Cloning the *sterol carrier protein 2* genes of Japanese toad (*Bufo japonicus formosus*) and Chinese toad (*Bufo gargarizans*) and its tissue expression analysis

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Abstract: In this study, to clarify the bioactive polypeptides included in the skins and secretions of *Bufo*, we screened the Japanese toad (*Bufo japonicus formosus*) skin cDNA library by colony polymerase chain reaction (PCR), and obtained a transcript of 1 075 bp consisting of 137 bp 5′ untranslated region (UTR), 515 bp 3′ UTR and a 423 bp open reading frame (ORF) encoding a polypeptide of 140 amino acid residues (GenBank accession number: KF359945). Homolog analysis showed a 70%–96% homology with sterol carrier protein-2 (SCP-2) present in other animals, which is implicated in lipid metabolism of other organisms. The gene *SCP-2* of Chinese toad (*B. gargarizans*) was cloned from a first strand cDNA of *Bufo* skin (GenBank accession number: KF381341) via PCR, whose encoding polypeptide has only one amino acid difference from that of Japanese toad. Tissue distribution analysis showed that *SCP-2* expressed in all organs tested, though in the liver and spleen it manifested lower expression than in other organs. These findings might indicate SCP-2 being one of the active ingredients in toad skin. These findings may in turn have implications for further drug development from traditional Chinese medicine sources.

Keywords: *Bufo gargarizans*; *Bufo japonicus formosus*; *SCP-2* cDNA cloning; Tissue expression

Amphibian skin and their secretions have been shown to contain large amount of biologically active compounds, suggesting a new potential source for drug discovery (Clark, 1997; Lai et al, 2002a, 2004; Novković et al, 2012; Rash et al, 2011; Zhao et al, 2014). The potential for novel therapeutics derived from these tissues is not unexpected; skin from the *Bufo* toad (Chan'pi), its cortex (Chan'yi) and secretions (Chan'su) have long been important components included in many prescriptions of traditional Chinese medicine (TCM) used in clinical treatments of several diseases, especially tumor control (Efferth et al, 2009; Liu et al, 2009; Tong, 2011; Xin et al, 2012). Previous reports showed that cinobufocini injection (water soluble extracts of toad skin) possessed excellent anti-tumor curative effects (Qi et al, 2010, 2011; Zhou et al, 2009), likely due to their unique polypeptides (Wu et al, 2012). Despite these promising findings, little has been done to advance the use of the active compounds in toad skin, especially in China.

China itself is in rich of amphibian species and has a long history of developing traditional medicine from unorthodox sources. Similarly, China is up and coming player in the pharmaceuticals and drug development. The key challenge in leveraging these two advantages is first gaining a clearer understanding of the underlying genetic and molecular mechanisms in many traditionally used

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treatments, and second, finding better ways to utilize the available resources more efficiently (Lai et al, 2002b). We previously sought to elucidate the active polypeptide components included in toad-skin and its related materials by first screening the skin plasmid cDNA library of Japanese toad (*Bufo japonicus formosus*) via colony polymerase chain reaction (PCR) (Yuan et al, 2013; Zhang et al, 2013; Zhuge et al, 2013). As part of our other research efforts, we have also begun cDNA cloning from Chinese toad (*B. gargarizans*) skin first strand cDNA (Hu et al, 2013). During these processes, we were able to isolate the cDNAs encoding sterol carrier protein-2 (SCP-2) from both Japanese toad and Chinese toad.

SCP-2 was initially segregated and purified from mouse (*Mus musculus*) liver tissue (Osumi et al, 1980), which is distributed in peroxidase, mitochondria, endoplasmic reticulum and cytoplasm, and functions as intracellular transporters such as cholesterol, lecithin, fatty acid, ester acyl CoA etc (Kriska et al, 2010; Schroeder et al, 2000, 2007). Here we cloned *SCP-2* from two *Bufo* species and conducted RT-PCR analysis on the Chinese toad, and found that SCP-2 is likely one of the effective polypeptides included in toad-skin origin materials. These findings confirm our earlier observation that toad skin (and potentially other tissues) may be viable targets for future development of drug treatments and novel therapeutics.

MATERIALS AND METHODS

Experimental materials and reagents

The proprietary Japanese toad skin plasmid cDNA library held by the Japan Advanced Industrial Science and Technology (AIST, Tsukuba, Japan) was authorized for use by Zhejiang Agricultural and Forestry University (ZAFU) for research as part of a Material Transfer Agreement. Concerning this library, pSD64TR (3 250 bp) has been used as a vector, and *Eco*R I and *Xho* I as cloning sites. The upstream primer of the vector is SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and the downstream one is S.D.A. (5′-TTATGTAGCTTAGAGACTC-3′), respectively. The cDNA length ranged from 500−2000 base pairs (bp). For further testing, Chinese toad individuals were obtained from the East lake Campus of ZAFU, and then ice anaesthetized prior to dissection, wherein the organs were removed and cut into small pieces before being frozen in liquid nitrogen. The resulting samples were kept in a –70 °C refrigerator prior to total RNA extraction.

