Oxidative stress and antioxidant defense in detoxification systems of snake venom-induced toxicity

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Abstract
Background: Snakebites remain a major life-threatening event worldwide. It is still difficult to make a positive identification of snake species by clinicians in both Western medicine and Chinese medicine. The main reason for this is a shortage of diagnostic biomarkers and lack of knowledge about pathways of venom-induced toxicity. In traditional Chinese medicine, snakebites are considered to be treated with wind, fire, and wind-fire toxin, but additional studies are required.

Methods: Cases of snakebite seen at the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine were grouped as follows: fire toxin – including four cases of bites by Agkistrodon acutus and three bites by Trimeresurus stejnegeri – and wind-fire toxin – four cases of bites by vipers and three bites by cobras. Serum protein quantification was performed using LC-MS/MS. Differential abundance proteins (DAPs) were identified from comparison of snakebites of each snake species and healthy controls. The protein interaction network was constructed using STITCH database.

Results: Principal component analysis and hierarchical clustering of 474 unique proteins exhibited protein expression profiles of wind-fire toxins that are distinct from that of fire toxins. Ninety-three DAPs were identified in each snakebite subgroup as compared with healthy control, of which 38 proteins were found to have significantly different expression levels and 55 proteins displayed no expression in one subgroup, by subgroup comparison. GO analysis revealed that the DAPs participated in bicarbonate/oxygen transport and hydrogen peroxide catabolic process, and affected carbon-oxygen lyase activity and heme binding. Thirty DAPs directly or indirectly acted on hydrogen peroxide in the interaction network of proteins and drug compounds. The network

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was clustered into four groups: lipid metabolism and transport; IGF-mediated growth; oxygen transport; and innate immunity.

Conclusions: Our results show that the pathways of snake venom-induced toxicity may form a protein network of antioxidant defense by regulating oxidative stress through interaction with hydrogen peroxide.

Background

Venomous snakebites have been a common and critical clinical illness since ancient times [1]. The popular story about the farmer and the snake from Aesop’s Fables illustrates that fatal snake bites were one of the major public health challenges. The World Health Organization estimated that about 4.5 to 5.4 million snake bites occur globally each year, resulting in 1.8 to 2.7 million cases of snakebite envenomings and 81,000 to 138,000 deaths [2]. Snakebites occur frequently in tropical, developing countries in South Asia, South-East Asia, Africa and Latin America [3–6]. Within China, there are approximately 200 species of snakes, including about 50 species of poisonous snakes and more than 10 species of highly virulent snakes [7]. Bites by dangerous snakes are greatly feared, and effective symptomatic diagnosis and treatment are poorly documented. Due to complex pathogenesis and interspecific variability of venom components, the mechanisms and pathophysiological changes are still not completely elucidated [8, 9].

