Proteomics and Traditional Medicine: New Aspect in Explanation of Temperaments

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Introduction

Traditional Iranian Medicine (TIM) is one of the oldest sources of medical science and a fusion of Greek, Egyptian, Indian, and Chinese doctrines. TIM, Ayurveda, and Traditional Chinese Medicine (TCM) are based on humorism as common theory [1–3]. This theory divides the human body fluids into 4 humors, each corresponding with the traditional 4 temperaments (yielding 9 categories [4–7]; fig. 1).

The foundation of TIM is based on natural philosophy. Semiology, experience-based treatments, and new therapeutic insights are the characteristics of this medicine. Disease prevention and health are built upon nutrition, weather, sleeping and awakening, motion and stasis, purification, and spiritual/mental states [5]. Patient treatment also involves approaches in nutrition and lifestyle changes and use of medications and manual therapy [1, 3, 4, 8]. All these efforts facilitate the way to move toward personalized medicine.

Some earlier studies have investigated the molecular and hormonal evidence that enables distinction of humors and temperaments in humans. To date, differences in levels of cytokines, interleukin-4, interferon-gamma, norepinephrine, epinephrine, cortisol, and types of antibodies have been reported [9, 10]. With the development of molecular approaches (high-throughput biotechniques) in the field of CAM, other molecular differences have also been described, such as the G-protein signaling pathway, T-cell proliferation, and oxidation/reduction in fatty acid metabolism [11–14].

According to traditional medical texts, it seems that the temperaments are consistent with basal metabolism [1, 4, 15]. Therefore, it would be plausible to track these phenotypical divisions in molecular markers associated with metabolism (e.g. in mitochondria organelles). Also, it should be noted that all biological processes could be followed up on protein levels [16]. Our study is aiming at distinguishing temperaments by mitochondrial proteins using the organelle proteomic approach.
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Fig. 1. Temperaments and humors. This schematic plot represents 9 temperaments (absolute number and combinations of 4 temperaments) and 4 traditional humors in TIM. These categories are generally or partially common in TIM, Ayurveda, and TCM.

Material and Methods

Materials

Materials and methods used in this project are reported in a previously published study in detail [17]. All of the materials were provided by Sigma-Aldrich (St. Louis, MO, USA) including EDTA, SDS, TEMED, TCA, PMSF, CHAPS, DTT, bisacrylamide, acrylamide, etc. Protein ladder was prepared from fermentase (page ruler unstained ladder). The main instruments used in this study were sonicator (Hilscher Ultrasonics GmbH, Teltow, Germany), refrigerated centrifuge (Kendro Laboratory Products, Asheville, NC, USA; D37520), electrophoresis tools (Paya Panjooresh Pars. Co. Theran, Iran), speed vacuum (LaboGene ApS, Lynge, Denmark), and high performance liquid chromatography (HPLC; Agilent Technologies, Santa Clara, CA, USA; 1,200 series).

Sample Preparation Procedure

The complete procedure was described in detail by Pooreydy et al. [17]. In a nutshell, peripheral blood mononucleated cells (PBMC) were extracted from 5 ml of human blood by Ficoll. The mitochondria were isolated from these cells, and mitochondrial proteins were extracted. Detergents and salts were removed by buffer exchange methods; and proteins were solved in 50% acetonitrile and stored at –80°C.

Volunteers

Based on questionnaire and physical examination, volunteers (aged from 18 to 24 years) were classified in 2 temperaments: hot/wet (n =4) and cold/dry (n =6). To determine temperaments in TIM, the following 9 indicators were used: tooth; body features; hair features; background color of the body; sleep/wake pattern; waste material (feces, urine, and sweat); organ size; temperamental impact of warmth; coldness, dryness, and moisture on function and behavior, mental states, and mood.

Hydrophilic Interaction Liquid Chromatography

Due to the complexity of the mitochondrial proteome (approximately up to 2,000 proteins [18]), we considered a two-dimensional liquid chromatography mass spectrometry technique (gel-free proteomics). In order to have maximum orthogonality, we applied hydrophilic interaction liquid chromatography (HILIC) [19] in the first dimension in combination with reversed-phase liquid chromatography in the second dimension. For the first time in gel-free proteomics, we introduced silica-based HILIC as a proper fractionation method on protein levels. Then, a HILIC column (250 × 4.6 mm, silica, 5µm) was applied for the fractionation of proteins in each sample in 3 technical replications.

