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TAGLN2 cDNA Cloning from *Bufo japonicus formosus* and its Diversity Analysis

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Abstract: *During the study of bioactive polypeptides included in Bufo skin, a cDNA segment with partial transgelin-2 (TAGLN2) ORF (open reading frame) was cloned from Japanese toad Bufo japonicus formosus skin cDNA plasmid library (accession number: JX197456). To confirm the expression of the full length TAGLN2, two primers (P1 and P2) were designed based on the partial TAGLN2 sequence, and two PCR (polymerase chain reaction) reactions (P1 and XhoTT, P2 and SP6 as a pair of primers, respectively) were performed to clone the 5'- and 3'-UTR (untranslated region) using the same cDNA library as templates, and the PCR products were cloned. Based on the newly cloned 5'- and 3'-UTR sequences of TAGLN2, two new primers (P3 and P4) were designed and the second round PCR was performed by pairing P3 with XhoTT, and P4 with SP6, respectively. As the result, several TAGLN2 cDNA including full length or partial ORF were cloned indicating the expression of full length TAGLN2 in Japanese toad. Concerning its diversity, one SNP (single nucleotide polymorphism sites) in ORF was found leading to no amino acid change, and different length of 5'-UTR as well as 6 SNP in 3'-UTR was indicated.*

Keywords: *Bufo japonicus formosus; Transgelin-2; cDNA cloning; Diversity*

1. INTRODUCTION

Chan'pi (skin), Chan'yi (cortex) and Chan'su (secretions) are all *Bufo* skin-origin materials having been widely used in many prescriptions of traditional Chinese medicine and showing nice effects on pain relief, swelling and tumor control as well as many other diseases [1-4]. Cinobufacini, aqueous extract of dried *Bufo* skin, is such a clinical drug mainly aimed at the advanced cancer [5,6]. Recent studies indicated that the polypeptides purified from Cinobufacini injection showed similar antitumor activity as the injection itself in vitro experiment [7]. During the analysis of polypeptides included in *Bufo* skin, a cDNA clone with partial *transgelin-2* (*TAGLN2*) ORF was isolated from Japanese toad skin cDNA plasmid library [8].

TAGLN2, also called SM22β (smooth muscle 22), is an actin binding protein which was purified from chicken gizzard smooth muscle for the first time [9,10] involving in cell proliferation and differentiation [11], and oncogenesis [12,13]. *TAGLN2* has become a potential marker of tumorigenesis, provides a reference for early diagnosis, treatment and monitoring of tumors [14]. To confirm the expression of *TAGLN2* with full length ORF, cDNA cloning was carried out from the skin cDNA plasmid library of Japanese toad based on the partial *TAGLN2* sequence cloned previously [8].

2. MATERIALS AND METHODS

2.1. Experimental Materials and Reagents

Japanese toad skin cDNA plasmid library held by the Japan Advanced Industrial Science and Technology (AIST, Tsukuba, Japan) was authorized Zhejiang A&F University (ZAFU) for research

as part of a Material Transfer Agreement. Concerning the library, as reported previously [15], whose vector is pSD64TR (3250 base pairs), upstream primer is SP6 and the downstream one is S.D.A., and *Eco*R I and *Xho* I are the cloning sites. cDNA length is ranged 500-2 000 base pairs (bp). PCR kit purchased from TaKaRa; pGM-T from Tiangen (Beijing, China); the primer synthesis and DNA sequencing were done by GENEWIZ (Suzhou, China).