Symptoms induced by snake venoms are multi-factorial and complex, which severely restricts therapeutic intervention after the snake bite [10]. Moreover, differing components of different venoms make it difficult to generate sufficient diagnostic markers for clinical practice in both Western medicine and Chinese medicine [11, 12]. Quantitative proteomic analysis of multiple venoms revealed that the venom proteome composition includes non-enzymatic and enzymatic proteins, such as three finger toxins (3FTx) and phospholipase A, PLA, [13–15]. In the absence of sufficient diagnostic markers, the clinical identification of a venomous snake is mainly based on the patient’s wound tooth distance, tooth marks, local symptoms, and consultations. Traditional Chinese medicine (TCM) classifies venomous snakes on the basis of toxicity symptoms into three types: wind toxin, fire toxin, and wind-fire toxin [16]. The fire toxin is similar to hemorrhagic venom. Clinical manifestations of snakebites by fire toxin snakes include local pain, immediately swelling, water or blood blisters around the bite on the affected limb, skin ecchymosis, ulceration, necrosis, and general hemorrhage. The wind toxin can be regarded as part of neurotoxic venoms, generating mild pain, minor blood loss, numbness, somnolence, difficulty in opening the mouth, confusion, respiratory distress, blurred vision, etc. The wind-fire toxin is similar to mixed venom, which presents a combination of neurotoxic and hemorrhagic symptoms. The wind-fire toxin provokes respiratory distress, drooping eyelids, blurred vision, muscle weakness, and paralysis of muscles in the extremities. In our TCM clinical work, fire toxin and wind-fire toxin are particularly prevalent, so that the predominant venomous snake species in China is deserving prior attention, including Agkistrodon acutus and Trimeresurus stejnegeri snakebite (fire toxin), and viper and cobra snakebite (wind-fire toxin). In TCM, the same treatment strategy is implemented for the same snakebite syndrome, even in different patients that were bitten by different snakes. At present, the identification of the snakebite syndrome in TCM relies on expert judgment and knowledge, such as diagnosis by exclusion, and speculative and empirical judgments. The emergency treatment of snakebites is based primarily on clinical practice guidelines, some of which lack scientific study. Furthermore, our understanding of the relationship between Compound Chinese Medicine and the effects of multiple components is still in its infancy [17]. Rapid and accurate identification of the snakebite etiology is also an important prerequisite for timely and effective treatment in TCM. It is urgent to correlate the pharmacological properties to clinical manifestations for a better understanding of wind-fire toxin and fire toxin. Investigation and analysis of the protein composition of serum from snakebite patients for diagnostic biomarkers will contribute to the clinical practice.

This study aims at solving diagnostic markers and investigating pathways of snake venom-induced toxicity from serum proteins of snakebite patients with four predominant venomous snake species in China. The serum protein profiles reflected the functional categorization. The differential abundance proteins (DAPs) supplied discovery biomarkers to distinguish snakebite by species. The network of interacting proteins and drug compounds contribute to understanding the complex pathogenesis of venom-induced toxicity. Serum proteome analysis could contribute to TCM diagnostic markers of snakebite syndromes, providing clinicians with a clear distinction between the wind-fire toxin and fire toxin, as well as early clinical identification of snake species.

Materials and Methods

Patient identification and eligibility

The experimental procedures were approved by the Ethics Committee of the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine (n. JZYFLL2017103012). Informed consent forms were obtained from all recruited participants in the surgical department of the Traditional Chinese Medicine of the Affiliated Hospital. All identified snakebite patients were reviewed for eligibility to inclusion and exclusion criteria. Patients were excluded for the following reasons:
**Results**

**Identification of serum proteins by LC-MS/MS**

Label-free quantitative proteomics of serum samples was performed on samples from 14 subjects with four types of snakebites and 15 healthy controls. Of the 14 patients, 7 were bitten by vipers (B) and three cases bitten by cobras (C) as the wind-fire toxin group; four cases bitten by *Agkistrodon acutus* (D) and three cases bitten by *Trimeresurus stejnegeri* (E) as the fire toxin group; and fifteen healthy volunteers as the control group. The serum samples from 5 healthy controls were pooled together, so that the individuals in the control group were divided into three experimental duplicates, named A1, A2, and A3. The wind-fire toxin group was coded B1, B2, B3, B4, C1, C2, and C3; while the fire toxin group was coded D1, D2, D3, D4, E1, E2, and E3.