The RNA extraction kit was purchased from Shanghai Bocai Biotechnology Company; Quantscript RT kit, pGM-T vector and *Escherichia coli* competent cells (DH5α) from Tiangen Biotechnology Limited Company; *Taq* PCR kit from TaKaRa Biotechnology Limited Company; and primer synthesis and DNA sequencing were commissioned by Shanghai Sang'ni Biotechnology Company.

B. japonicus formosus SCP-2 screening

Japanese toad cDNA screening was performed as described previously (Yuan et al, 2013). In brief, Japanese toad skin plasmid cDNA library was transformed into *E. coli* (DH5α), and colony PCR was performed using colony suspension as templates, and SP6 and XhoTT (5′-AGATCTCTCGAGTTTTTTTTTTT-3′, a self-designed primer complementary with the area compassing the connection point of cDNA polyA tail and the downstream cloning site of *Xho* I) as primers. Following this process, the recombinant plasmids were collected and double enzyme digested with *Eco*R I and *Xho* I to further confirm positivity, and then sent for sequencing with vector primers SP6 and S.D.A.

B. gargarizans SCP-2 cloning

For *SCP-2* cloning from Chinese toad, total RNA was extracted from its dorsal skin, and a first strand cDNA synthesized based on the manufacturer's protocols. Meanwhile, based on the Japanese toad *SCP-2* sequence, an upstream primer (SCP-2-S: 5′-CGTGGTCGTTACG TTATACAAG-3′) and a downstream primer (SCP-2-R: 5′-GAAATTAGTGGCTTTTATTAAGTG-3′) were designed for use in RT-PCR. The PCR product was ligated into a pGM-T vector and then sequenced with vector upstream primer T7 and downstream primer SP6.

Sequence Analysis

DNAstar/EditSeq was used to find the open reading frame (ORF) and deduce their encoding protein amino acid sequence. Potential phosphorylation sites were predicted via Net Phos 2.0. A further 17 SCP-2 protein sequences from other animals were downloaded by NCBI blast program (http: //blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with DNAstar/MegAlign. Phylogenetic tree based on SCP-2 amino acid sequences was constructed using the neighbor-joining method by MEGA5.1 (bootstrap with 1 000 replications).

***SCP-2* tissue expression analysis by RT-PCR**

Total RNA samples were extracted from different Chinese toad organs including brain, heart, lung, liver, spleen, kidney, stomach, intestines, fallopian tube and skin, and their first strand cDNA were synthesized as mentioned above. Samples of the different tissue were analyzed via PCR for *SCP-2* expression using a reference gene of β-actin (upstream primer: 5′-TTGAGAC CTTCAACACC-3′; downstream primer: 5′-CTTGATGT CACGCACAA-3′).

RESULTS

SCP-2* screening and sequence analysis of *B. japonicus formosus

Restriction enzyme digestion with *Eco*R I and *Xho* I showed one recombinant plasmid screened from Japanese toad skin plasmid cDNA library had a cDNA insert of about 1000 bp, which was later confirmed by sequencing analysis (Figure 1). The transcript is 1 075 bp consisting of 137 bp 5′UTR (untranslated region), 515 bp 3′ UTR and a 423 bp ORF encoding a polypeptide of 140 amino acid residues, which showed high homology with sterol carrier protein-2 (SCP-2) found in other animals. The clone we screened in the present study (named *B. japonicus formosus SCP-2*), has been deposited into GenBank (accession number: KF359945).

SCP-2* cloning and sequence analysis of *B. gargarizans

From Chinese toad skin first strand cDNA, a 920 bp transcript was obtained consisting of 22 bp 5′ UTR, 475 bp 3′ UTR and 423 bp ORF encoding a polypeptide consisting of 140 amino acid residues (Figure 2). The only difference between two *Bufo* SCP-2 proteins is that Thr130 in Chinese toad was substituted by Ser in the Japanese toad. This clone was designated as *B. gargarizans SCP-2* and deposited into GenBank (accession number: KF381341).

