**A survey of the venom of the spider *Lycosa vittata* by biochemical,
pharmacological and transcriptomic analyses**

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Abstract

Lycosa vittata, mainly distributed in the southwest of China, is a medium-sized and venomous spider, whose venom remains unexplored so far. This study aims to present an overview of the venom. It mainly consisted of diverse peptides and exhibited inhibitory effects on voltage-gated ion channels in rat dorsal root ganglia neurons, with a strongest inhibition on tetrodotoxin-sensitive and tetrodotoxin-resistant voltage-gated Na⁺ channels. Interestingly, it exerted cytotoxicity to cancer cells, with approximately 10-fold selectivity on PC-3 over others, implying the existence of selective anti-PC-3 agents in the venom. Moreover, 51 toxin-like peptides were deduced from the venom gland transcriptome. Bioinformatic analyses suggested their structures might have some distinguished properties and their predicted functions were consistent with the venom activities. This study suggests that the venom is an attractive source of neurotoxins with therapeutic significance, and provides references for the structure and function investigation of specific toxins in the future.

Key words: *Lycosa vittata*; spider venom; peptide toxins; bioactivity; transcriptome

1. Introduction

Spiders are the most diverse and successful terrestrial invertebrates excluding insects, and are also the most abundant terrestrial predators (Coddington *et al.*, 1991; Saez *et al.*, 2010). There are 44,906 described species in approximately 114 families to 2014, with an even greater number awaiting characterization (Platnick, 2014. The world spider catalog, version 13.5. American Museum of Natural History, is available online at <http://research.amnh.org/iz/spiders/catalog/COUNTS.html>). Spiders roam the world and have conquered most terrestrial environments (Dos Santos *et al.*, 2009; Hu *et al.*, 2014). One of the major contributors to the evolutionary success of spiders is their ability to produce complex venoms for predation and predator deterrence (King, 2004). Spider venoms are complex cocktails composed of a variety of chemical compounds, including salts, small organic molecules, peptides and proteins, but the major components are small and disulfide-rich peptides (Saez *et al.*, 2010; Klint *et al.*, 2012). These peptides target a diverse range of ion channels, cell receptors and enzymes in a wide range of vertebrate and invertebrate species (Liang, 2008; Saez *et al.*, 2010). Therefore, the spider venom peptides exhibit attractive pharmacological activity, such as antiarrhythmic, analgesic, cytolytic, haemolytic, insecticidal, antimicrobial, antiparasitic, antitumor and enzyme inhibitory activity. In the past two decades, these fascinating bioactivities of venom peptides make spider venom an ideal natural source for the discovery of novel therapeutic leads (Escoubas and Bosmans, 2007).

The geographic distribution of *Lycosa vittata* is mainly the southwest of China, such as Tibet, Yunnan and Guizhou provinces. In this study, the spiders were collected in Nyangtri of Tibet. *L. vittata* mainly lives in valley and paddy fields, and feeds on insects. It is a medium-sized spider (Fig. 1A). The females are 14.5-16.5 mm in body length and 19-26 mm in leg span, and the males are 10.5-15 mm in body length and 18-25 mm in leg span. The spider is dark brown in color with tawny specks of its carapace. The chelicerae are brown and with long brown hairs.

The potential of spider venom components as pharmacological tools and drug leads attracts more and more researcher's attention, and this potential is conformed by

an increasing number of publications (Escoubas and King, 2009; Diego-García *et al.*, 2010; Escoubas and Bosmans, 2007; King *et al.*, 2008; Klint *et al.*, 2012; Windley *et al.*, 2012; Pineda *et al.*, 2014; Kalia *et al.*, 2015). However, the investigation of the gold mine of spider venom is only the tip of the iceberg. On this point in time, we investigated the biochemical and pharmacological properties of *L. vittata* venom and the transcriptome of the venoms glands. In the present study, we showed that the venom contains diverse peptides and possesses neurotoxic and anticancer activities, and we reported 51 peptide toxins deduced from the venom gland cDNA library of the wolf spider *L. vittata*. These results revealed the significant pharmacological activity and peptides diversity of the venom, and also provided the clues for screening pharmacological tools and drug leads from these peptides in future studies.