**Protein separation and LC-MS/MS analysis**

All of the serum samples were processed as described previously [18]. Briefly, high abundance proteins were first depleted by multiple affinity removal according to the Agilent protein purification protocol [19]. Then, each depleted protein sample was separated on 12% gradient tris-glycine polyacrylamide gel using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method [20]. The gel was stained with Coomassie blue, then re-suspended with dithiothreitol and digested with the sequence grade modified Trypsin (Promega) [21]. The digested proteins were passed through a C18 cartridge to be desalted. Finally, the dried peptides were resuspended in 40µL of 0.1% formic acid prior to LC-MS/MS. Then 2 µL peptide injection volume was loaded via a direct inject column configuration onto an Easy-nano LC 1200 instrument (Thermo Fisher Scientific) coupled online to a Q-Exactive Plus (Thermo Fisher Scientific). The resuspended peptides were first separated on Thermo Acclaim PepMap RSLC nanoViper C18 column (50 µm×15 cm) using solvent A (0.1% formic acid in water) and solvent B (80% acetonitrile in water containing 0.1% formic acid) as mobile phases for gradient elution at an auto-injector temperature of 30°C and a flow rate of 300 nL/min for 120 min [22]. Peptide elution was initiated with sustaining 6% solvent B (80% acetonitrile with 0.1% formic acid) for minutes 0-5, ramping solvent B 6-28% for minutes 5-105, ramping solvent B 28-38% for minutes 105-110, ramping solvent B 38-100% for minutes 110-115, and maintaining solvent B at 100% for minutes 115-120. The eluted peptides were introduced to the mass spectrometer and a full MS scan was acquired in the m/z range of 350-1,800 with a mass resolution of 70,000 at m/z 200 in the positive ion mode. The top 10 most intense peaks with a charge state ≥2 were fragmented with normalized collision energy 27 and isolation window 2 m/z. Tandem mass spectra were generated with a mass resolution of 17,500 at m/z 200. The maximum ion accumulation times were 50 ms and 45 ms for the survey scan and the MS/MS scans, respectively.

**Protein identification and quantitative analysis**

The LC-MS/MS data were analyzed using Andromeda and MaxQuant (v 1.3.0.5) for peptide identification and quantification [23, 24]. The UniProt human isofrom protein database was downloaded in August 2017, and contained 159,615 entries at this time. For the searches, the first search and mass tolerance of 20 ppm and two max missed cleavages were allowed. Fixed modification was set to carboxymethyl (C), while variable modifications were oxidation (M) and acetylation (protein N-terminal). A false discovery rate (FDR) of 0.01 was set to filter the results at the peptide and protein levels. Matching and alignment time window in match between run options were set to 0.7 and 2 minutes, respectively. Furthermore, only unique proteins identified with more than unique razor peptides were considered [24]. Finally, a label-free quantification (LFQ) approach was carried out for normalization of protein intensities across runs with a minimum of one ratio count [23]. The LFQ measuring intensities were assigned to the relative abundance for each protein and used in the subsequent statistical analysis.

**Bioinformatics analysis**

For the statistical analysis, p-values were calculated by Student’s t-test in R 3.5.0 under the null hypothesis to identify differentially expressed proteins between the two groups. At least two non-null values of data in each group were required for calculation of fold change and p-values. Proteins with expression changes with a p-value < 0.05 and |log2fold change| > 1 were selected as significant candidates. In addition, we took into account a comparison of all missing values in one group with expression values in another group. Proteins that were expressed in one group and not expressed in another group were considered to have a significant difference between the two groups.

For the functional annotation of identified proteins, the Gene Ontology (GO) and UniProt databases were used. GO enrichment analyses of differentially expressed proteins were performed with Fisher’s exact test. An adjusted p-value <0.05 was chosen as the threshold for significance. A heat map of hierarchical clustering of expression proteins was generated using the web tool ClustVis ([https://biit.cs.ut.ee/clustvis/](https://biit.cs.ut.ee/clustvis/)) [25]. Protein-protein interaction (PPI) networks were conducted using the STITCH 5 database [26, 27].
diagnosed by TCM to have wind-fire toxin including 4 snakebites by vipers (B) and 3 snakebites by cobras (C), and 7 were diagnosed by TCM to have fire toxin including 4 snakebites by *Agkistrodon acutus* (D) and 3 snakebites by *Trimeresurus stejnegeri* (E). The fifteen healthy controls were pooled into three samples as described in the methods. After preprocessing LC-MS/MS runs, the peptide intensities were obtained in MaxQuant analysis (Additional file 1). A total of 735 proteins with one or more unique peptides were quantified by LC-MS/MS analysis (Additional file 2). The representative proteins for each group were comprised of 471, 514, and 680 proteins in healthy control, wind-fire toxin, and fire toxin groups, respectively. When we filtered the results to satisfy the condition of getting at least two non-null intensities in replicates, we identified 474 unique proteins between patients with snakebites and healthy control.