Then, we separated the samples on the column using 120-min long effectual linear gradient of a) methanol, b) water, and c) acetonitrile (ACN) in 0.05% trifluoroic acid (TFA) at flow rate of 1 ml/min. The gradient conditions were as follows: 95% A and 5% B for 10 min; 95–90% A and 5–10% B for 10 min; 90–75% A and 10–25% B in 10 min; 75–55% A and 25–45% B for 10 min; 55% A and 45% B in 10 min; 55–100% A and 45–0% B for 10 min; 100% A in 15 min; 100% C for 5 min; and 100% C in 20 min. Data was determined at a wavelength of 218 and 280 nm. This method was used as a first fractionation dimension in a gel-free proteomic study.

Data Analysis and Statistical Methods

We numbered all separated peaks (in first dimension chromatogram) manually, based on retention times. To study the distribution of data, we utilized the quantile-quantile (QQ) plot and pairwise scatter plot [20]. The pairwise correlations within groups and between groups were also calculated to assess the quality of grouping. Finally, we evaluated the differences between the 2 groups statistically by a non-parametric test (Mann-Whitney, P-value ≥ 0.05) [21, 22]. In the next step, the second dimension of chromatography (reversed phase, C18) was performed using tandem mass spectrometry (thermo LCQ ion trap).

Results and Discussion

Ten peaks with acceptable resolution and reproducibility of the chromatographic results were obtained. Interestingly, at the beginning of this study, we observed the typical differences in the first dimension of the fractionation profile between the 2 groups. As represented in figure 2a, chromatograms of cold/dry temperament show the 4 particular peaks of this temperament. Also, the chromatograms of hot/wet temperament with its 6 sequestered peaks (fig. 2b) are shown. In panel c (fig. 2c), the intensity of peaks in each group was compared statistically (by Mann-Whitney test).

Nine of these 10 peaks are differently expressed in both groups, of which 3 peaks (i.e. peak numbers 1, 3 and 9) are significantly overexpressed and 6 others (i.e. peak numbers 2, 4–8) are underexpressed in hot/dry temperament in comparison with cold/dry group. In fact, peak numbers 4, 5, 7, and 8 appeared in cold/dry temperament only and were not detected in hot/wet group.

The differences concerning the separation of the hydrophilic interaction mechanism [19, 23] strengthen the hypothesis of the impact of glycoside and phosphate groups (post-translational modification, PTM) on the protein samples in the cold/dry group. This kind of PTM directly influences protein activity/stability and consequently biological active process in cells. This might be also true for phenotypes of the diverse temperaments in healthy individuals, i.e. same proteins with various kinetic properties. It should be noted that each peak in the first dimension is separated into 30–40 identical
Fig. 2. Chromatograms and statistical analysis. Absorbance of samples in 2 wavelengths (red and black lines indicate absorbance at 280 and 218 nm, respectively) and 120 min; a) chromatograms of cold/dry temperament in 2 magnifications. The 10 peaks of this group are numbered individually; the red arrows indicate the 4 particular peaks in cold/dry temperaments; b) chromatograms of hot/wet temperament in 2 magnifications. The 6 peaks of this group are numbered individually; c) statistical significance of differences of these peaks (Mann-Whitney test); overexpressed peaks (high intensity) in both groups.

Fig. 3. Pairwise scatter plot and correlation: a) pairwise scatter plot and the correlation coefficients of the intra-group of cold/dry temperament; b) pairwise scatter plot and the correlation coefficients of the intra-group of hot/wet temperament; c) correlation coefficients of the inter-group of cold/dry and hot/wet temperaments.

Before statistical analysis, we used pairwise scatter plot and correlation analysis to corroborate inter- and intra-group homogeneity. The results showed that all intra-group correlation coefficients are higher than 0.7 in both groups, and that inter-group correlation coefficients are below 0.1 (fig. 3). Statistical analysis (Mann-Whitney test) confirms the significant difference (p-value is less than 0.05) between the groups with 9 peaks (fig. 2). The multivariate Mann-Whitney test was also performed for high-amount peaks. This test confirmed multivariate statistical differences with a p-value of < 10–6.
We would certainly be speaking about molecular (proteins) differences between temperaments after a complete fractionation and mass spectrometry analysis, that is important for the identification of biomarkers of these temperaments and is necessary to explain the mechanism of these differences [11, 24]. Further investigation by means of other high-throughput biotechniques, such as genomics and metabolomics, and comparison of other temperaments (fig. 1), can be an important step toward personalized CAM [13, 14].

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Disclosure Statement

The authors declare that there is no conflict of interests.

References