2.2. Primer Design and Cloning of *TAGLN2* with Whole ORF from Japanese Toad

Based on the partial Japanese toad *TAGLN2* cDNA (GenBank accession number: JX197456) [8], two primers (P1, P2) were designed (Table 1). P2 was paired with SP6 for the cloning of the 5′-UTR (Group 1), and P1 paired with XhoTT (a self-designed primer complementary with the area compassing the connection point of cDNA poly(A) tail and the downstream cloning site of *Xho* I) for the cloning of the 3′-UTR (Group 2) (Table 2). Based on the sequences newly cloned 5′-UTR and 3′-UTR, two other primers (P3, P4) were designed (Table 1) for the second round PCR. P4 paired with SP6 (Group 3) and P3 paired with XhoTT (Group 4) for the cloning of cDNA with original ORF (Table 2). Japanese toad skin cDNA plasmid library was used as template in the current study. All four PCR constitutions are the same except the primers as indicated in Table 2. PCR parameters are following: 94℃/3 min; (94℃/30 s, 52℃/30 s, 72℃/2.5 min) x 35 cycles. PCR products were ligated into pGM-T, and the candidates of positive clones were sequenced.

Table1. *Primers used in current study*

Name	Sequences
SP6	5′-ATTTAGGTGACACTATAGAA-3′
XhoTT	5′-AGATCTCTCGAGTTTTTTTTTTT-3′
P1	5′-GCTAAAATCCAGACATC-3′
P2	5′-GATGAGTGGATGATCTG-3′
P3	5′-AACCACCAACCACTAAAATGG-3′
P4	5′-TAAACATAGATTGGTTTTATT-3′

Table2. *PCR constitutions for TAGLN2 cloning from Japanese Bufo*

Components	Vol (μl)
10x*Taq* buffer	2.0
10 mM dNTPs	0.8
Taq (5U/μl)	0.2
Primer 1(2 μM)*	1.0
Primer 2(2 μM)*	1.0
Template**	0.5
H_2O	14.5

Notes: * SP6 paired with P2, P1 with XhoTT, SP6 with P4 and P3 with XhoTT in Group 1, 2, 3 and 4, respectively. ** Japanese toad skin plasmid cDNA library

2.3. Homology Analysis of TAGLN2 Amino Acids

After sequencing, DNAstar/EditSeq was used to find out ORF and deduce the amino acid sequence of the encoding protein, then the amino acid sequence was applied NCBI blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The homolog sequences of 8 other animals were downloaded and aligned by DNAstar/MegAlign, which was also used to construct the phylogenetic tree of TAGLN2.

3. RESULTS

3.1. *TAGLN2* Cloning from *B. japonicus formosus*

The first round PCR (lane 1 and 2 in Fig.1) was to clone *TAGLN2* cDNA with complete 5'- and 3'-UTR, and the second round PCR (lane 3 and 4 in Fig.1) was to clone the whole *TAGLN2* ORF with either complete 5'-UTR or complete 3'-UTR. Sequencing analysis indicated that 6 different clones were obtained in Group 1, 4 in Group 2, 7 in Group 3, and 6 in Group 4, totally 23 different clones were obtained. For easy description, these clones were designated as *TAGLN2*-M-N (M: Group No., N: Clone No. in each group).

