Identifying quality-markers from Shengmai San protects against transgenic mouse model of Alzheimer's disease using chinmedomics approach

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Abstract

Background: Shengmai San (SMS), a Chinese classic herbal formula, has been widely used for the treatment of Qi-Yin deficiency syndrome in Asia. Modern pharmacological studies have shown that SMS improves the cognitive function. However, the quality markers (Q-markers) for SMS still need further research.

Purpose: Using chinmedomics strategy to systematically evaluate the efficacy of SMS in the treatment of APPswe/PS1dE9 (APP/PS1) transgenic model of Alzheimer's disease (AD) and to discover the efficacy-related Q-markers.

Methods: The effect of SMS on APP/PS1 mice was evaluated by behavioral test, immunohistochemistry and urine metabolic profile, and the urine marker metabolites associated with SMS treatment of AD were characterized using metabolomics method. In the premise of efficacy, Serum Pharmacochemistry of Traditional Chinese Medicine was applied to investigate the in vivo constituents of SMS. A correlation analysis between marker metabolites of therapeutic effects and serum constituents was completed by chinmedomics approach.

Results: SMS had a therapeutic effect on APP/PS1 mice, and 34 potential urine biomarkers were reversed by SMS treatment. A total of 17 in vivo constituents were detected, including 14 prototype components and 3 metabolites. The correlation analysis showed that eight constituents were extremely correlated with protective effects of SMS in AD, and considered as potential Q-markers of SMS, including schisandrin, isoschisandrin, angeloxygomin M2, ginsenoside Rg3.

Conclusion: This study has demonstrated that chinmedomics is novel strategy for discovering the potential effective constituents from herbal formula, which are recognized as Q-markers.

Keywords:
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Efficacy
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Introduction

Traditional Chinese medicine (TCM) has been applied in clinical for thousands of years in Asia, with multi-component and multi-target characteristics (Zhang et al., 2016). Elucidation of the efficacy of TCM is important to understand the scientific value of TCM, and promote the innovation and development of TCM (Qiu et al., 2014; Zhang et al., 2014). However, due to complexity of formula and vagueness of syndrome or disease, it is difficult to understand its efficacy (Wang et al., 2016). Moreover, there is a challenge in selecting appropriate components for the purpose of quality control. Previously, those components at relatively higher concentrations are preferentially selected as the quality markers (Q-markers), rather than those with better efficacy (Liu et al., 2017). Hence, it is necessary to establish an efficacy evaluation and quality control system of TCM in an objective and systematic way.

Chinmedomics, a newer theory and methodology proposed by our team (Wang et al., 2012), is organic integration of serum pharmacology of TCM and “Omics” technology. In brief, using metabolomics technology to clarify the biomarkers of syndrome, take the biomarkers as targets to evaluated the efficacy of TCM formulation, under the condition of effective treatment to identify the active form of
constituents in vivo originated from formulation, and further analyzing the correlation between the exogenous constituents in vivo and endogenous biomarkers to discover the constituents which are highly associated with formulation efficacy as the active constituents (Zhang et al., 2015). This strategy has been successfully applied to research on TCM, such as Wen-Xin formulation, AS1350, and Kai-Xin-San (Liu et al., 2016; Cao et al., 2014; Chu et al., 2016; Wang et al., 2017).

Alzheimer’s disease (AD) is a neurodegenerative disease which is difficult to recover after onset, characterized by accumulation of Aβ into senile plaques and hyperphosphorylated tau into neurofibrillary tangles (Spires-Jones et al., 2014). APP/PS1 double-transgenic mouse model, with mutations in the genes for amyloid precursor protein (APP) and presenilins 1 (PS1), are preferable for experimental use because of the early appearance of AD-like pathology and cognitive decline (Bonardi et al., 2011). Shengmai-San (SMS), a traditional Chinese herbal formula, is composed of Radix Ginseng, Radix Ophiopogonis, and Fructus Schisandrae. It has long been used for the treatment of Qi-Yin deficiency. The chemical components of SMS were identified mainly including ginsenosides, lignoids, steroid saponins and homoiso-flavanones (Wu et al., 2011; Yu et al., 2017). It has been reported to have a protective effect on the brain by reducing inflammatory cytokines and nitric oxide formation (Wang et al., 2005a,b). Experiments have shown that it alleviates amyloid beta (Aβ)-induced cytotoxicity of PC-12 cell (Nishida et al., 2007). However, little is known about the effect of SMS on AD transgenic mice model and the bioactive constituents in SMS. Therefore, in this study, we attempted to explore the effects of SMS on APPsw/PS1dE9 mice, and identify the effective components as Q-markers using chinmedomics approach.