Phosphorylation site prediction of *Bufo* SCP-2

From the analysis of phosphorylation site prediction, 10 potential sites (Ser10, Ser11, Ser19, Ser77, Ser79, Ser82, Ser91, Ser93, Thr66 and Tyr44) were found in both *Bufo* species (Table 1), suggesting that *SCP-2* expression might be regulated by the upstream factors.

Homology analysis of *SCP-2* amino acids

Phylogenetic analysis showed that two *Bufo* species had a homology as high as 96% with *Xenopus (Silurana) tropicalis* and 92% with *Xenopus laevis*, with lower homology ranging from 70% to 91% among 15 other animals (Figure 3). The phylogenetic tree we constructed showed 9 different mammals (*Homo sapiens*, *Bos taurus*, *Rattus norvegicus*, *Mus musculus*, *Mesocricetus auratus*, *Capra hircus*, *Camelus ferus*, *Ictidomys tridecemlineatus* and *Sus scrofa*) gathered in a branch, 5 different birds (*Gallus gallus*, *Falco peregrinus*, *Falco cherrug*, *Pseudopodoces humilis* and *Melopsittacus undulatus*) in a branch, 1 reptile (*Anolis carolinensis*) in another branch, 2 frogs (*X. laevis* and *X. Silurana tropicalis*) in a branch, 2 toads (*B. gargarizans* and *B. japonicus formosus*) in a branch, and 2 fish in a branch (Figure 4),

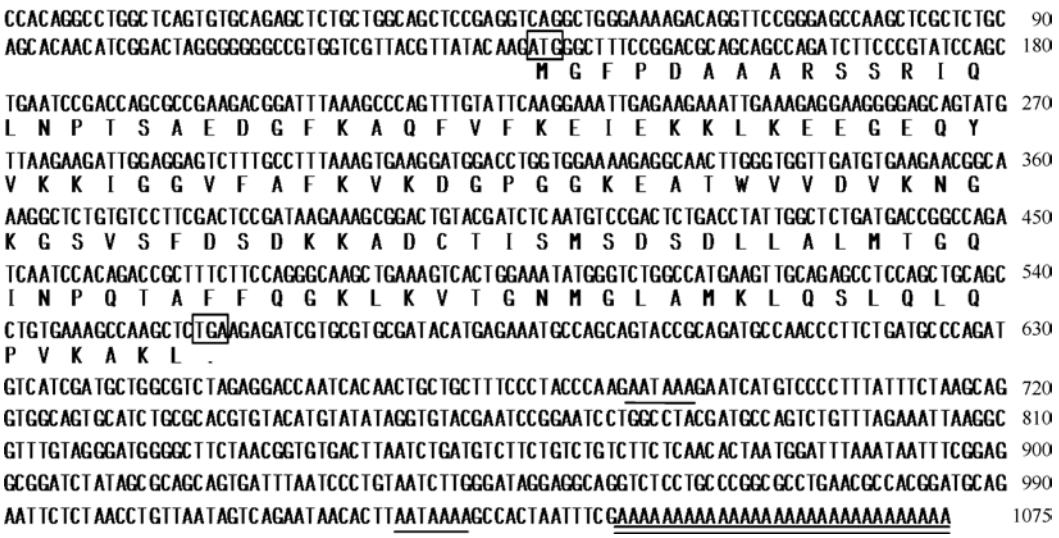

Figure 1 *SCP-2* cDNA and its deduced amino acid sequence of *Bufo japonicus formosus*

Start and stop codons were enclosed by line box. —: Polyadenylation signal; =: Poly(A) tail.

