Genetics and Molecular Research

Characteristics of *cathelicidin-Bg*, a novel gene expressed in the ear-side gland of *Bufo gargarizans*

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ABSTRACT. The traditional Chinese medicine Chan Su (toad venom) comprises dried secretions of the ear-side gland of *Bufo gargarizans*. Chan Su is known for its small molecular components, which include telocinobufagin, marinobufagin, and bufalin, while in other amphibians, studies mainly focus on peptide components. Until recently, no genes expressed in the ear-side gland of *B. gargarizans* gland had been cloned. In this study, *cathelicidin-Bg*, a coding sequence of anti-microbial peptide (AMP), was cloned. The predicted amino acid sequence of *cathelicidin-Bg* was very similar to that from other amphibians, with a 34-amino acid mature peptide predicted in the C-terminus. The functions of this mature peptide were verified by microbe and tumor

cell inhibition assays. Our results showed that the mature peptide of cathelicidin-Bg could inhibit the proliferation of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The mature peptide was also shown to selectively inhibit tumor cells. These results indicate that the identified coding sequence represents an active peptide of Chan Su.

Key words: Cathelicidin-Bg; *Bufo gargarizans*; Anti-microbial peptide; Tumor

INTRODUCTION

Peptides secreted from amphibian skin have been the focus of many studies (Miller, 1990; Simmaco et al., 1990; Lee et al., 2005; King et al., 2008). In recent years, a large number of anti-tumor peptides have been reported in different toad and frog species (Bevins and Zasloff, 1990; Bechinger et al., 1993; Westerhoff et al., 1995). Members of the maganin family of peptides have been isolated from the skin of African clawed frog *Xenopus laevis* (Ohsaki et al., 1992; Baker et al., 1993). In addition, the 23-amino acid peptides maganin 1 and 2 have been reported to inhibit proliferation of NCI-H82 and NCI-H526 cells at very low doses (Baker et al., 1993; Giacometti et al., 2004). Because maganin 2 is highly cytotoxic, its derivative, formed via amino acid modification, has been approved as a novel cancer treatment (Shin et al., 2000; Takeshima et al., 2003). Furthermore, peptides belonging to the maximin and bombinin families in *Bombina maxima* and *Bombina orientalis* have been reported to inhibit the proliferation of tumor cells (Gibson et al., 1991).

In contrast to the anti-tumor activity of peptides from other amphibians, studies on the anti-tumor activity of skin secretion from *Bufo gargarizans* have focused on small molecules. In traditional Chinese medicine, dried secretions from the skin gland of *B. gargarizans* (Chan Su) have been used as an anti-inflammatory agent in China for thousands of years (The State Pharmacopoeia Commission of PR China, 2010). Recent studies have reported that toad venom has anti-tumor activity. For example, toad venom extracts were shown to inhibit the growth of non-small-cell lung cancer (Meng et al., 2009; Zhang et al., 2013b). Active components of Chan Su such as telocinobufagin, marinobufagin, bufalin, bufotalin, and resibufogenin were reported to have anti-tumor activity (Qiao et al., 2008; Li et al., 2010; Zhang et al., 2013a). Bufalin and cardiotonic steroids isolated from Chan Su were found to promote apoptosis in human prostate and breast cancer cells by up-regulating the expression of caspase family genes (Zhu et al., 2012; Hong et al., 2013). Bufalin can also suppress cellular proliferation and inhibit the migration and invasion of tumor cells, although the molecular mechanisms underlying these effects remain unknown (Zhai et al., 2013; Chang et al., 2014; Chen et al., 2015). Furthermore, arenobufagin, another major component of Chan Su, was reported to be a specific inhibitor of vascular endothelial growth factor-mediated angiogenesis (Zhang et al., 2013a).

Although no coding sequence has been cloned from the ear-side gland, which secretes Chan Su, the coding sequence of anti-microbial peptide (AMP) has been identified from the skin of *B. gargarizans*, e.g., cathelicidin (Sun et al., 2015), which is a homolog identified in other amphibians. In this study, we constructed a cDNA library using total RNA isolated from the ear-side gland of *B. gargarizans*, and attempted to use it to identify the coding sequences of AMPs in order to identify novel components of Chan Su.