Material and methods

Drugs and reagents

SMS is composed of Radix Ginseng, Radix Ophiopogonis, and Fructus Schisandrae. These three crude drugs were purchased from the Harbin Tongrentang Drug Store, and authenticated by Professor Xijun Wang of the Department of Pharmacognosy of Heilongjiang University of Chinese Medicine (Harbin, China). The reference standards: schisandrin, schizandrin A and schizandrin B were of HPLC grade (≥ 98%) were purchased from Sichuan Wikeqi Bio-Technology Co. Ltd. Anti-beta Amyloid 1-42 antibody was obtained from Abcam (Abcam Plc., Cambridge, UK). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Deionized water was purified by the Milli-Q system (Millipore, Bedford, USA). Leucine-enkephalin and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). As our previous work, the SMS sample was prepared by water extraction (Wu et al., 2011). The aqueous extract was lyophilized. The freeze-dried powder of SMS was added distilled water to the appropriate amount.

Animals and treatment

2-month old male APPsw/PS1dE9 transgenic mice (APP/PS1, J004462) and littermate wide-type controls (WT, J004462) were obtained from the Animal Research Centre of Nanjing University. All animals were kept in a controlled environment, with a 12:12 h light schedule, temperature (23 °C ± 1 °C), and relative humidity (55 °C ± 5 °C). The mice had free access to food and water. Twenty-four male APP/PS1 mice were randomly assigned to receive systemic administration of either SMS (4.0 mg/kg) or vehicle (equivalent volume of distilled water), for 12 mice in the APP/PS1 + SMS group and 12 mice in the APP/PS1 group, and 12 male wild-type littermates, the WT group, were administrated vehicle (equivalent volume of distilled water). The mice began to receive SMS treatment at 3 months of age, and the treatment was continued until they reached 10 months of age, with the SMS administration lasting for 8 months.

Preparation of urine and plasma samples

The 12 h night urine samples were collected at the end of dosing period. The urine samples were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was diluted 8-fold with water, then screened with 0.22 µm filter membrane for UPLC-Q-TOF-MS analysis. The next day, the blood and brain tissue were collected after 1 h of administration. The brain tissue samples were immersed in 10% neutral paraformaldehyde for immunohistochemical staining. For plasma samples, after centrifugation at 4000 rpm for 15 min at 4 °C, the supernatant was separated out. The samples were stored at –80 °C until analysis. 4 µl of phosphoric acid was added into 200 µl plasma sample, and then vortexed for 30 s. The mixed solution was applied to preactivated OASIS HLB SPE columns (Waters, USA). Before that, the column was successively washed with 1 ml of methanol and water, respectively. Then, 1 ml water was eluted, and the eluent was discarded, then eluted by 100% methanol, collected the eluent and dried under N2 at room temperature. 200 acetonitrile was used to redissolve the residues, vortexed for 60 s and sonicated for 60 s. The mixed solution was centrifuged at 13,000 rpm for 15 min at 4 °C. All samples were filtered through the 0.22 µm filter membrane before analysis, and the injection volume was 3 µl.