2. Materials and methods

2.1. Venom collection

The spider *L. vittata* were captured from the valley of Nyangtri of Tibet province in the southwest of China. Adult female *L. vittata* spider were maintained in plastic pails, and fed weekly with worms and given water daily. The venom was collected by using an electro-pulse stimulator to chelicere every two weeks as reported in our previous study (Wang *et al.*, 2007). Venom was lyophilized and stored at -80°C prior to analysis.

2.2. Toxin purification

The venom powder was dissolved with water to a final concentration of 10 mg/mL, centrifuged at 14,000 rpm for 30 min at 4°C and filtered using a 0.22 µm microfilter (SJGVL04NS, Millipore). The venom solution was analyzed by RP-HPLC on a C18 column (phenomenex 100Å, 4.6 mm×250 mm, 5 µm) using a Waters Alliance 2695 (Milford, MA, USA). Venom components were eluted using a linear acetonitrile gradient (5%-50% acetonitrile/0.1% TFA in 45 min) at a flow rate of 1.0 mL/min (Liu *et al.*, 2006). Fractions (1 mL/tube) were collected manually by monitoring the absorbance at 215 nm. Each fraction was lyophilized and reconstituted in 10 µL of 50% acetonitrile in water/0.1% TFA prior to MALDI-TOF-MS analysis.

2.3. MALDI-TOF-MS analysis

The venom was desalted with a ZIP-TIP and then analyzed using MALDI-TOF-MS (Ultraflex, Bruker Daltonics, Bremen, Germany). Ionization was achieved by irradiation with a nitrogen laser (337 nm) with a 20 kV acceleration voltage. α -Cyano-4-hydroxy-cimamic acid (CCA) was used as the matrix. Regarding the analysis the fractions from RP-HPLC, 1 μ L of each fraction was applied to a well overlaid with 1 μ L of CCA. Data were acquired using Bruker Daltonics Flexcontrol software (Bremen, Germany) and exported with Flex analysis software (Bremen, Germany). All analyses were conducted in a liner mode (Wang *et al.*, 2013).

2.4. SDS-PAGE Analysis

The venom powder was dissolved with water to a final concentration of 10 mg/mL, centrifuged at 14,000 rpm for 30 min at 4°C and filtered using a 0.22 μ m microfilter (SJGVL04NS, Millipore). The venom solution was analyzed by SDS-PAGE (15% separation gel and 5% stacking gel) using standard protocols to check the MW range and stained with Coomassie blue G-250 (Sigma-Aldrich, St. Louis, MO, USA) (Hu *et al.*, 2014).

2.5. Electrophysiological studies

Whole-cell voltage-clamp recordings of voltage-gated ion currents were made in rat DRG neurons which were acutely dissociated from 30-day old Sprague-Dawley rats and maintained in short-term primary culture according to the method described by Xiao and Liang (2003). DRG neurons with a large diameter (>45 μ m) and those with a relatively small diameter (<20 μ m) were selected for measuring tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) Na⁺ currents, respectively. Meanwhile, TTX (final concentration: 200 nM) was used to separate TTX-R Na⁺ currents from TTX-S Na⁺ currents. Electrophysiological recordings were performed at room temperature. Suction pipettes (2.0-4.0 M Ω) were made from borosilicate glass capillary tubes using a two-step pulling on a vertical micropipette puller. For Na⁺ current recording, the pipette solution contained (in mM): CsCl 145, MgCl₂·6H₂O 4, hydroxyethyl piperazine ethanesulfonic acid (HEPES) 10, ethylene glycol tetraacetate (EGTA) 10, D-glucose 10, adenosine-triphosphate (ATP) 2 (pH 7.2); and the external solution contained (in mM): NaCl 145, KCl 2.5, CaCl₂ 1.5,