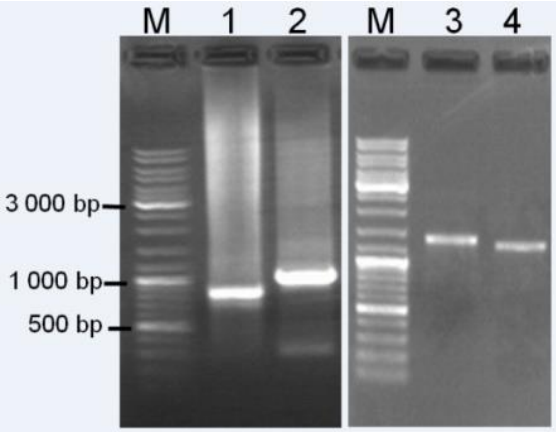

Fig1. *PCR products of TAGLN2 amplified from B. japonicus formosus*

M: DNA Ladder; 1: SP6 and P2; 2: P1 and XhoTT; 3: SP6 and P4; 4: P3 and XhoTT

3.2. *TAGLN2* cDNA of *B. japonicus formosus*

Among the above 23 clones, *TAGLN2*-3-5 is 1 267 bp in length with 86 bp 5'-UTR, 594 bp ORF and 587 bp 3'-UTR encoding a protein consisting of 197 amino acid residues (Fig. 2), whose molecular weight is 21.953 kD, closed to that of other animal TAGLN2. Homology analysis of TAGLN2 of Japanese toad indicated 82% homology with *Xenopus laevis* (NP_001080783.1), 81% with *Xenopus* (*Silurana*) *tropicalis* (NP_989354.1), and 71%-78% among 6 other animals (*Rattus norvegicus*, NP_00101314 5.1; *Crotalus adamanteus*, AFJ51813.1; *Mus musculus*, NP_848713.1; *Homo sapiens*, AAH02616.1; *Gallus gallus*, XP_003643901.1; *Danio rerio*, NP963870.1) (Fig. 3). So the clone was deposited into GenBank (accession number: KC820703) as *TAGLN2* of *B. japonicus formosus*. The phylogenetic tree is basically consistent with the traditional animal taxonomy (Fig. 4).

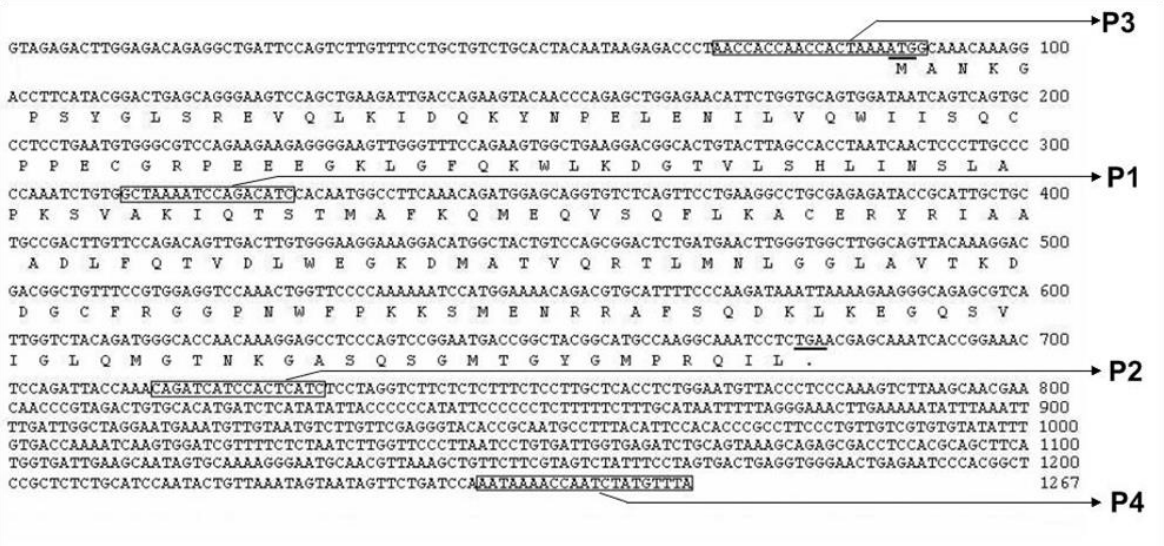

Fig 2. *TAGLN2 cDNA of B. japonicus formosus with the whole ORF and the deduced amino acid sequence of the encoding protein*

Underline: Start and stop codons; P1-P4: the locations of 4 primers

3.3. Diversity of *TAGLN2* cDNA of *B. japonicus formosus*

As describes above, 23 clones were obtained. Due to the use of SP6 as the upstream primer, 5'-UTR sequences of the clones included in Group 1 and 3 should represent the original 5'-UTR in vivo. Meanwhile because of the use of XhoTT as the downstream primer, 3'-UTR sequences of the clones included in Group 2 and 4 should represent original 3'-UTR in vivo. Additionally, because the clones of Group 1, 3 and 4 were derived from the PCR products using SP6 paired with P2/P4, or /XhoTT paired with P3, while P2, P3 and P4, are all located in either 5'-UTR or 3'-UTR as indicated in Fig. 2, therefore, the clones from these 3 groups are supposed to have original ORF in tissue. Here, the *TAGLN2* cDNA diversity was analyzed according to ORF, 5'-UTR and 3'-UTR, respectively.