We initially examined whether the serum proteins could discriminate between the two snakebite groups and the healthy control group. We identified 474 proteins in both patient groups, constituting 64.6% of the total proteins identified (Figure 1A). Based on the serum protein expression profiles, the three groups were differentiable by principal component analysis (PCA) (Figure 1B). We observed that the healthy control group was clearly separated from the bulk of snakebite patients at the first principal component (PC1), accounting for 64.8% of the observed variation. In contrast, the PC2 discriminated 35.2% of variability between the wind-fire toxin and fire toxin groups (Figure 1B). We next evaluated the hierarchical clustering, represented as a heat map of the relative protein abundance in each protein group. In the dendrogram, the clustering exhibited a similar expression trend across the wind-fire toxin and fire toxin groups, which appeared distinct from the protein expression patterns in the healthy control group (Figure 1C). This interpretation was consistent with the PCA results. Therefore, we conclude that the three main groups were clearly differentiable by their serum protein expression profiles.

**Differential abundance of proteins among the snakebite subgroups and the healthy control group**

To investigate the differential expression of proteins in the snakebite groups and the healthy control group, four subgroups on the basis of snake species were individually analyzed to identify differential abundance proteins (DAPs) as compared with those in the healthy control group. The distributions of upregulation and downregulation of 93 DAPs in the four snakebite subgroups are shown in Additional file 3. Collectively, 38 proteins were expressed in groups representing two snake species and selected as significant candidates with a *p*-value < 0.05 and |log2 fold change| > 1. Of these DAPs, 14 proteins were upregulated and 28 were downregulated relative to the peptide intensities of the healthy control group in Additional file 3.

Further comparisons between the four snakebite subgroups were detailed in a heat map (Figure 3). Unsupervised hierarchical clustering revealed different protein expression profiles among the four subgroups of snake species. However, no strong clustering was observed to separate wind-fire toxin and fire toxin (Figures 3A and 3B). Given the low overlap and lack of strong clustering of proteins between the four snakebite subgroups, great care should be taken in defining biomarker discovery for snakebites.

**Functional categorization of proteins identified in the serum**

To identify potential molecular processes associated with serum proteins that are characteristic of snake venom-induced toxicity, all 474 proteins were categorized by their Gene Ontology (GO)
terms. The GO annotations identified 395 proteins that were assigned to GO terms. GO analysis revealed that the biologic processes included defense/immunity response, cell motility, wounding healing, vesicle-mediated transport, lipid and gas transport, coagulation, protein metabolic process, and more (Additional file 4). The DAPs were categorized into fewer biological processes, including bicarbonate/oxygen transport and hydrogen peroxide catabolic process (Figure 4). The molecular function classes included carbon-oxygen lyase activity and heme binding.
The interaction network among proteins and chemicals

To understand the complex pathogenesis induced by snake venoms, the network of interacting proteins and drug compounds was constructed from the STITCH database [26]. Both chemicals and proteins were taken as nodes, and the linkage was taken as edges assigned weight scores to measure the strength of the association. In the constructed network of DAPs, we found one candidate compound, hydrogen peroxide, was highly associated with many DAPs (Figure 5). Forty-one proteins out of the 93 DAPs were matched with annotation in STITCH. In Additional file 5, 30 proteins of the DAPs showed complex interactions with 9 other proteins from STITCH, including BAG1, CFTR, DNAJB1, HBA1, HSP90AA1, IGFBP2, MAP3K5, STUB1, and UBC. One protein cluster consists of several apolipo proteins, such as APOC2, APOC3, APOC4, APOD, and APOF, which are associated with lipid metabolism and transport. Another cluster, including IGF2, IGFBP2, IGFBP3, IGFBP7, and HRG, is highly related to IGF-mediated growth. The third cluster, including HBA1, HBB, HBD, and SLC4A1, was associated with oxygen transport. The largest cluster was the ubiquitin mediated network, associated with innate immunity, protein degradation, and autophagy to eliminate toxic protein aggregates.