MgCl₂·6H₂O 1.2, HEPES 10, and D-glucose 10 (pH 7.4). For K⁺ currents recording, the pipette solution contained (in mM): KCl 135, KF 25, NaCl 9, CaCl₂ 0.1, MgCl₂ 1, EGTA 1, HEPES 10 and ATP-Na₂ 3, adjusted to pH 7.4 with 1 M KOH; and the external bath solution contained (in mM): NaCl 150, KCl 30, CaCl₂ 5, MgCl₂ 4, TTX 0.3, HEPES 10 and D-glucose 10, adjusted to pH 7.4 with 1 M NaOH. For Ca²⁺ current recording, the pipette solution contained (in mM): Cs-methane sulfonate 110, phosphocreatine 14, HEPES 10, EGTA 10, ATP-Mg 5, adjusted to pH 7.3 with CsOH; the external solution contained (in mM): BaCl₂ 10, tetraethylammonium (TEA)-Cl 125, TTX 0.3 and HEPES 10, adjusted to pH 7.4 with TEA-OH. To eliminate the potential influence of differences in osmotic pressure, all internal and external solutions were adjusted to 280 ± 5 mosmol/L with sucrose. Experimental data were collected and analyzed with Pulse/Pulsefit 8.0 (HEKA Electronics, Germany) and Sigmaplot 10.0 (Sigma). Macroscopic currents were filtered at 10 kHz, digitized at 3 kHz with an EPC-10 patch-clamp amplifier (HEKA Electronics, Germany). Series resistance was kept near 5 MΩ and compensated 65%-70%. Linear capacitive and leakage currents were digitally subtracted using the P/4 protocol. All data are presented as means ± S.E., and *n* is the number of independent experiments.

2.6. Anticancer cells proliferation analysis

K562, U937 human leukemia cell lines, PC-3 prostate cancer cell line, MDA-MB-231 breast cancer cell line, and the control cell line HEK-293 were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Science. K562 and U937 cells were cultured in RPMI 1640 medium (Invitrogen/Gibco) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Gibco). PC-3 cells were cultured in Kaighn's modification of Ham's F12 medium (F12K) (Invitrogen/Gibco) with 10% FBS, and HEK293T cells were cultured in DMEM medium (Invitrogen/Gibco) with 10% FBS. All cell lines were grown under standard cell culture conditions (5% CO₂ and 37 °C).

The five different cell lines were seed at a density of 1×10⁴ cells/100 μL in 96-well sterile plates. Then cells were treated with the venom (6.5-500 μg/mL) and the inhibitory effect of the venom on cell growth was determined after 48h. The cell

viability was assessed by MTT assay as described by Mai *et al.* (2001). All assays were conducted in sextuplicate. The concentration of the venom causing 50% inhibition (IC₅₀) was determined by plotting toxin concentrations vs percentages of inhibition (on a Probit scale) as calculated from the absorbance data.

2.7. cDNA library construction, DNA sequencing, and sequence analysis

The venom glands of 6 adult female spiders were isolated and immediately homogenized in liquid nitrogen. The total RNA was extracted with Trizol Reagent (Invitrogen) and used for library construction. The full-length cDNA library was made following the instructions for the Creator SMART cDNA Library Construction Kit. Colony PCR was performed with the universal M13 forward and reverse primers to rapidly screen recombinant clones. The PCR products were resolved by agarose gel electrophoresis to determine the size of each product. Selected clones with the cDNA insert >350 base pairs were sequenced using an automated ABI PRISM 3730 sequencer (Perkin-Elmer). The clustering and assembling of ESTs were carried out with a universal tool of the EGAssembler (<http://egassembler.hgc.jp/>). cDNA sequences were translated and processed by the MEGA software (Kumar *et al.* 2008). According to sequence identity, peptide toxin precursors can be classified into different families and related families. The above methods were referenced from Tang *et al.* (2010).