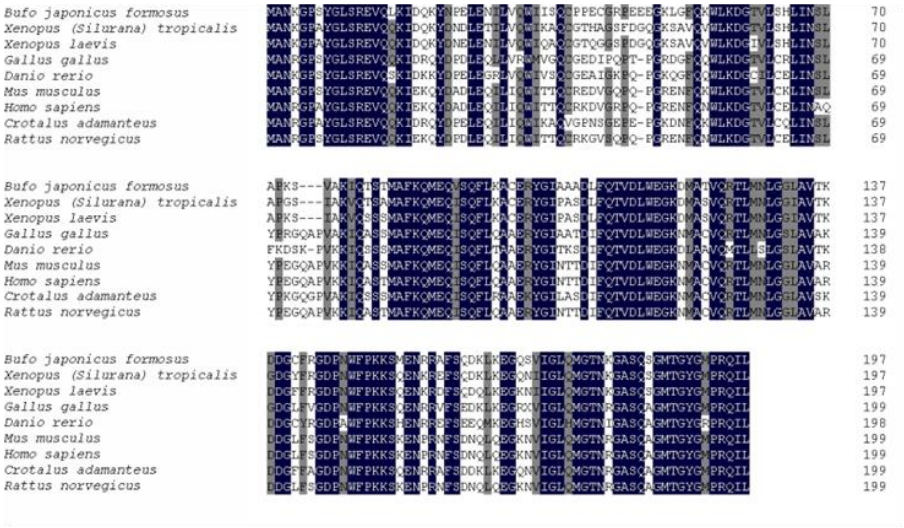

Fig3. *Homology analysis of TAGLN2 of B. japonicus formosus*

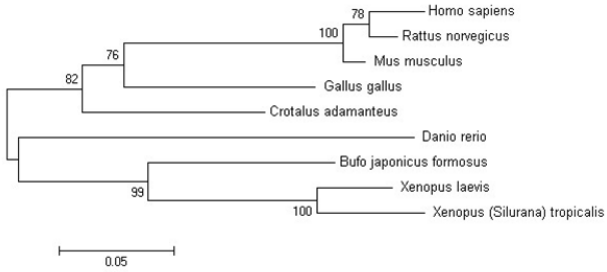

Fig4. *Phylogenetic tree of TAGLN2 between B. japonicas formosus and 8 other animals*

Concerning ORF, 16 out of 19 clones from Group 1, 3 and 4 have 597 bp ORF with 7 SNP (single nucleotide polymorphism site), the remaining 3 clones have shorter ORF being the same as that reported previously [8]. Among 7 SNP, 5 appeared only once, which might be introduced by PCR. However, the remaining 2 are located at the 117th and 564th positions of ORF, which appeared repeatedly in different experiments indicating their objectivity, while they did not lead to amino acid change because of their synonymous mutations (transition between thymine and cytosine). Therefore, all 16 clones encode the same TAGLN2 of Japanese toad.

