.1) (Fig. 2). Phylogenetic tree showed two *Bufo* species get together, two *Xenopus* species gather together, while all these 4 amphibians converge in one large branch (Fig. 3), which is consistent with the traditional animal taxonomy. Further analysis indicated 5 amino acids are different between two *Bufo* species (asterisks in Fig. 2), the first two locate in signal peptide (dot-line

in Fig. 2), the 3rd in non-conserved area, the 4th in X position of the 3rd N-glycosylation area (Asn-X-Ser/Thr), and the last one in unimportant domain (Fig. 2). Other important characteristics of CLU are conserved completely between Chinese and Japanese toads including 5 disulfide bonds, 6 N-glycosylated structures and 2 coiled coil regions (Fig. 2).

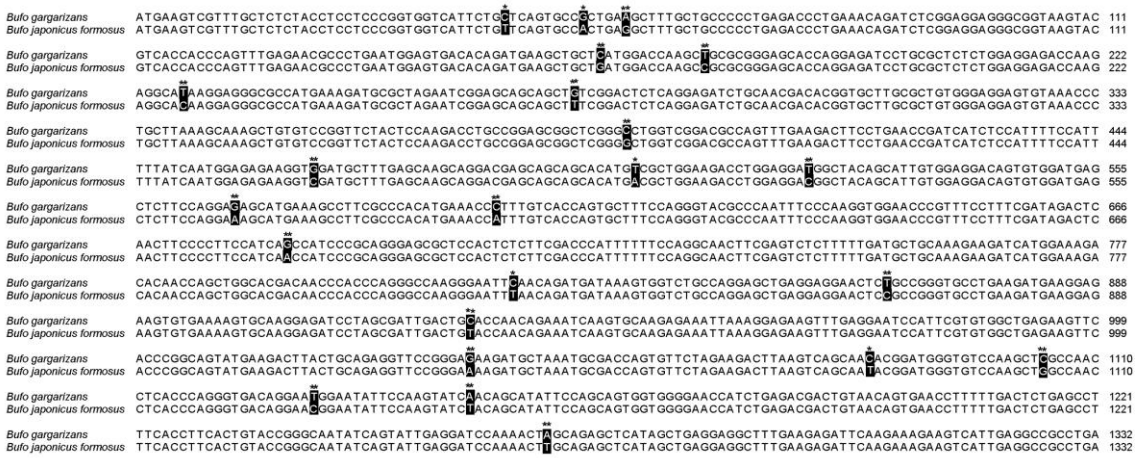

Fig1. *Comparison of CLU ORF between B. gargarizans and B. japonicas formosus*

Shade: Different nucleotide between two *Bufo* species. *: Missense. **: Synonymous

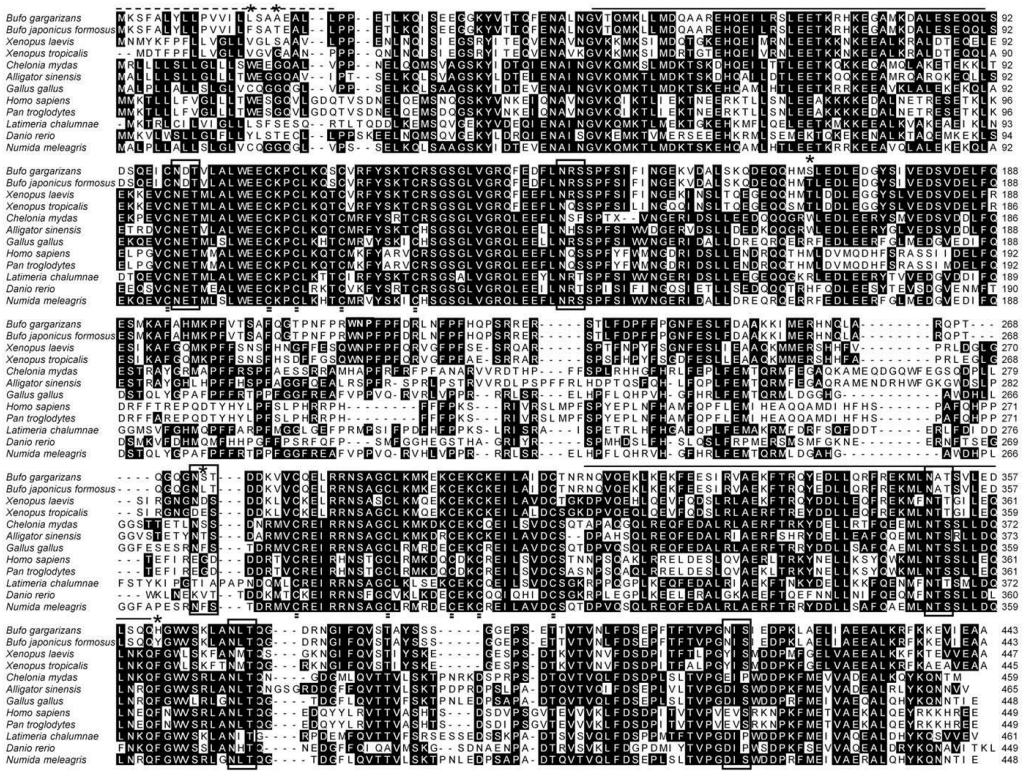

Fig2. *Alignment of CLU amino acid sequence between B. gargarizans and other animals*

Dot-line: Signal peptide. Line: coiled coil region. *: Different amino acid residue between two *Bufo* species. Double-line: Cysteine residue. Rectangle: Location of N-glycosylation (Asn-X-Ser/Thr, X indicates any amino acid residue).