Morris water maze test

Spatial learning and memory function of mice were assessed using Morris water maze test (Morris, 1984). The system consisted of a circular water tank (120 cm diameter × 50 cm high). The water temperature was maintained at 22 ± 1 °C. Mice received place navigation test for 5 consecutive days and probe trial on Day 6. For the place navigation test, a transparent platform (10 cm in diameter) was located in the Northeast quadrant and submerged 1–2 cm below the water surface. Mice were trained once in each quadrant into the water per day. In each trial, they were given 120 s to find a hidden platform, and were terminated when the mice climbed onto the platform or after 120 s. Each one was allowed to remain on the platform for 20 s. In the probe test on the sixth day, the platform removed, and the mice were allowed to swim freely for 120 s. The time required for finding the hidden platform (escape latency) and the percent time spent in target quadrant were recorded and analyzed using Ethovision XT 11.5 (Noldus, Wageningen, Netherlands).

Immunohistochemistry

Sections were first incubated with 3% H2O2 in 0.01 mol/l PBS for 30 min at room temperature to quench endogenous peroxidase activity, heated for 20 min in 10 mM citrate buffer (PH 6.0) for epitope retrieval, and then incubated overnight at 4 °C with rabbit mAb anti-Aβ1-42 (1:1000; Abcam, London, UK). After rinsing, sections were incubated with biotinylated secondary antibody for 30 min at 37 °C, then processed using 3,3′-diaminobenzidine (DAB) and hematoxylin (Peking, China).

Metabolomics analysis condition

Chromatography was performed on an ACQUITY™ UPLC system (Waters, Milford, USA) with quaternary pump, vacuum degasser, autosampler, and diodearray detector. The separation was carried out on an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm i.d., 1.8 µm; Waters, Milford, USA). The column temperature was maintained at 40 °C and the flow rate was 0.4 ml/min. The analysis was performed with gradient elution using (A) acetonitrile with 0.1% formic acid and (B) water with 0.1% formic acid as the mobile phase. The gradient elution condition was: 0–4 min, 1–20% A; 4–7.5 min, 20–40%; 7.5–8.5 min, 40–99%; 8.5–10.5 min, 99% A isocratic. The Mass spectrometry system was quadrupole-time-of-flight mass spectrometer.
(SYNAP G2-Si HDMS, Water, Milford, USA) equipped with an ESI ion source that operates in positive ionization mode (ESI+®) and negative ionization mode (ESI−®). In the ESI+, the capillary voltage 3000 V, sampling cone voltage 40 V, source temperature 110 °C, desolvation temperature 350 °C, cone gas flow 50 l/h, and desolvation gas flow 600 l/h. In the ESI−, capillary voltage 2200 V, sampling cone voltage 40 V, source temperature 110 °C, desolvation temperature 350 °C, cone gas flow 50 l/h, and desolvation gas flow 600 l/h. All data was acquired in centroid mode from 50 to 1000 m/z. For accurate mass acquisition, a lock-mass of leucineenkephalin at 0.2 ng/ml under a flow rate of 15 μl/min via a lockspray interface, for positive mode [M + H]+ = 556.2771 and negative mode [M − H]− = 554.2615.

Constituent analysis condition

The instruments and column used for chromatography separation and mass spectrometric detection were the same as those in metabolomics analysis methods. The column temperature was maintained at 45 °C and the flow rate was 0.5 ml/min. The analysis was performed with gradient elution using (A) acetonitrile with 0.1% formic acid and (B) water with 0.1% formic acid as the mobile phase. The gradient elution condition was: 0–2.5 min, 1–20% A; 2.5–5.5 min, 20–32%; 5.5–8.5 min, 32–43%; 8.5–12.5 min, 43–55%; 12.5–20 min, 55–99%; 20–22 min, 99% A isocratic. The mass spectrum parameters were as follows: the capillary voltage 3000 V, sampling cone voltage 40 V, source temperature 110 °C, desolvation temperature 350 °C, cone gas flow 50 l/h, and desolvation gas flow 650 l/h in the ESI+; capillary voltage 2500 V, sampling cone voltage 40 V, source temperature 110 °C, desolvation temperature 350 °C, cone gas flow 50 l/h, and desolvation gas flow 650 l/h in the ESI−. All data was acquired in MS6 continuum mode from 50 to 1200 m/z. Lower collision energy and higher collision energy were 6 V and 15–40 V, respectively. For accurate mass acquisition, a lock-mass of leucineenkephalin at 0.2 ng/ml under a flow rate of 15 μl/min via a lockspray interface, for positive mode [M + H]+ = 556.2771 and negative mode [M − H]− = 554.2615.