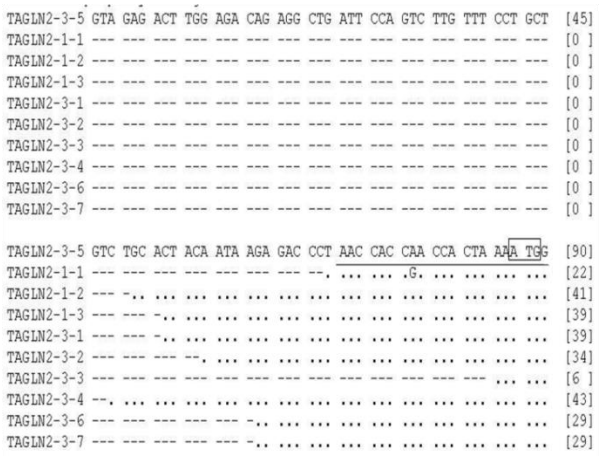

Fig5. *Alignment of multiple TAGLN2 5'-UTR sequences of B. japonicus formosus*

- : absence of corresponding nucleotide; · : identical nucleotide, Underline: Location of P3 primer; □: Start codon

With regard to 5'-UTR, 10 clones with full length ORF were analyzed. As shown in Fig. 5, the length is quite different among different clones, the longest one is 86 bp (*TAGLN2*-3-5), and the shortest one is only 2 bp (*TAGLN2*-3-3). But the nucleotides of the overlapped area are the same (Fig. 5). About 3'-

UTR, 11 sequences were compared and 15 SNP appeared, among which 9 appeared only once or in a certain group, most likely introduced by PCR. The remaining 6 were appeared in different experiments indicating their objective existence. Additionally, the length of poly (A) tail was different among clones (Fig. 6).