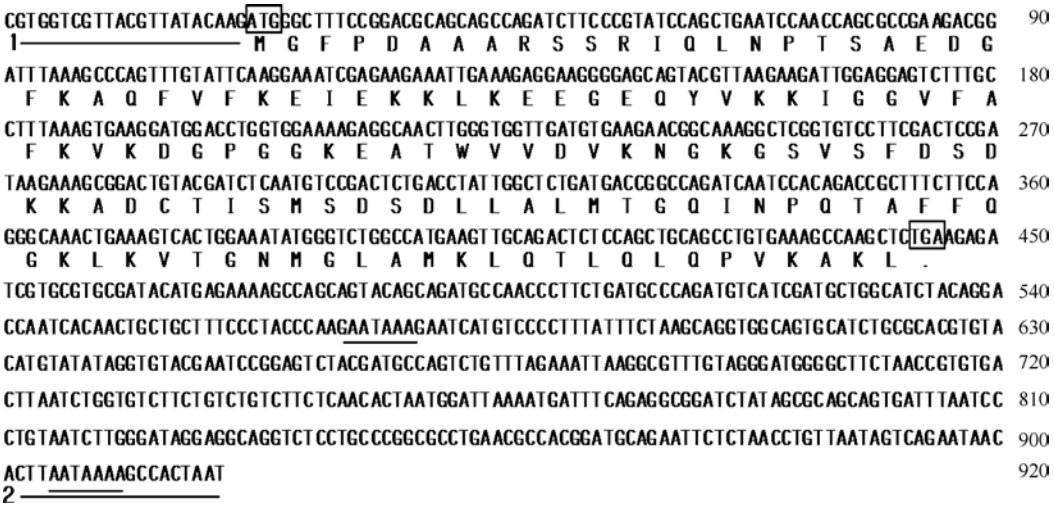

Figure 2 *SCP-2* cDNA and its deduced amino acid sequence of *Bufo gargarizans*

Start and stop codons were enclosed by line box. 1: Upstream primer, SCP-2-S; 2: Downstream primer, SCP-2-R; —: polyadenylation signal.

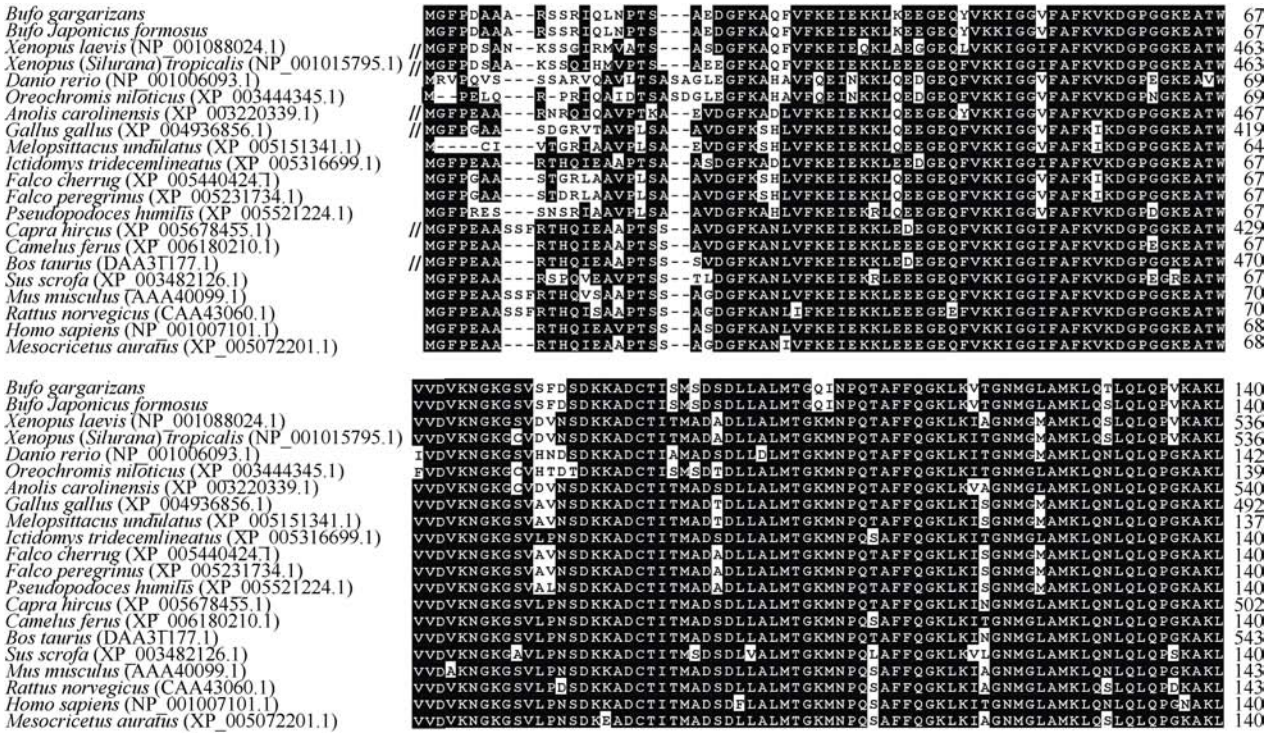

Figure 3 *Bufo* SCP-2 amino acid sequence alignment with other animal species

which is consistent with the traditional animal taxonomy.

SCP-2* expression in different organs of *B. gargarizans

RT-PCR detection of *B. gargarizans SCP-2* in the brain, heart, lung, liver, spleen, kidney, stomach, intestines, fallopian tube and dorsal skin (Figure 5) showed that *SCP-2* was expressed in all tested organs, though the expression was lower in both liver and spleen as compared with other organs.