Correlation analysis of marker metabolites and serum constituents

A plotting of correlation between marker metabolites and serum constituents (PCMS) was used to correlate the constituents absorbed into blood after oral administration of SMS and the urine biomarkers of therapeutic effects. Datasets of the two variables were imported into PCMS software. The correlation coefficient γ was set to 0.8 and the correlation coefficient γ2 was set to 0.9. Then, a heatmap of the correlation analysis results was calculated. In this experiment, we selected |γ| ≥ 0.9 as the standard for screening the constituents contributed most to the protective effects of SMS in AD.

Results and discussion

SMS improves the cognition impairment of APP/PS1 mice

To determine the effects of SMS on cognition functions of mice, after 8 months of drug administration, APP/PS1 mice and WT mice were evaluated in Morris water maze test. Among them, the navigation tests were performed to assess the spatial learning function of mice. As showed in Fig. 1A, the latency of each group was demonstrated to decrease over time. As expected, compared with WT mice, the latencies of the APP/PS1 mice was significantly increased on each day (F = 0.02, P < 0.05, repeated measures ANOVA). Notably, compared with APP/PS1 mice, APP/PS1 + SMS mice were remarkably shorter (F = 0.03, P < 0.05, repeated measures ANOVA), relative to WT mice. In addition, the probe tests were conducted to assess the spatial memory function of mice on day 6. As shown in Fig. 1B, APP/PS1 mice got shorter percent time spent in target quadrant than WT mice (F = 0.04, P < 0.05, one-way ANOVA). Specially, the percent time spent in target quadrant was increased in APP/PS1 mice treated with SMS than APP/PS1 mice (F = 0.04, P < 0.05, one-way ANOVA), which was approximate to the outcomes of WT mice. These results indicated that SMS significantly improved the spatial learning and memory deficits of APP/PS1 mice.

SMS reduced Aβ plaques in APP/PS1 mice

Immunohistochemical staining for Aβ1-42 in the brain was used to investigate the effect of SMS on neuropathological changes in APP/PS1 mice. Anti-Aβ1-42 antibody immunostaining showed that WT group had no Aβ1-42 deposition in the cerebral hippocampus and cortex, while the APP/PS1 mice showed a large number of Aβ1-42 positive plaque (Fig. 2A). The percentage of the area occupied by Aβ plaque of the APP/PS1 + SMS mice was lower than that in the APP/PS1 mice (F = 0.047, P < 0.05, one-way ANOVA) (Fig. 2B). The Aβ1-42 positive plaque number of APP/PS1 + SMS mice was lower than that in APP/PS1 mice (F = 0.017, P < 0.05, one-way ANOVA) (Fig. 2C). These results indicated SMS may play a role in inhibiting Aβ1-42 production.

Protective effects of SMS on APP/PS1 mice using metabolomics analysis

The urine UPLC-MS raw data were imported into Progenesis QI for data preprocessing, including peak alignment, data normalization, grouping, and peak picking. Then, the preprocessed datasets were further subjected to multivariate statistical analysis using EZInfo 2.0 software. The principal component analysis (PCA) score plot showed an obvious separation between the WT mice and APP/PS1 mice (Fig. 3A and B), suggesting that the endogenous metabolites were disturbed by pathological changes caused by APP and PS1 genotype in APP/PS1 mice. The OPLS-DA was employed to find the potential biomarkers (Fig. 3C and D). The R2Y and Q2 values of the model (R2Y (cum) = 0.918159, Q2 (cum) = 0.780247 in positive mode and R2Y (cum) = 0.974468, Q2 (cum) = 0.786354) indicated that the models have good quality and predictability.

Features with importance in the projection (VIP) > 1 from OPLS-DA and P-value < 0.05 were selected for further identification as the potential biomarkers (Fig. 3E and F). The exact molecular mass was measured using high resolution Q-Tof mass spectrometry platform and the elementary composition of the compound was calculated. Then, the database in the Progenesis QI software was used to screen the compounds. Finally, the MS/MS spectrum of metabolites was compared to its standard reference in HMDB database or related literatures to ultimately determine the metabolite. According to the above procedure, 41 differentiating metabolites between APP/PS1 mice and WT mice were tentatively identified (Table S1), and the relative intensity of each metabolite was shown in Fig. 4.