MATERIAL AND METHODS

Toad samples

B. gargarizans weighing 100-130 g were collected from Hangzhou, China on the first day of each month from April to November in 2015. Toads were surface-sterilized using 75% ethanol and ear-side glands were subsequently dissected. Total RNA was extracted from the ear-side glands using the TRIzol (Invitrogen™, USA) method. Then, first-strand cDNA was synthesized by ReverTra Ace qPCR RT Kit (TOYOBO, Japan). For gene cloning, a mixed sample of samples from each month was used and individual samples were used to analyze gene expression profiles.

Sequencing of a partial DNA sequence

To obtain a partial cDNA sequence, primers (Table 1) were designed based on *cathelicidin* sequences conserved in other amphibian species. Synthesized cDNA was used as a template, and PCR was conducted in the following manner: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 1 min, and 72°C for 7 min. A generated DNA fragment of the expected size was used to construct a recombinant plasmid with a pMD-19 T vector (TaKaRa, Japan), which was transferred to *Escherichia coli* DH5α cells, and sequenced by Sangon Biotech Co. Ltd. (Shanghai, China).

Table 1. Primers used for gene cloning and differential expression of *Cathelicidin-Bg*.

Primers	Primer sequences	Application	Accession No.
RACE-Cat-5'	3'-CTTGGACCTCAGGCTCTGCAGT-5'	RACE	KU556733
RACE-Cat-3'	3'-GGATCCTGAGGATATAATGTGCACC-5'	RACE	KU556733
q-Cat-F	3'-ATTAAAGAGACGGTGTGCCTCAAAT-5'	qPCR	KU556732
q-Cat-R	3'-TGTTGGACCGCTTCACACGAAC-5'	qPCR	KU556732
q-actin-F	3'-GAGCTATGAGTTGCCTGATGGACAG-5'	qPCR	EU661596
q-actin-R	3'-AATCCTTACGAATATCCACATCACAC-5'	qPCR	EU661596

Rapid amplification of cDNA end (RACE)

The full-length *cathelicidin-Bg* cDNA sequence was cloned by the RACE method. Primers (Table 1) were designed from partial *cathelicidin-Bg* sequences (accession No. KU556733), as described above. Synthesized cDNA was used as a template, and 3'- and 5'-RACE was performed using a SMART™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer instructions. PCR fragments were subcloned into the pMD-19 T vector, transferred to *E. coli* DH5α cells, and sequenced by Sangon Biotech.

Analysis of cDNA sequence

BLASTn and BLASTp were used to identify the nucleotide and deduced amino acid sequences of the cloned cDNA sequence. A putative signal peptide was predicted by the SignalP 4.1 Server online software. Multiple alignments between *cathelicidin-Bg* and its homologs were conducted using the DNAMAN software, and nucleotide and deduced amino acid sequence profiles were analyzed by the DNASTAR software. A phylogenetic tree was constructed using the neighbor-joining algorithm in the MEGA software.

Quantitative PCR (qPCR)

To normalize differences in the amount of reverse-transcribed RNA used in each reaction, β-actin from *B. gargarizans* (GenBank accession No. EU661596) was used as an endogenous control. All qPCR assays were performed using an ABI PRISM® 7500 Fast Real-Time PCR System (Applied Biosystems, USA) in 20-μL reactions. Each 20-μL reaction contained 2 μL template cDNA, 10 μL THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Japan), and 0.5 μM each forward and reverse primer. Three replicates were included, and qPCR Ct values were analyzed using the $2^{-\Delta\Delta Ct}$ method (Shi et al., 2013) and further tested by performing a one-way analysis of variance (ANOVA).

Anti-microbial assay

Bacteria - *Bacillus subtilis* (ATCC6633), *E. coli* DH5α (ATCC69925), *Staphylococcus aureus* (ATCC2592), and *Pseudomonas aeruginosa* (ATCC9027) and fungus - *Monilia albican* (ATCC10231) and *Saccharomyces cerevisiae* (ATCC9763) were obtained from the American Type Culture Collection. *B. subtilis*, *S. aureus*, and *P. aeruginosa* were cultured in nutrient broth medium, *E. coli* DH5α cells were cultured in Luria-Bertani medium, *M. albican* were cultured in Martin modified medium, and *S. cerevisiae* was cultured in Sabouraud dextrose and yeast extract medium.