CLU tissue distribution to clarify the tissue distribution of *CLU* in Chinese toad, qPCR was performed (Fig. 4), which indicated higher expression in testicle, lower in heart, liver, lung, kidney, intestine and stomach relative to the expression level in skin, but no expression was detected from spleen.

4. DISCUSSIONS

In current study, *CLU* ORF of *B. gargarizans* (Fig. 1) was successfully cloned, whose encoding protein showed 98% homology with that of Japanese toad (Fig. 2 and 3) [12]. All-important characteristics of CLU are completely conserved between Chinese toad and Japanese toad including 5

disulfide bonds, 6 N-glycosylated structures and 2 coiled coil regions suggesting the same function of CLU of both *Bufo* species. qPCR analysis discovered higher expression in testis (Fig. 4).

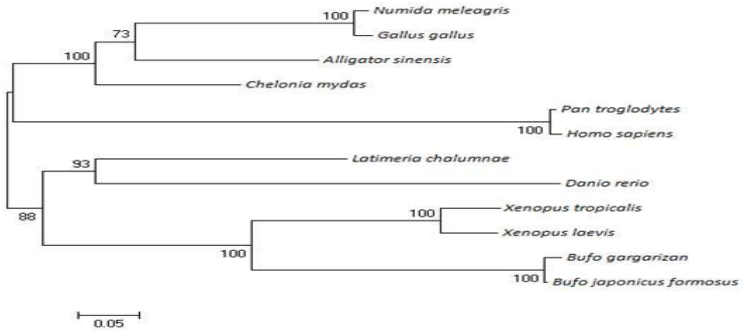

Fig3. *Phylogenetic tree based on CLU amino acid sequences of B. gargarizans and 11 other animals*

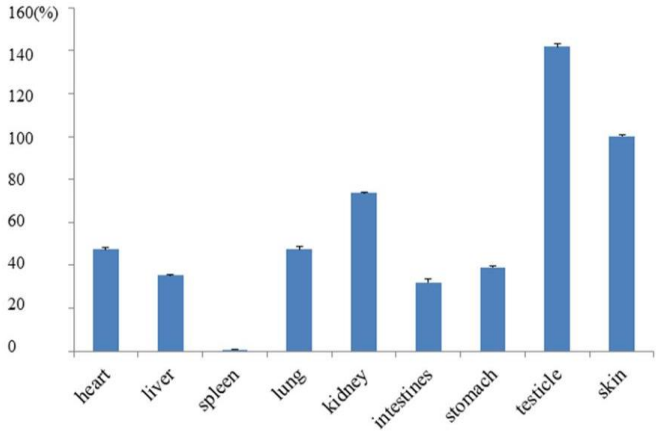

Fig4. *qPCR analysis of CLU expression in different organs of B. gargarizans*

Abscissa: Organs of *B. gargarizans*. Ordinate: *CLU* expression level relative to skin. Error bars: Standard deviation from the data of 3 individuals.

Concerning the mechanism of *CLU* expression, although it is not so clear currently, but influenced by cytokines, growth factors as well as stress ones such as oxidative stress, heat shock, chemotherapy and radiotherapy [14,17]. According to previous reports, the tolerance to chemotherapy and radiotherapy is closely related with *CLU* expression in several cancers, and *CLU* up-regulation has been confirmed in many cancers including prostate, kidney, bladder, breast, head and neck, colon, cervical, pancreatic, lung, melanoma lymphoma [16,18]. Therefore, *CLU* has been considered as a biomarker for tumour malignancy, and it is believed that improvement of tumour cell sensitivity to chemotherapy and radiotherapy is promising way to control cancer by reducing *CLU* expression [14,19-21].

Cinobufacini as an anticancer drug, its polypeptide mixture has been demonstrated to have the same antitumor activity as the injection itself, whose molecular weight is 3~5 kDa [7]. In fact, Cinobufacini also works as oral liquid, tablet and capsule. These studies indicated toad skin protein might function in short peptides instead of intact protein. For example, Buforin I and II are antimicrobial poly-peptides consisting of 1-39 and 16-36 amino acid residues of *B. gargarizans* H2A, respectively [22]. Abhisin is 1-40 amino acid residues of *Haliotis discus* H2A having antibacterial activity as well as anti-endotoxin and anti-tumor activity [23]. NuBCP-9 (the 489-497 amino acid residues of Nur77) had good antitumor activity [24,25]. Although there is no direct evidence shown CLU or CLU-derived peptide included in toad skin or Cinobufotini, *CLU* ORF does have been cloned from skin first-strand cDNA of *B. gargarizans* here and *CLU* full-length cDNA from Japanese toad skin cDNA library [12]. Therefore, CLU is probably one of the anti-tumor peptides included in toad skin, which might function as an antagonist of native *CLU*. The expression of *CLU* in most organs of Chinese toad (Fig. 4) further indicates the promise for the importance of *Bufo* skin origin materials in anti-tumor drug development.

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