To determine whether SMS could influence the metabolic pattern induced by AD, the PCA score plot from the metabolic profiling of WT mice, APP/PS1 mice and APP/PS1 + SMS mice was shown in Fig. 5B. From the PCA score plot, APP/PS1 + SMS mice were relatively close to the WT mice while away from APP/PS1 mice. These results suggested that SMS could restore the pathological process of AD on a global metabolite level. Furthermore, the relative intensities of the differential metabolites were compared among the WT, APP/PS1 and APP/PS1 + SMS mice. Of note, 34 potential biomarkers were reversed to varying degrees by SMS (Fig. 5A and Table S1). These regulated metabolites were involved in nicotinate and nicotinamide metabolism, tryptophan metabolism, catecholamine biosynthesis, vitamin B6 metabolism, phenylalanine and tyrosine metabolism, oxidation of branched chain fatty acids, beta oxidation of very long chain fatty acids, tyrosine metabolism, protein biosynthesis, purine metabolism and citric acid cycle.

In vitro and in vivo analysis of SMS using UNIFI™ software

UPLC-Q-TOF-MS combined with UNIFI™ software was used to
Fig. 1. SMS improves behavioral performance in APP/PS1 mice. The spatial learning function of mice was evaluated by escape latency in the navigation tests (A). Each data point indicates the mean ± SEM for 8 mice in each group. **P < 0.01 WT mice compared with APP/PS1 mice, and ###P < 0.01 APP/PS1 + SMS mice compared with APP/PS1 mice (repeated measures ANOVA). The spatial memory performance of mice was analyzed in the probe test by % time spent in the target quadrant (B). *P < 0.05 WT mice compared with APP/PS1 mice, and #P < 0.05 APP/PS1 + SMS mice compared with APP/PS1 mice. Each datum presents the mean ± SEM. (one-way ANOVA).

Fig. 2. SMS reduced Aβ1-42 plaques in APP/PS1 mice. Aβ1-42 immunohistochemical staining in brain hippocampus (×40) and cortex (×100) (A). (B) and (C) showed % area occupied by Aβ plaques and the number of plaque in each group, respectively. *P < 0.05 WT mice compared with APP/PS1 mice, and #P < 0.05 APP/PS1 + SMS mice compared with APP/PS1 mice. Each datum indicates the mean ± SEM. (one-way ANOVA).
accomplish rapid discovery and global characterization of multiple constituents from SMS. The UNIFI™ software can automatically complete chromatographic peak detection, molecular formula prediction, TCM database retrieval, MS/MS fragment matching, and preliminary chemical characterization (Ma et al., 2016). The analysis parameters are as follows: The adducts were \([M + K]^+ / [M + Na]^+ / [M + H]^+\) and \([M + COOH]^- / [M - H]^-\) in positive ion mode and negative ion mode, respectively; The target match tolerance is less than 10 ppm, while the fragment match tolerance is less than 5 mDa. As a result, a total of 73 compounds were identified at 20 min acquisition time. Among them, 20 compounds derived from *Radix Giseng*, 17 compounds derived from *Radix Ophiopogonis* and 36 compounds derived from *Fructus Schisandrae* (Table S2).

The strategy by integrating UPLC-Q-TOF-MS and UNIFI™ software was also used for screening the absorbed constituents and metabolites of SMS. All the data acquired by MS² mode, including SMS in *vitro* samples, control samples and dosed samples, were imported into UNIFI™ software (Fig. S1A). Then, the datasets were processed by using the metabolite identification-MSe method. Compounds were selected for identification which their response value in the dosed samples had to be 10 times greater than that of control samples (Fig S1B). Finally, a total of 17 compounds in *vivo* were tentatively identified of SMS by automatically matching the exact mass and MS/MS fragment of the compounds in the UNIFI database (Fig. 6C), among them 12 compounds were detected in ESI⁺ mode (Fig. 6A) and 5 compounds were detected in ESI⁻ mode (Fig. 6B). The 14 prototype components and 3 drug metabolites were also summarized in Table 1.

