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Combined venomics, antivenomics and venom gland transcriptome analysis of the monocoled cobra (*Naja kaouthia*) from China

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ABSTRACT

We conducted an omics-analysis of the venom of *Naja kaouthia* from China. Proteomics analysis revealed six protein families [three-finger toxins (3-FTx), phospholipase A_2 (PLA_2), nerve growth factor, snake venom metalloproteinase (SVMP), cysteine-rich secretory protein and ohanin], and venom-gland transcriptomics analysis revealed 28 protein families from 79 unigenes. 3-FTx (56.5% in proteome/82.0% in transcriptome) and PLA_2 (26.9%/13.6%) were identified as the most abundant families in venom proteome and venom-gland transcriptome. Furthermore, *N. kaouthia* venom expressed strong lethality (i.p. LD_{50}: 0.79 μg/g) and myotoxicity (CK: 5939 U/l) in mice, and showed notable activity in PLA_2 but weak activity in SVMP, L-amino acid oxidase or 5′ nucleotidase. Antivenomic assessment revealed that several venom components (nearly 17.5% of total venom) from *N. kaouthia* could not be thoroughly immunocaptured by commercial *Naja atra* antivenom. ELISA analysis revealed that there was no difference in the cross-reaction between *N. kaouthia* and *N. atra* venoms against the *N. atra* antivenom. The use of commercial *N. atra* antivenom in treatment of snakebites caused by *N. kaouthia* is reasonable, but design of novel antivenom with the attention on enhancing the immune response of non-immunocaptured components should be encouraged.

Biological significance: The venomics, antivenomics and venom-gland transcriptome of the monocoled cobra (*Naja kaouthia*) from China have been elucidated. Quantitative and qualitative differences are evident when venom proteomic and venom-gland transcriptomic profiles are compared. Two protein families (3-FTx and PLA_2) are found to be the predominated components in *N. kaouthia* venom, and considered as the major players in functional role of venom. Other protein families with relatively low abundance appear to be minor in the functional significance. Antivenomics and ELISA evaluation reveal that the *N. kaouthia* venom can be effectively immunorecognized by commercial *N. atra* antivenom, but still a small number of venom components could not be thoroughly immunocaptured. The findings indicate that exploring the precise composition of snake venom should be executed by an integrated omics-approach, and elucidating the venom composition is helpful in understanding composition-function relationships and will facilitate the clinical application of antivenoms.

1. Introduction

The evolution of venoms in advanced snakes allows them to transit from a mechanical (constriction) to a chemical (venoms) means of predation, and play an important role in the diversification of the snakes involved [1]. The variability in snake venoms is caused by recruitment of new toxin genes or explosive diversification of existing toxin genes, which occurred before and across the evolutionary process of the advanced snakes [2,3]. The rapidly evolved toxins are speculated to be heavily driven by natural selection [4,5]. Generally, the complexity in functional role is highly correlated with the variability in venom composition and abundance [6–9], and thus elucidating the venomics and venom gland transcriptomics of snakes would be helpful for clinical management of snakebites, filtration of medical components and preparation of antivenoms.

There are some 60 species of venomous snakes in China, and the envenomation burden caused by snakebites is heavy in the country [10,11]. Here, we studied the monocled cobra (*Naja kaouthia*), a clinically important cobra that is widely distributed in Northeast India, Bangladesh, Malaysia, Indochina Peninsula, Nepal and Southwest China (Yunnan, Sichuan and Guangxi Provinces) [12]. Envenomation by *N. kaouthia* is capable of inducing several very serious symptoms including ptosis, dysphagia and increased salivation, even leading to coma and death from respiratory paralysis [13–15]. One study conducted recently on the proteomic profile and potential role of *N. kaouthia* venom identified

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12 protein families from crude venom, among which 3-FTx and PLA_2 were the predominant components and accounted for 77.5% and 13.5% of the total venom, respectively [16]. Similar proteomic profiles have also been identified in *N. kaouthia* venoms from Malaysia, Thailand and Vietnam, although there are apparent geographical variations in venom composition and abundance that may correlate with the differences in venom lethality [17]. However, as the venom gland transcriptomic profile of *N. kaouthia* has not yet been studied, we still cannot have a deeper understanding of venom proteomic profile.

As is well known, antivenoms are most efficient for treatment of snakebites caused by venomous snakes, and it has been recommended that a pan-regional polyvalent, regional monovalent or monoclonal antivenom should be designed and prepared [18–22]. However, the conflict between high investment in development and relatively low medical market demand of antivenom leads to a low diversity in commercial antivenoms, and victims envenomed by a snake sometimes have to be injected with a antivenom not raised against that snake but a phylogenetically related or congeneric species. For example, snakebites caused by *N. kaouthia* in China have been treated with a monovalent antivenom against the Chinese cobra *Naja atra*. Actually, in China the snakebites caused by *N. kaouthia* and *N. atra* are not strictly discriminated, because these two cobras have an overlapping range of distribution, are morphologically similar to each other, and even induce similar clinical symptoms. To our knowledge, there is no commercially available antivenom specifically raised against *N. kaouthia* in China, and there is no adequate proof of preclinical assessment for clinical use of commercial *N. atra* antivenom in treatment of envenomation caused by *N. kaouthia*.

Here, we used a combined proteomic strategy and Illumina sequencing technology to resolve and compare the venom proteomic and venom gland transcriptomic profiles of *N. kaouthia* from China. To understand the potential role of venom on envenomation and assess the efficacy of commercially available *N. atra* antivenom on clinical treatment of envenomation caused by *N. kaouthia* in China, we also evaluated the toxicological and enzymatic activities of crude venom, and conducted second-generation antivenomics based on *N. kaouthia* venom and *N. atra* antivenom.