3. Result and discussion

3.1. RP-HPLC, MALDI-TOF-MS and SDS-PAGE analysis of the venom

The spider *L. vittata* is able to expel venom from the chelicerae after electrical stimulation. The venom is a clear and colorless liquid, easily soluble in water, and approximately 1-3 μ L venom collected per spider. Finally, 16.2 mg dry venom was obtained from 120 *L. vittata* spiders. A typical RP-HPLC chromatogram of the venom of the spider *L. vittata* was shown in Fig. 1B. More than 45 fractions eluted and were monitored at 215 nm. Most of the fractions were eluted with retention time between 30-50 min, corresponding to 30%–50% acetonitrile gradient. Peptides are the most abundant components of most spider venoms (Kuhn-Nentwig *et al.*, 2011). Therefore, we attempted to analyze the mass distribution of the venom peptides by MALDI-TOF

MS. As shown in Fig. 1C, approximately 20 peaks were detected in a mass range from 1000 Da to 10,000 Da. Moreover, these peaks are localized in the range of 2000-3500 Da and 7000-9000 Da. To explore the molecular weight distribution of the venom proteins, the venom was analyzed by SDS-PAGE using standard protocols. Except for peptides with molecular weights below 10 kD, the venomous proteins were mainly distributed in three bands on SDS-PAGE corresponding to the molecular weights of approximately 30 kD, 66.2 kD and 116 kD, respectively (Fig.1D).

Fig. 1. A female *L. vittata* and the complexity of the venom peptides. **A.** A female *L. vittata* has a body length of 15 mm and a leg span of 25mm. **B.** RP-HPLC separation of 1 mg of soluble venom from *L. vittata* in an analytical C18 column equilibrated with solution A (distilled water in 0.1% TFA), using a gradient from 5% to 50% of solution B (acetonitrile in 0.1% TFA) over 45 min with a flow rate of 1 mL/min. Absorbance was read at 215 nm. **C.** MALDI-TOF spectrum of *L. vittata* venom. **D.** SDS-PAGE of the venom of the spider *L. vittata*. 1, marker; 2 and 3, 70 and 140 µg venom, respectively.

3.2. The venom inhibits voltage-gated ion channels in rat DRG neurons.

Voltage-gated sodium channels (VGSCs) are transmembrane proteins that form ion channels, conducting Na^+ through the cell plasma membrane upon activation (Cestèle *et al.*, 2000). VGSCs are responsible for the generating and propagating nerve

impulses and play a key role in many electrophysiological processes (Ogata *et al.*, 2002). As for the fundamental physiological significance, VGSCs are the most widely ion channels targeted by toxins from venomous organisms (Bosmans and Swartz, 2010). The effects of the *L. vittata* venom on VGSCs in rat DRG neurons were therefore examined. Both TTX-S and TTX-R VGSCs are present in rat DRG neurons. Large DRG neurons (>45mm diameter) predominately express rapidly activating and inactivating TTX-S currents, whereas small neurons (< 20mm diameter) primarily exhibit slow TTX-R currents (Baker and Wood, 2001; Patrick and Waxman, 2007; Ho and O'Leary, 2011). Therefore, the large and small diameter neurons were selected for measuring TTX-S and TTX-R VGSCs, respectively. The venom displayed inhibitory effect on VGSCs in rat DRG neurons. As shown in Fig. 2, 100 µg/mL of venom could inhibit $63.2 \pm 4.5\%$ ($n = 5-8$) (Fig. 2A, *upper panel*) of TTX-S currents and $36.2 \pm 5.4\%$ ($n = 5-8$) (Fig. 2B, *upper panel*) of TTX-R currents, respectively. Please note that 200 nM TTX was used to block the TTX-S currents from mixed currents in order to confirm the inhibitory effect on TTX-R currents. The current-voltage (I-V) curves showed that the venom changed activation of TTX-S currents by a depolarizing shift of the activation voltage of the peak current from -25 mV to -15 mV (Fig. 3A, *lower panel*, $n = 5-8$), while no evident change of the I-V relationships of TTX-R currents (Fig. 3B, *lower panel*, $n = 5-8$). In rat DRG neurons, Nav1.7 is the main subtype of TTX-S currents, while Nav1.8 and 1.9 constitute TTX-R currents. These three VGSC subtypes are potential targets for pain therapeutics because of their established role in pain, selective expression in nociceptive neurons and their regulation in pain states (Lewis, *et al.*, 2012). Our data indicate that it is possible to identify their antagonists with therapeutic potentials from the venom of *L. vittata*.