Discovering Q-markers by correlation analysis

To discover the Q-markers that actually play a role in the treatment of disease, a correlation analysis between marker metabolites and serum constituent of SMS was performed using PCMS technology. In this experiment, the correlation degree was defined according to the range of the correlation coefficient \(\gamma\): \(0.8 \leq |\gamma| < 0.9\) indicates high correlation and \(0.9 \leq |\gamma| \leq 1\) represents extremely high correlation. The results of correlation analysis showed that eight compounds were extremely correlated with protective effects of SMS in AD, including schisandrin, isoschisandrin, angeloylgomisin Q, gomisin D, angeloylgomisin H, gomisin M2, ginsenoside F1, 20(R)-ginsenosideRg3 (Fig. 7). 6 of which were derived from *Fructus Schisandrae*, and 2 of which were derived from *Radix Ginseng*. The abnormal levels of L-tyrosine and homovanillic acid sulfate have been observed in patients with Alzheimer’ disease (Soininen et al., 1981), and isoschisandrin were extremely correlated with them.

Schisandrin, isoschisandrin, angeloylgomisin Q, gomisin D, angeloylgomisin H and gomisin M2 belong to lignin. It has been showed that the lignan-rich of *Fructus Schisandrae*, which contains schizandrin, gomisin A and angeloylgomisinH, could improve insulin sensitivity (Kwon et al., 2011). Studies suggested that schisandrin ameliorates Aβ1-42-induced memory impairment through antioxidative action (Hu et al., 2012). Gomisin M2 was a prototype component of *Fructus Schisandrae* and was also a demethylation product of \(\gamma\)-schizandrin in vivo. \(\gamma\)-Schizandrin may inhibit the production of Aβ1-42 by inhibiting \(\gamma\)-secertase (Liu et al., 2006). Ginsenoside F1, 20(R)-ginsenoside Rg3 were components of ginsenoside. Ginsenoside Rg3 could play a role in
Fig. 4. The relative intensity of urine metabolites between the WT mice and APP/PS1 mice. *P < 0.05, **P < 0.01 WT mice compared with APP/PS1 mice. Blue represents WT mice, and red represents APP/PS1 mice.

Fig. 5. Protective effects of SMS in APP/PS1 mice based on metabolomics analysis. The relative intensity of urine metabolites associated with SMS treatment of AD (A). *P < 0.05, **P < 0.01 WT mice compared with APP/PS1 mice. #P < 0.05, ##P < 0.01 APP/PS1 + SMS mice compared with APP/PS1 mice. The PCA score plot for SMS treatment of AD (B). Blue represents WT mice; red represents APP/PS1 mice; green represents APP/PS1 + SMS mice.
antioxidative action, decreasing the levels of nitric oxide (Wei et al., 2012). Moreover, ginsenoside F1 may fight against oxidant damage of mitochondrial function (Wang et al., 2016). As we known, more and more attention has been applied to antioxidant drugs for the treatment of AD (Liu et al., 2017). Therefore, these eight compounds contributed most to the protective effect and may be identified as Q-markers of SMS.

Conclusion

In this work, the chinmedomics strategy was applied to assess the efficacy of SMS against APP/PS1 mice, and to discover the potential quality-markers of SMS. The results of correlation analysis between 33 urine marker metabolites of therapeutic effects and 17 serum constituents showed that schisandrin, isoschisandrin, angeloylgomisin Q, gomisin D, angeloylgomisin H, gomisin M2, ginsenoside F1 and 20(R)-ginsenoside Rg3 were extremely correlated with the effect of SMS on...
AD. Eight compounds may be considered as potential Q-markers of SMS, which was also required for further validation. It also indicated that chimedonomics may be an effective strategy to discovering the Q-markers and play an important role in quality control of TCM.

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Conflict of interest

The authors declare no competing financial interests.

Supplementary materials

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