To determine the anti-microbial activity of cathelicidin-Bg, stock cultures of microbes (in -80°C) were grown in medium for 18 h at 37°C, diluted to OD_{600} = 0.2, and then added to a 96-well plate at 100 μL/well. Each microbe was treated with cathelicidin-Bg at a final concentration of 0, 0.1, 1, 10, or 100 mg/mL. Absorbance was measured at 630 nm in a Mustikcan FC scanning multiwell spectrophotometer (Thermo Scientific, USA). Ampicillin (Sigma, USA) at a final concentration of 50 ng/mL was used as a positive control. The experiments were repeated three times.

Tumor inhibition assay

Human HEK293 and HepG2 cells were purchased from the typical cell culture collection Committee of the Chinese Academy of Sciences Library (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (Hyclone, USA) supplemented with 10% fetal calf serum (Hyclone, USA) and antibiotics (Sigma, USA) at 37°C under a 5% CO_2 and 95% air humidified atmosphere in a carbon dioxide cell incubator (Sanyo, Osaka, Japan). The medium was replaced every other day.

Human HEK293 and HepG2 cells were seeded on 96-well plates (Corning, USA) at an initial density of 1.0 x 10^4 cells/well and incubated at 37°C for 24 h. Cells were treated with cathelicidin-Bg solution at 0, 0.5, 5, 50, or 500 mg/mL, at 37°C for 24 h. According to the manufacturer protocol (Sangon, Shanghai, China), methylthiazolyldiphenyl-tetrazolium bromide (MTT), whose reducing capacity is an indicator of cellular activity, was added to each well and incubated at 37°C for 4 h. Formazan solution was added to each well to resolve MTT formazan crystals. Absorbance at 490 nm, indicating cellular activity, was determined on a Mustikcan FC scanning multiwell spectrophotometer (Thermo Scientific, USA). The experiments were repeated three times.

Data analysis

Significant differences between treatments were identified using ANOVA in SPSS 17.0, and significance was set at $P < 0.05$.

RESULTS

cDNA sequence of *Cathelicidin-Bg*

Full-length *cathelicidin-Bg* cDNA (accession No. KU556732) was 909-bp long and contained a 498-bp open reading frame encoding a 166-amino acid residue protein. A 132-bp 3'-untranslated region (UTR) and a 279-bp 5'-UTR were identified at the 3'- and 5'- ends, respectively, of the cDNA sequence (Figure 1). The deduced amino acid sequence was then used to search the National Center for Biotechnology Information database, which revealed a high degree of identity with the *cathelicidin* sequences of amphibians and other vertebrates. A putative signal peptide was predicted at the N-terminal, and a mature peptide "RPCRGRSCSPWLRGAYTLIGRPAKNQNRPKYMWV" was predicted at the C-terminal (Figure 1).

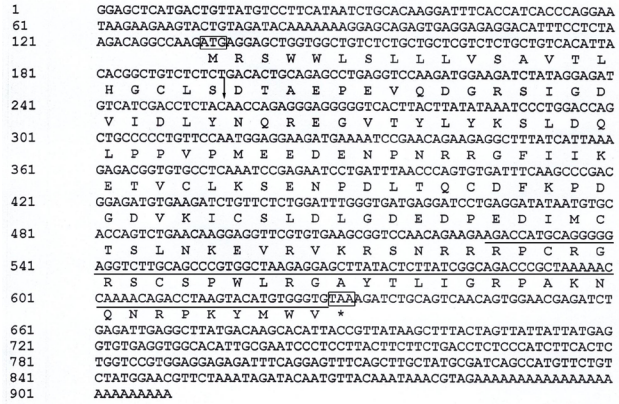

Figure 1. Nucleotide sequence of *cathelicidin-Bg* and its deduced amino acid sequence. Initiation and termination codons are indicated in boxes. The cleavage site of the predicted signal peptide is indicated by an arrow. The mature peptide of the sequence is underlined.