Fig. 2. Effects of *L. vittata* venom on VGSC currents in rat DRG neurons. The upper panel indicates the current traces before and after the treatment of 100 µg/mL of venom, while the lower panel shows the corresponding I-V curves. Na⁺ currents were induced by a 50 ms depolarized potential of -10 mV from a holding potential of -80 mV in DRG neurons. **A.** TTX-S currents amplitude were blocked by $63.2 \pm 4.5\%$ ($n = 5-8$) by 100 µg/mL venom. **B.** With the use of 200 nM TTX, the remnant TTX-R currents were inhibited by $36.2 \pm 5.4\%$ ($n = 5-8$) in the presence of 100 µg/mL venom. For I-V curves, the currents were elicited by a series of 50-ms depolarizations from a holding potential of -80 mV to +40 mV in -5 mV steps. The data was expressed as mean±S.E. ($n = 5-8$).

DRG neurons express many types of K⁺ channels including voltage-gated (Kv), inwardly rectifying (Kir), Ca²⁺ activated (K_{Ca}) and background (leak, K2P) K⁺ channels. These channels contribute to the regulation of membrane repolarization, resting membrane potential, frequency of firing, and excitability of sensory neurons (Rasband *et al.*, 2001; Takeda *et al.*, 2011). Among various K⁺ channels, voltage-gated K⁺ channels (VGPCs) regulate many fundamental physiological processes, including action potential repolarization, hormone secretion, and

neurotransmitter release, making them fascinating pharmacological targets for binding distinct channel modifiers (Wickenden *et al.*, 2002; Shieh *et al.*, 2000). As shown in Fig. 3A, 100 $\mu\text{g/mL}$ venom inhibited K^+ currents in rat DRG neurons by approximately $18.1\% \pm 3.2\%$ ($n = 5-8$). The I-V curves obtained before and after the venom treatment indicated that the venom caused no significant alteration of the activation of K^+ currents (Fig. 3B). The result demonstrates that the venom should actually contain some neurotoxins acting on VGPCs although its inhibitory potency was weaker compared with that on VGSCs.

Fig. 3. Effects of *L. vittata* venom on VGPC currents in rat DRG neurons. **A.** K^+ currents were evoked by a 500-ms depolarized potential of 10 mV from a holding potential of -80 mV. 100 $\mu\text{g/mL}$ venom inhibited approximately $18.1\% \pm 3.2\%$ K^+ currents ($n = 5-8$). **B.** Normalized current-voltage curves of K^+ currents before (filled circles) and after (open circles) the venom treatment. Currents were elicited by a series of 500-ms polarizations from a holding potential of -80 mV to +60 mV in 10-mV steps. The curve was fitted with the equation $G = G_{\text{max}}/[1+\exp (V_{0.5}-V)/k]$, where G_{max} is the peak membrane conductance at +60 mV. The data were expressed as mean $\pm$ S.E. ($n = 5-8$).

Voltage-gated Ca^{2+} channels (VGCCs) mediate many physiological processes including neurotransmitter release, neurosecretion, neuronal excitation, survival of neurons, and regulation of gene expression. Currently, five main types of VGCCs (T, L, N, P/Q, and R) have been identified in vertebrate cells based on their physiological and pharmacological properties (Escoubas *et al.*, 2000; Catterall *et al.*, 2011).