2. Materials and methods

2.1. Venom and antivenom

We collected two adult *N. kaouthia* in 2014 in Baise, Guangxi, South China (Fig. 1), and brought them to our laboratory in Hangzhou for venom collection. The venom was milked by snake biting on parafilm-wrapped jars. Fresh venom was centrifuged to remove impurities for 15 min at 10000*g* 4 °C, lyophilized, pooled equally and stored at −80 °C until use. Commercial monovalent *N. atra* antivenom (batch 20,070,601, expiry date: 06/2010; 1000 IU/vail) purchased from Shanghai Serum Biological Technology Co., Ltd. (Shanghai, China) was prepared from the plasma of horses, and consisted of purified $F(ab')_2$ fragments. The antivenom was lyophilized and stored at −80 °C, and re-dissolved when used. Protein concentrations of venom fractions were determined according to Bradford [23]. Concentration of antivenom was determined spectrophotometrically using 1 cm light pathlength cuvette based on an extinction coefficient (ε) of 1.4 for 1 mg/ml protein at 280 nm.

2.2. Isolation and characterization of venom proteins

Crude venom (5 mg) was dissolved in 0.1% trifluoroacetic acid (TFA, solution A), centrifuged for 10 min at 10000*g* 4 °C, then the supernatant was collected and loaded on a Kromasil 300 C18 reverse phase column (250 × 4.6 mm, 5 μm particle, AkzoNobel, Sweden) using a Waters E600 HPLC system (Waters, USA). The flow-rate was set to 1 ml/min, and the venom proteins were separated with a mobile phrase system of 0.1% TFA in water and acetonitrile (ACN, solution B) at the following steps: isocratically (10% B) for 5 min, followed by 10–25% B for 15 min, 25–45% B for 80 min, and 45–60% B for 20 min. Protein detection was performed at 215 nm. Fractions were collected manually, and concentrated in a Centrifugal Concentrator (CentriVap®, Labconco, USA). The proteins were dissolved in loading buffer, and applied to 18% or 12% SDS-PAGE for separation. The gels were stained in 0.2% Coomassie brilliant blue (CBB) R-250, and scanned by UMAx2100 densitometer (Umax Technologies, China).

Protein bands in the CBB-stained gels were excised, and subjected to automated reduction with DTT and alkylation with IAA, then digested by trypsin gold (Promega). The tryptic peptides were dried in centrifugal concentrator, and re-dissolved in 1.5 μl of 30% ACN and 0.1% TFA. The solutions were spotted onto a sample holder, air-dried to 0.5 μl, mixed with an equal volume of 5 mg/ml α-cyano-4-hydroxycinnamic acid (ABI) in 50% ACN and 0.1% TFA, dried completely, and subjected to MALDI-TOF/TOF-MS (Autoflex speed™, Bruker Dalton, Germany) according to the manual instructions. While for LC-MS/MS analysis, the tryptic peptides were dissolved in 2% ACN and 0.1% FA, then loaded on a C18 reverse phase column (100 μm × 10 cm, 3 μm resin, Michrom Bioresources, CA) and subjected to nESI-MS/MS (LTQ-Orbitrap, Thermo Electron, Germany) according to the manual instructions. The MS spectra were interpreted by FlexAnalysis or Xcalibur software, and the assignment of amino acid sequence similarity was performed against a non-redundant protein sequence database in NCBI (strict to the taxa Serpentes) using Mascot Search Engine 2.3.02. The mass tolerance was set at 0.6 Da. Carbamidomethyl (C) was set as fixed modification, and Acetyl (N-term) and Oxidation (M) was set as variable modification.

We followed Calvete et al. [24] to estimate the relative abundance of protein family. The relative abundance of fractions was calculated by peak area measurement using Empower software (Waters, USA). When the fractions present one protein band in SDS-PAGE, the relative abundance was directly obtained from the peak area measurement; while the fractions present more than one protein band, the relative abundance of each band was estimated by densitometry using Tan4100 software (Tanon Science & Technology, China).

2.3. Venom gland cDNA synthesis and sequencing

Four days after venom milking, the aforementioned two snakes were anesthetized with sodium pentobarbital (s.c. 30 mg/kg). Venom glands of both sides were removed from each snake, rinsed with RNase-free water, and pooled. Total RNA of venom gland from each snake was extracted using TRIzol (Life Technologies, USA) following the manufacturer's protocol. RNA was purified and dissolved in 100 μl THE RNA storage solution (Ambion, Inc., USA). RNA degradation and contamination was assessed using 1% agarose gel electrophoresis. RNA purity and concentration was measured using the Nanophotometer (Implen, USA) and Qubit 2.0 fluorometer (Life Technologies, USA), and RNA integrity was evaluated using the Agilent 2100 system (Agilent Technologies, USA). The RNA for sequencing was pooled equally from the above two RNA samples.

RNAseq libraries were constructed with TruSeq™ RNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's instructions. Briefly, mRNA was purified and enriched using magnetic bead with oligo (dT). The mRNA was treated with fragmentation buffer, and used as a template to synthesize first-strand cDNA with reverse transcriptase and random hexamer primers. Second-strand cDNA was synthesized using dNTPs, DNA polymerase I and RNase H. Double-stranded cDNA was then purified with AMPure XP system (Beckman Coulter, USA), and underwent the processes of end pair, ligation of poly (A) tail and sequencing adapters. The adaptor ligated fragments were selected for PCR-amplification and purified using AMPure XP system (Beckman Coulter, USA) to create the final cDNA libraries. Deep sequencing with the Illumina HiSeq™ 2500 (Illumina, USA) platform was