Discussion

Snakebites from highly venomous species can be fatal [4, 28]. It is often difficult to find the appropriate antivenom for optimal clinical management, due to the misidentification of the snake species. Previously, there was a lack of careful studies investigating the protein profiles of the serum from snakebite patients. Thus, we used label-free protein expression profiling to identify potential protein biomarkers in the serum of patients bitten by four different snake species. We found that one candidate compound, hydrogen peroxide, was highly associated with many proteins in the interaction network of proteins and drug compounds.
Figure 5. The network of protein and drug compound interactions. Thirty DAPs were found to be directly or indirectly associated with hydrogen peroxide. Colored nodes represent query DAPs, and small nodes represent those with an unknown 3D structure. Large nodes represent proteins with a known or predicted 3D structure. The weight of edges indicates the confidence score, wherein a thicker line indicates stronger association. Protein-protein interactions are represented by a gray edge, while chemical-protein interactions are depicted in green.
The label-free method is an effective technique for the simultaneous identification and quantification of thousands of proteins, providing potential biomarkers in a highly focused pool [29, 30]. In this study, we quantified 735 non-redundant proteins in serum by LC-MS/MS analysis. Due to interpersonal variability, the expression proteins were qualified to satisfy the condition of at least two non-null intensities in replicates of each subgroup. This resulted in 474 unique proteins that showed high reproducibility between snakebite patients and the healthy control group. The distinct reduction in the number of shared proteins may result from biased estimates in the clinic in identifying similar snakes [31]. However, the wind-fire toxin patients that were bitten by vipers and cobras are distinguished from the fire toxin patients bitten by *Agkistrodon acutus* and *Trimeresurus stejnegeri* in TCM. Based on serum protein expression profiles, we observed a significant distinction in PCA and hierarchical clustering between the wind-fire toxin and fire toxin groups (Figure 1B and 1C). We observed two obvious clusters of significantly upregulated proteins, presented in Figure 1C. Therefore, exploring differences in the serum protein expression profile is effective to distinguish snakebites, as well as for biomarker and antivenom discovery.

Discovering the pathways the identified serum proteins are associated with offers an opportunity for biomarker discovery. We found that the venom-induced toxicity impacted defense/immunity response, cell motility, wound healing, and especially oxidative stress response. DAPs were associated with the hydrogen peroxide catabolic process, carbon-oxygen lyase activity, and heme binding. Previous studies have demonstrated that venom-induced toxicity elevates reactive oxygen species (ROS) and hydrogen peroxide levels, implying potential cytotoxicity of venom [11, 32]. The compound hydrogen peroxide is an important component of ROS, which regulates many cellular functions in limited concentrations, but can also cause damage of cellular organelles, DNA, and proteins when present in excessive concentrations, an effect known as oxidative stress [33]. The interaction network of proteins and drug compounds showed that hydrogen peroxide was highly associated with many DAPs (Figure 5). These interacting DAPs were grouped into four clusters, including lipid metabolism and transport, IGF-mediated growth, oxygen transport, and innate immunity. The venom-induced toxicity activates various oxidases and respiratory burst due to inflammation [34]. Phospholipases A2 (PLA2s) are abundant components of venom in many snake species, and have the effect of blocking neuromuscular transmission and inducing acute muscle damage [35]. However, the physiological roles of PLA2s remain unknown. We discovered that a large cluster in the protein interaction network is concentrated in the innate ubiquitin-proteasome. Protein degradation may potentially be the cellular targets of PLA2s. The molecular composition of venom and serum from snakebite patients may be the most direct and credible way to evaluate the molecular mechanisms underlying the venom-induced toxicity process.