Phylogenetic analysis and multiple alignment

The EditSeq function in DNASTAR revealed that cathelicidin-Bg was 166-amino acid residues long. The cathelicidin proteins of all selected sequences were classified into two major groups; the cathelicidin-Bg protein clustered with *Duttaphrynus melanostictus*, *Nanorana yunnanensis*, *Xenopus tropicalis*, and *Rana catesbeiana*, which are all amphibian species (Figure 2A). These results suggest that the results of our analysis were accurate. The amino acid sequence was then compared with those from *D. melanostictus*, *N. yunnanensis*, *X. tropicalis*, and *R. catesbeiana* by multiple alignments. The results showed that the cathelicidin-Bg amino acid sequence possessed high similarity with those from the other species, suggesting that they have similar functions (Figure 2B).

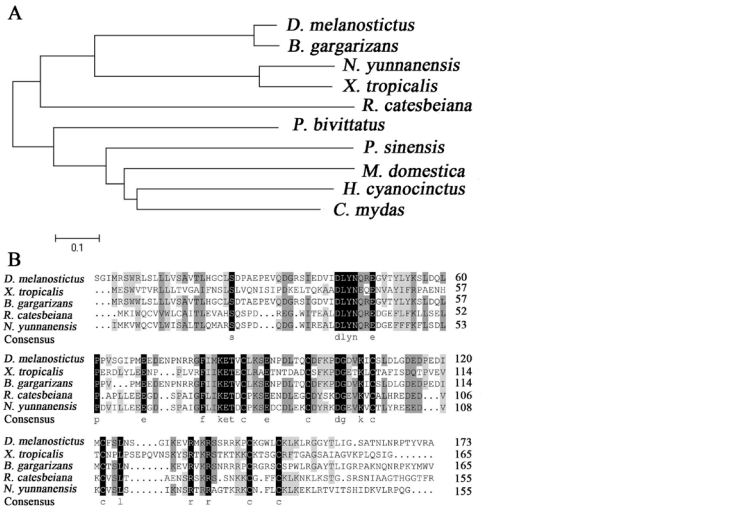

Figure 2. Phylogenetic analysis and multi-alignment of *cathelicidin-Bg* and its homologs from other species. **A.** Phylogenetic analysis of *cathelicidin-Bg* and its homologs. All species names are listed on the left of the graph. The following sequences were used in the phylogenetic analysis cathelicidins of *Duttaphrynus melanostictus* (AJQ20790.1), *Bufo gargarizans* (KU556732), *Nanorana yunnanensis* (AFX61592.1), *Xenopus tropicalis* (XP_004915576.1), *Rana catesbeiana* (AHW58221.1), *Python bivittatus* (XP_007443269.1), *Pelodiscus sinensis* (XP_006129687.1), *Monodelphis domestica* (XP_003341768.1), *Hydrophis cyanocinctus* (AKJ54480.1), and *Chelonia mydas* (XP_007066508.1). **B.** Multi-alignment of *cathelicidin-Bg* and its homologs. The amino acid sequences of cathelicidin proteins were obtained from GenBank as follows: *D. melanostictus* (AJQ20790.1), *B. gargarizans* (KU556732), *N. yunnanensis* (AFX61592.1), *X. tropicalis* (XP_004915576.1), and *R. catesbeiana* (AHW58221.1).

Expression profile of *cathelicidin-Bg*

The expression profile of *cathelicidin-Bg* in eight cDNA samples was determined by the qPCR method. Our results showed there was dynamic expression of *cathelicidin-Bg* over time from April to November. The expression level of *cathelicidin-Bg* in April, May, and November was relatively low, while that in August and September was the highest (Figure 3).

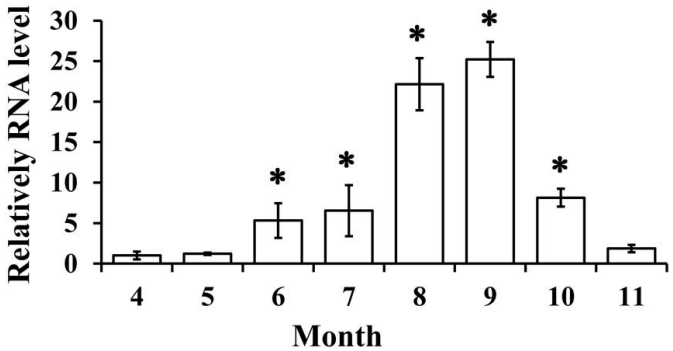

Figure 3. Expression profile of *cathelicidin-Bg* over time. Data are reported as means ± SE from three independent experiments. *P < 0.05.