High-voltage-activated (HVA) L-, N-, P/Q- and R-type Ca^{2+} channels require strong depolarization for activation and are essentially implicated in neurotransmitter release. Low voltage-activated (LVA) T-type Ca^{2+} channels currents activated by small depolarization are rapidly inactivated, and modulate pacemaker type repetitive activity in cardiac cells and neurons (Nelson *et al.*, 2006). As shown in Fig. 4A, the addition of 100 $\mu\text{g}/\text{mL}$ venom exerted very weak inhibition on total Ca^{2+} currents in rat DRG neurons, which were activated by a 150-ms step depolarization to +10 mV from a holding potential of -90 mV. Then, we investigated whether the venom had inhibitory activity against both types of VGCCs in rat DRG neurons. As shown in Fig. 4B and C, the application of 100 $\mu\text{g}/\text{mL}$ venom led to an approximately $12.2\% \pm 2.5\%$ ($n = 5-8$) reduction of LVA currents elicited by a 150-ms step depolarization to -30 mV from a holding potential of -90 mV, while the treatment with 100 $\mu\text{g}/\text{mL}$ venom had no effect on HVA currents ($n = 5-8$) activated by a 150-ms depolarization to +10 mV from a holding potential of -40 mV. These data implied that the venom might contain toxin components selectively targeting LVA currents. Since the inhibitory potency was not strong, we guessed that the antagonists of T-type VGCCs in this venom were either weak or low abundance. T-type VGCCs are widely distributed in pain pathway neurons and implicated in pain modulation. Since no peptide antagonist of T-type VGCCs has been identified to date, it is significant to identify their selective inhibitors from the venom of *L. vittata*.

Fig. 4. Effects of *L. vittata* venom on whole-cell VGCC currents in rat DRG neurons. **A.** The application of 100 $\mu\text{g}/\text{mL}$ of venom showed very weak inhibition of total Ca^{2+} currents evoked by a 150-ms depolarization to 0 mV from a holding potential of -90 mV. **B.** The application of 100 $\mu\text{g}/\text{mL}$ of venom inhibited $12.2\% \pm 2.5\%$ ($n = 5-8$) of LVA- Ca^{2+} currents evoked by a 150-ms depolarization to -30 mV from a holding

potential of -90 mV. **C.** The application of 100 $\mu\text{g/mL}$ of venom showed little inhibition of HVA- Ca^{2+} currents elicited by a 150-ms depolarization to +10 mV from a holding potential of -40 mV. The data was expressed as mean $\pm$ S.E. ($n = 5-8$).

3.3. The venom inhibits the growth of cancer cells

The animal venoms possess anticancer activities through apoptosis inducement or cell cycle arrest. Recently, proteins, peptides and non-protein compounds with anticancer activities have been identified from animal venoms and some of them have been studied intensively (Heinen *et al.*, 2011; Gupta *et al.*, 2010; Liu, *et al.*, 2012). Here, the anticancer potential of the *L. vittata* venom was examined on the growth of PC-3, U937, K562, MDA-MB-231 cancer cell lines, using HEK293T as control. As shown in Fig. 5, the venom caused an evident reduction of the viability of the four

cancer cell lines in a dose-dependent manner. The IC_{50} values were calculated to be 31.6 $\pm$ 2.5 $\mu\text{g/mL}$ for PC-3, 303.5 $\pm$ 3.8 $\mu\text{g/mL}$ for U937, 338.2 $\pm$ 4.2 $\mu\text{g/mL}$ for K562 and 396.1 $\pm$ 2.9 $\mu\text{g/mL}$ for MDA-MB-231. Interestingly, the venom showed a selective cytotoxicity on the five cell lines. The rank of susceptibility of the five cell lines to the venom was PC-3 > U937 $\approx$ K562 $\approx$ MDA-MB-231 > HEK293T. The venom had very low inhibitory effect on HEK293T cells with 500 $\mu\text{g/mL}$ venom resulting in only less than 30% loss of cell viability; it exhibited the most potent inhibition on PC3 cells and the IC_{50} value for PC-3 is about 10-fold lower than other cancer cell lines. These data indicated that it is possible to identify anticancer compounds, especially agents selectively targeting PC-3, from this venom.

Fig. 5. Effects of the venom on cancer cell growth. **A.** The venom treatment induces cell growth inhibition. Treatment of four cancer cell lines (PC-3, U937, K562,

MD-MBA-231) with 500 µg/mL of venom caused more than 50% cell death. In contrast, treatment of the non-cancer cell HEK-293T with 500 µg/mL of venom displayed less than 30% cell death. *bars*, mean±SEM. **B**. The IC₅₀ values of the venom on the four cancer cell lines.