Comparison of DAPs in snakebites from different snake species identifies potential biomarkers and provides theoretical support for TCM on the molecular level. A few proteins participating in the networks were upregulated in the serum of the fire toxin group as compared to the wind-fire toxin group, such as TXN (Thioredoxin), CETP (Cholesteryl ester transfer protein), IGFBP7 (Insulin-like growth factor binding protein 7), MPO (Myeloperoxidase), and SLC4A1 (Solute carrier family 4). One protein, YWHAG (Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein), was only upregulated in the B subgroup (vipers), while one protein IGF-2 (Insulin-like growth factor 2) was only upregulated in the C subgroup (cobras). Two proteins, FabP6 (Fatty acid binding protein 6) and B3GNT1 (Beta-1,3-N-acetylglucosaminyltransferase 1), were only upregulated in the D subgroup (*Agkistrodon acutus*), while four proteins, HspA8 (Heat shock 70kDa protein 8), CKM (Creatine kinase), SEMA4 (semaphorin 4B), and SAA1 (serum amyloid A1) were only unexpressed in the E subgroup (*Trimeresurus stejnegeri*). These potential protein biomarkers well illustrated in toxin classification of TCM theory, and provided more biomarkers for identification of snakebite by different snake species. Importantly, the combined DAPs from four snake species suggested that they were mainly involved in the interaction network of proteins and hydrogen peroxide. Envenomation has been shown to induce redox status imbalance in a few snakes [36]. Some of these upregulated proteins, such as TXN, CETP, IGFBP7, and IGF-2, have been found to coincide with an increase of reactive oxygen species, which could act as antioxidants by reducing glutathione ratio (GSSG/GSH) [37–40]. Thus, the DAPs associated with oxidative stress and antioxidant action could be regarded as prior consideration for experimental validation in the future.

**Conclusion**

Five proteins (TXN, CETP, IGFBP7, MPO, and SLC4A1) were potential biomarkers to distinguish fire toxin and wind-fire toxin in classification of TCM theory, and a few proteins could identify snakebite of different snake species. These potential protein biomarkers were connected into the interaction network of proteins and hydrogen peroxide, which indicated that the venom-induced toxicity of snakebites was associated with oxidative stress and antioxidant defense. In the future, these potential biomarkers will need to be screened in a larger cohort of patients for clinical application.

**Availability of data and materials**

The original peptide intensities obtained in LC-MS/MS are in Additional file 1. The serum protein quantifications are available in Additional file 2.
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Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
WW conceived this research and designed experiments. DD, ZD and ZY wrote the manuscript. WM, JY and MS contributed to proteomics analysis. QL, JC, QC and LL contributed to interpretation of results. XW and XH participated in the discussion. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The experimental procedures were approved by the Ethics Committee of the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine (n. JZFYLL2017103012). The informed consent forms were obtained from all recruited participants (14 snakebite patients and 15 healthy controls) in the surgical department of Traditional Chinese Medicine of the Affiliated Hospital.

Consent for publication
Not applicable.

Supplementary material
The following online material is available for this article:

Additional file 1. Peptide identification reported using MaxQuant analysis.

Additional file 2. Protein quantification with LFQ intensity profiles obtained after normalization by the MaxLFQ algorithm.

Additional file 3. The distribution of 93 differential abundance proteins (DAPs) among each snakebite subgroup and the healthy control group. Upregulation and downregulation of 38 DAPs are statistically significant in expression. Expression and no expression of 55 proteins represent only detection in one subgroup. The total number is larger than the number of DAPs because of the overlap of DAPs in the four snakebite subgroups as compared to the healthy control group. The columns “B vs. A”, “C vs. A”, “D vs. A”, and “E vs. A” indicated the snakebite subgroup comparison with the healthy control group.

Additional file 4. Gene Ontology (GO) analysis of all 474 identified proteins revealed the following significantly enriched terms in biological processes: defense/immunity response, cell motility, wounding healing, vesicle-mediated transport, lipid and gas transport, coagulation, protein metabolic process, and others.

Additional file 5. List of the differential abundance proteins (DAPs) participating in the network of protein and drug compound interactions.

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