Anti-microbial activity

Six microbes were used to verify the anti-microbial activity of cathelicidin-Bg. The results showed that cathelicidin-Bg could significantly inhibit the proliferation of two strains of Gram-positive bacteria, *B. subtilis* and *S. aureus*, but not that of the other four strains (Figure 4A). The inhibitory effect of cathelicidin-Bg on Gram-positive bacteria has also been shown by the growth curve of *B. subtilis* and *S. aureus*, in which cathelicidin-Bg almost completely inhibited the growth of these two bacterial strains (Figure 4B).

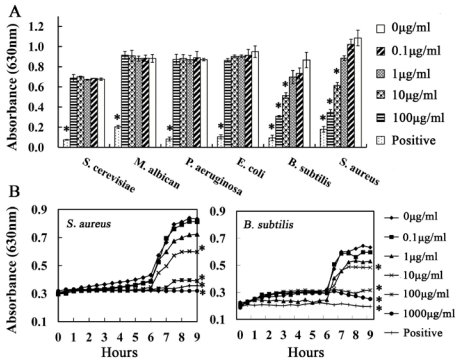

Figure 4. Anti-microbial activity of cathelicidin-Bg. **A.** Proliferation inhibition of six microbes by cathelicidin-Bg. **B.** Growth curves of *Staphylococcus aureus* and *Bacillus subtilis* treated by cathelicidin-Bg. Data are reported as means ± SE from three independent experiments. *P < 0.05.

Anti-tumor activity

The anti-tumor activity of cathelicidin-Bg was verified, since many anti-microbial peptides act as cancer inhibitors. Two kinds of tumor cells were used in this experiment, HEK293 and HepG2 cells. Interestingly, cathelicidin-Bg was able to selectively inhibit the proliferation of tumor cells. The results showed that cathelicidin-Bg could inhibit the proliferation of HEK293, but not HepG2 cells (Figure 5).

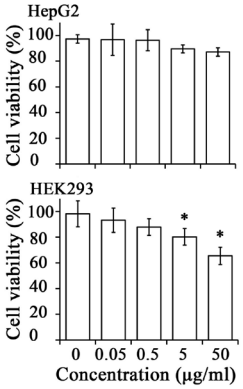

Figure 5. Proliferation inhibition of HEK293 and HepG2 cells by cathelicidin-Bg. Data are reported as means ± SE from three independent experiments. *P < 0.05.

DISCUSSION

For a long time, studies on Chan Su have focused on ethanol extracts and many of its components have been identified. However, studies in other Amphibian species have indicated that water extracts, especially for peptides, are the most significant components, which possess anti-microbial and anti-tumor activity. In this study, we identified a gene expressed in the ear-side gland whose product (a mature peptide) has anti-microbial and anti-tumor activities. The anti-microbial activity differed from that of cathelicidin from *Bufo gargarizans* (Sun et al., 2015), indicating that AMPs from amphibians possess various functions.

A rapid and effective response to pathogens is essential for the survival of all living organisms. Amphibians live in environments that are full of microbes and have therefore evolved to meet this challenge by producing a large variety of AMPs. AMPs are often secreted from the skin glands of amphibians, such as the serous glands. In the present study, we constructed a cDNA library using total RNA isolated from the ear-side gland of *B. gargarizans* and verified the expression of AMP-encoding genes. Finally, a cDNA, *cathelicidin-Bg*, was cloned and was found to possess a conserved proregion and a variable C-terminal antimicrobial domain similar to other cathelicidins (Zanetti et al., 1995). In further studies, the mature peptide of cathelicidin-Bg was shown to inhibit the growth of Gram-positive but not Gram-negative bacteria and fungi. In addition, many AMPs showed anti-tumor activity as observed in previous studies (Ohsaki et al., 1992; Lee et al., 2008; Ferreira et al., 2013). In this study, we showed that cathelicidin-Bg could inhibit the proliferation of HEK293 cells but not HepG2 cells; the mechanism(s) involved should be clarified in future studies.

Conflicts of interest

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

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