3.4. Transcriptomic analysis of venom peptides from *L. vittata*

L. vittata venom gland library was constructed and approximately 500 clones were selected for sequencing. After discarding the poor-quality sequences, 51 toxin-like peptide precursors were deduced from the high-quality ESTs (Fig. 6). The lengths of complete cDNA encoding toxin peptides range from 0.41 to 0.58 kb corresponding amino acid sequence length ranging from 83 to 121 residues. According to precursor sequence identity, these peptide precursors could be categorized into eight families (families A-H) (**Fig. 6**). A phylogenetic tree was constructed by MEGA 3.1 using neighbor-joining method. The result showed that the eight families formed five independent clads in the phylogenetic tree, the family A, C and E, the family B and D, as well as the other three families belonging to the five distinct five clades, respectively (**Fig. 7**).

Fig. 6. Sequence alignment of toxin precursors of families A-H. Cysteine residues are shaded in black. The signal peptides and propeptides are shown in boxes. Homologs from other spiders are marked with black star.

Toxin precursors within the same family share very high sequence identity, while those from different families are rather distinct, however, some common properties are still observed as follows (Fig. 6). (1) All precursors consist of a signal peptide, a

propeptide and a mature peptide, expect those from the family H. (2) These mature peptides are rich of positively charged residues, making their PI values in the basic range. (3) These mature peptides include 8-10 cysteine residues forming 4-5 disulfide bonds; the mature peptides from the family A-E and the family G share the consensus cysteine arrangement (“-C-C-CC-CXC-CXC-”), while the family F toxins have eight cysteine residues in an arrangement of “-C-CXC-CXC-CXC-C-” and the only one family H toxin contains 10 cysteine residues (“-C-C-CC-CXC-C-C-”). (4) The toxins from the families A-E have long, basic and flexible C-terminal segments lack of disulfide bond constraints, which might be directly involved in their functions, same as *Cupiennius salei* toxins (CSTXs) from the venom of the wandering spider *Cupiennius salei* (Kuhn-Nentwig *et al.* 2000; Schalle *et al.* 2001; Wullschleger *et al.* 2004; Kuhn-Nentwig *et al.*, 2012). Some CSTXs are L-type VGCC blockers but also cytolytic peptides through destroying cell membrane. They also contain such kind of C-terminal sequences that are predicted to form α -helixes and attributed to their higher toxicity (Kubist *et al.* 2007; Kuhn-Nentwig *et al.*, 2012; Kuhn-Nentwig *et al.* 2000). (5) Most toxin precursors contain a consensus propeptide processing enzyme cleavage signal “EQ/ET/M/PR”, such motif was commonly observed in many other spider toxin precursors (Diego-García *et al.* 2010; Zhang *et al.* 2015; Zhang *et al.*, 2010). (6) Most mature toxins have a consensus “G” in the C-terminals, which are trimmed through post-translational amidation modification (Tang *et al.*, 2010; Jiang *et al.* 2013).

Since the venom collected from the spider *L. vittata* was very limited, it is hard to analyze the structures and functions of individual toxins in the peptide level. Alternatively, we attempted to predict such information through bioinformatics analysis including cysteine residue arrangement and blast search. The toxins from the families A-E have a consensus cysteine arrangement (“-C-C-CC-CXC-CXC-”) which is typical for spider toxins with an inhibitor cysteine knot motif (ICK) (Tang *et al.*, 2010). The eight cysteine residues are linked in the 1-4, 2-5, 3-8 and 6-7 pattern where the former three disulfide bonds form a cysteine knot. Therefore, same as that of CSTXs, the structure of these toxins from families A-E might be described as