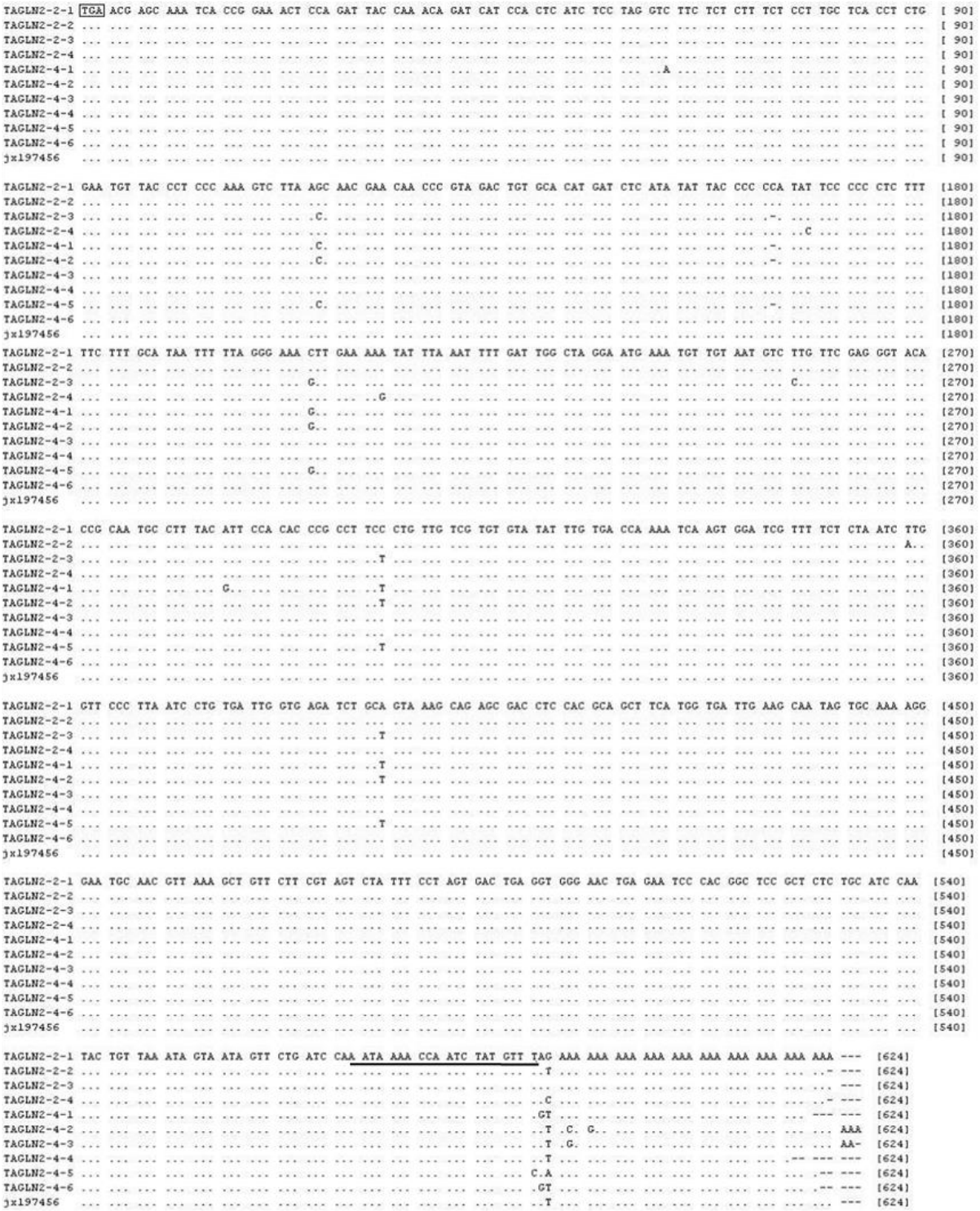

Fig6. *Alignment of multiple TAGLN2 3′-UTR sequences of Bufo japonicus formosus*

-: absence of corresponding nucleotide; ·: identical nucleotide; Underline: Location of P4 primer; □: Stop codon

4. DISCUSSION

Current study clarified the expression of full length *TAGLN2* in Japanese toad skin (Fig. 2), and confirmed the existence of partial *TAGLN2* expression [8]. At the protein level, there is only one amino

acid difference between TAGLN2 of Japanese *Bufo* and or Chinese *Bufo* (*B. gargarizans*) showing homology as high as 99%. The different amino acid is located in the unimportant area, therefore, both should have the same characteristics as reported in previous study [16]. *TAGLN2* diversity of Japanese toad is mainly reflected in the length of 5'-UTR (Fig. 5), while ORF and 3'-UTR are relatively conserved (Fig. 6). Homology analysis of Japanese toad TAGLN2 showed 71-82% with other 8 vertebrates (Fig. 4), indicating its high conservancy in the process of evolution.

As an actin binding protein, TAGLN2 is involved in many physiological and pathological processes by reorganization of cytoskeleton, microfilaments [17-19]. Concerning the molecular mechanism of the antitumor activity of TAGLN2, there have been some reports that TAGLN2 noncoding region affects its posttranscriptional translation, and plays a role in inhibiting cancer metastasis together with miRNA [20-22]. But, the mechanism is still necessary to be further studied in future.

In fact, cDNA cloning either from Japanese *Bufo* or Chinese *Bufo* has been our main study to address the polypeptide ingredients working on tumor control as well as other diseases, and the expression such as *Mcl-1*, *Gal-3*, *EDF-1*, *PPDPF* and *Clu* was clarified, all of which are related with oncogenesis or metastasis, and supposed to be the anti-tumor ingredients included in *Bufo* skin-origin materials [23-27]. One more issue is that partial cDNA of *Mcl-1* [23], *Gal-3* [24] has also been cloned. So far, several peptides derived from protein have been reported including Buforin I and II [28], Abhisin [29] and NuBCP-9 [30], either with antimicrobial activity or antitumor activity. Combined all these studies with the report that polypeptides from Cinobufacini has antitumor activity, and the fact that most tradition Chinese medicine is taken orally, it might be not unreasonable to suggest that proteins included in tradition Chinese medicine play their pharmaceutical functions by short polypeptides. The polypeptides included in Chan'pi, Chan'yi and Chan'su, are urgent to be studied.

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