DISCUSSION

Previous studies found that SCP-2 is involved in adjusting concentrations of cholesterol inside and outside cell membranes via activation of cholesterol hydrase, and participating in regulating cholesterol transport in the cell culture system (Kriska et al, 2010; Schroeder et al, 2000, 2007). Many human diseases—diabetes, arteriosclerosis, Zellweger, NPC disease and gallstones—have similarly

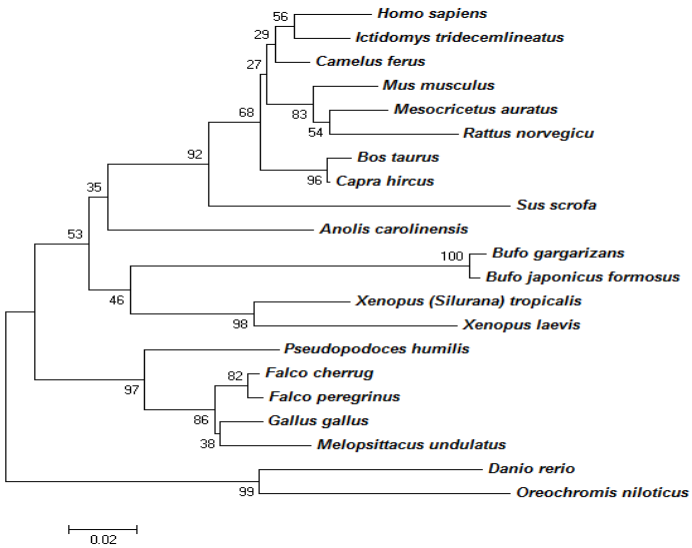

Figure 4 Phylogenetic tree of amino acid of SCP-2 between two *Bufo* species and 17 other species

Figure 5 RT-PCR detection of *SCP-2* expression in different organs of *Bufo gargarizans*

M: DNA ladder; 1: brain; 2: lung; 3: heart; 4: liver; 5: spleen; 6: kidney; 7: stomach; 8: intestines; 9: fallopian tube; 10: dorsal skin.

Table 1 *Bufo* SCP-2 phosphorylation site prediction

Position	Context	Score	Prediction
10	AAARSSRIQ	0.552	*S*
11	AARSSRIQL	0.598	*S*
19	LNPTSAEDG	0.995	*S*
77	NGKGSVSFD	0.943	*S*
79	KGSVSFDSD	0.902	*S*
82	VSFDSDKKA	0.994	*S*
91	DCTISMSDS	0.992	*S*
93	TISMSDSDL	0.955	*S*
66	GKEATWVVD	0.577	*T*
44	EGEQYVKKI	0.926	*Y*

been found to have associations with abnormal expression of SCP-2 (Castelli, 1984; McLean et al, 1996). For example, NPC (Niemann-Picktype C) disease seems to be caused by NPC-peak C protein mutations, and a corresponding drop in liver SCP-2 expression was indicated (Schroeder et al, 2007). In diabetic mice models induced by streptozotocin, the level of SCP-2 in the liver was reduced 60%−90%, alongside a 60% reduction in ovarian SCP-2 (McLean et al, 1996). SCP-2 expression is also related to the formation of cholesterol calculus (Cui et al, 2011).

In the present study, our successful cloning of sterol carrier protein-2 (*SCP-2*) genes from both *B. gargarizans* and *B. japonicus formosus* (Figures 1, 2) indicated that SCP-2 expressed in toad skin as well as in other organs (Figure 5) has a high homology with that in other animals (Figure 3, 4). Due to the function of SCP-2 in adjusting lipid metabolism in numerous animal species and many *SCP-2* expression-related disorders among humans, it may be reasonable to predict that SCP-2 is one of potentially several important ingredients within toad skin.

Our study extends the basic knowledge necessary to assess the potential for *Bufo* skin and other organs for potential drug development. Previously, numerous reports summarized several descriptions on the clinical efficacy of Chan'su, such as detoxification, analgesia, anti-inflammation, antidiarrheal, and antitumor, etc. (Liu et al, 2009; Xin et al, 2012). Curiously though, we found that SCP-2, as a potentially important element in toad skin, is largely involved in adjusting lipid metabolism. However, few reports have ever noted the use of *Bufo* skin in treating lipid metabolism related diseases, aside from an old description concerning Chan'su that pointed out that administration of the medicine could make the symptoms of stasis and stagnation disappear in "BenCaoHuiYan". This reference itself is rather intriguing, given that it appears in a Ming Dynasty era

book written by Zhu-mo NI published in 1624. Presuming our finding survive replication and further targeted studies are conducted to answer some of the remaining questions this research poses, our present results offer a potentially novel way of looking at cholesterol-related disease therapeutics using *Bufo* origin materials.

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