follows: the N-terminal cysteine residue-contained segment might adopt an ICK motif serving as the core structure, while the C-terminal cysteine residue-free segment might form an α -helix that is flexible and appended to the core structure. This unique structure property is closely correlated with the functions of these toxins. The 10 cysteine residue-contained toxin (LVTX-H368) from the family H shares a common cysteine arrangement to U19-CNTX-Pn1a from the Brazilian armed spider *Phoneutria nigriventer* and MIT1, mamba intestinal toxin 1 (MIT1) isolated from *Dendroaspis polylepis* venom (Boisbouvier *et al.* 1998; Szeto *et al.*, 2000). The ten cysteine residues form five disulfide bonds as 1-4, 2-5, 3-7, 6-9 and 8-10. Speculatively, LVTX-H368 might share the same disulfide linkage and structural fold as MIT1, whose structure is a similar global fold with respect to colipase, a protein involved in fatty acid digestion. The three toxins from the family F share sequence identity (44%) and identical cysteine arrangement with ω -agatoxin-1A, a blocker of VGCCs isolated from the North American funnel-web spider *Agelenopsis aperta* (Scott *et al.*, 1990). This cysteine arrangement is also adopted by some toxins from the wolf spider *Lycosa singoriensis* and the fishing spider *Dolomedes mizhoanus* (Zhang *et al.*, 2010; Jiang *et al.*, 2013). It is obvious that such kind of cysteine arrangement is distinct from that of ICK toxins, implying the family F toxins and other spider toxins containing the same cysteine arrangement might adopt novel structural fold different from ICK motif. Taken together, according the data available, three kinds of structural folds might be present in the toxins from this venom, although ICK motif is the main structural fold adopted by the toxins. Determination of the other two novel structural folds will expand our understanding of structure and function relationships of spider peptide toxins.

Generally speaking, the toxins from *L. vittata* show sequence identity with those from *L. singoriensis*, implying the close evolution relationship between the toxins from the two wolf spiders which are mainly distributed in the southwest and northwest of China, respectively (Zhang *et al.*, 2010). It should note that the structures and functions of toxins from *L. singoriensis* have not experimentally examined yet, while some other homologs of these toxins are functionally known. Therefore, these

homologs can be considered as references for the function prediction of the toxins from *L. vittata*. For example, the toxins in the families F and G show high sequence identity with ω -agatoxin-1A and ω -LSP-1A, two blockers of VGCCs, respectively (Pluzhnikov *et al*, 2007; Scott *et al*, 1990), indicating their functions might be potentially correlated with VGCC blockage. This prediction was consistent with the data that the venom was able to suppress the Ca^{2+} currents in rat DRG neurons. In a similar way, the cytotoxic activity of the venom on cancer cells might be explained that some toxins in the families A-E share sequence identity and properties mentioned above with CSTXs and therefore were predicted to have inhibitory activity on cancer cells. On the other hand, patch clamp analysis revealed that the venom potently inhibited the TTX-S and TTX-R Na^+ currents in rat DRG neurons. However, our venom gland transcriptomic data presented no homologs known to target VGSCs. We estimated that the *L. vittata* venom should contain novel VGSC toxins that were distinguished from known ones and probably had different action mechanisms.

In summary, the novel venom of the spider *L. vittata* shows attractive pharmacological activities, including neurotoxicity and anticancer activity. The venom contains neurotoxins targeting on voltage-gated Na^+ , K^+ and Ca^{2+} channels in rat DRG neurons, with the strongest inhibition on TTX-S and TTX-R VGSCs. We expected our future studies will identify selective Nav1.7 or Nav1.8 channel antagonists with therapeutic implications. The venom also displays a potent and selective cytotoxicity to PC-3, suggesting that it is possible to screen anticancer agents affecting PC-3. Furthermore, the venom gland transcriptomic analysis yielded 51 toxin-like peptide sequences that are of diversity and exhibit some unique properties in amino acid sequence, cysteine residue arrangement and spatial structures. The bioinformatic analyses predicted potential spatial structures and functions for individual toxins, which provides a starting point for the structure and function elucidation of specific toxins in the future.

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462 **Fig. 7.** Phylogenetic tree of the toxins precursors from *L. vittata* glands. The
463 phylogenetic analysis was conducted by using the Neighbor-Joining method of the
464 MEGA 3.1 software package.

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466 **Acknowledgements**

467 This work was supported by the National 973 Project of China under contract
468 (2012CB22305); the National Science Foundation Project (31370854); the Science
469 Found for Distinguished Young Scholars of Hunan Province (No. 14JJ1018); the
470 Cooperative Innovation Center of Engineering and New Products for Developmental
471 Biology of Hunan Province (No. 20134486).

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629 M114.635458

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