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Metabolomics for characterization of gender differences in patients infected with dengue virus

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ABSTRACT

Objective: To determine the metabolic response associate with dengue infection based on human gender metabolic differences by means of ^{1}H NMR-spectrometry.
Methods: The mid-stream urine collected from both male and female patients diagnosed with dengue fever at Penang General Hospital and fourty-three healthy individuals were analyzed with ^{1}H NMR spectroscopy, followed by chemometric multivariate analysis. NMR signals which highlighted in the OPLS-DA S-plot were further selected and identified using Human Metabolome Database, Chenomx Profiler.
Results: The results pointed out that NMR urine profiling was able to capture human gender metabolic differences that are important for the distinction of classes of individuals of similar physiological conditions; infected with dengue. Distinct differences between dengue infected patients versus healthy individuals and subtle differences in male versus female infected with dengue were found to be related to the metabolism of amino acid and tricarboxylic acid intermediates cycle.
Conclusions: The ^{1}H NMR metabolomic investigation combined with appropriate algorithms and pattern recognition procedures, gave an evidence for the existence of distinct metabolic differentiation of individuals, according to their gender, modulates with the infection risk.

1. Introduction

Gender differences are of interest from a variety of perspectives and have been the focus of a number of studies for many years. Females and males are also known to have different abilities for managing diseases. For instance, the pulmonary scientific community is exploring how gender may impact the diagnosis, treatment and surveillances of chronic obstructive pulmonary disease [1]. The impact of gender differences in the prevalence, manifestation and management of cardiovascular diseases have been widely studied [2]. In infectious diseases, sex-specific pattern were seen in cutaneous leishmaniasis, tuberculosis, leprosy, leptospirosis and schistosomiasis [3]. Taking such differences between male and female into account, it is possible to improve the understanding of the epidemiology and the clinical outcome of the diseases.

Most of the reported metabolite profiling studies of gender have relied on ^{1}H NMR spectroscopy. NMR based metabolite profiling has had success in characterizing gender differences due to its ability to measure close to hundreds metabolites in a single analysis of the biological sample [4]. Method of choice performed in characterizing gender differences in dengue-infected patients is a combination of ^{1}H NMR spectroscopy with spectral binning (0.04 ppm width) together with pattern recognition tools principle component analysis (PCA) and orthogonal partial least square-discriminant analysis (OPLS-DA). The *P*-values for the individual metabolites were determined based on the normalized metabolites quantities using the Shapiro–Wilk test analysis. Metabolites showing Mann Whitney U test *P*-values smaller than 0.05 were considered to be statistically different between the female and male groups (IBM SPSS Statistic 20).

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2. Materials and methods

2.1. Equipments

All one-dimensional ^{1}H NMR spectra of the urine samples was acquired on AVANCE III 500 MHz Bruker spectrometer with BBO broadband probe using TSP (δ 0.00 ppm) as an internal standard and D_2O as the frequency lock at 300 K. The pulse sequence used included an excitation sculpting routine for the suppression of the water signal [5].

2.2. Biological samples

Sample size is calculated according to the yearly report published by Rancangan Kawalan Penyakit Bawaan Vektor, Penang by using the Power and Sample Size Program (http://www.kck.usm.my/ppsg/stats_resources.htm).

Ninety-six patients were successfully collected from Penang General Hospital, Penang from February to July 2011. However only fifty-two patients were serologically confirmed infected with dengue fever, another forty-four patients were IgM negative indicating non-dengue fever. Fifty healthy volunteer individuals were self-identified as healthy without major illnesses, recruited from Pangsapuri Pauh Damai and Taman Pauh, Bukit Mertajam, Penang, Malaysia. However, after initial screening with principle component analysis and consultation of the urine samples revealed the presence of precipitation/cellular debris in seven samples, resulted only 43 samples of healthy individual were valid to be used for further analysis.

Inclusion criteria include all dengue-infected patients and healthy individual who had voluntarily agreed to participate in this study. While exclusion criteria include: menstruated girls; persons who already had or recently had any medical conditions; persons with a recent history of drug or alcohol abuse; pregnant women; persons who have taken any medication; persons who have the genetic or/and auto-immune disease background.

Fifty-two urine samples were collected from patients whom have been serologically confirmed with dengue infection and forty-three urine samples were collected from healthy individuals whom have self-identified as healthy without major illnesses and fulfill the listed inclusion and exclusion criteria. All the samples were collected in the urine container by the subjects. Subjects were asked to fill in the container until half of the container via mid-stream method. All the urine samples were collected in the morning from 09:00 to 11:00 am.

2.3. Chemicals

All chemicals used in this research were of analytical grade and purchased from Sigma–Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

2.4. Methods

Permission to perform this research has been approved by Medical Review and Ethics Committee, Ministry of Health Malaysia (Protocol No.: NMRR-10-297-5392). Signed informed consent was obtained from each volunteered patients and healthy individual after a full explanation of the study is provided. All data has been handled confidentially and anonymously.

2.5. Sample preparation

Urine samples were collected from both male and female in urine containers separately and kept in the cold box prior to be processed. Samples were centrifuged at 1 500 × *g* for ten minutes to remove any cellular debris. One milliliter of urine aliquots were transferred to 1.5 mL microtubes. The aliquots were stored −80 °C prior to NMR spectrometry [5].

To prepare samples for NMR, 540 μL aliquot of urine samples were added with 60 μL of 1.5 M potassium phosphate buffer (pH 7.4) in D_2O containing 0.1 percent of TSP as frequency reference prior to analysis and 2 mM sodium azide as bacteriostatic reagent. Six hundreds microliters of the mixture was transferred to five millimeter NMR tube prior to be analyzed by proton NMR [5].

2.6. NMR file processing

Proton NMR spectra of urine samples from the subjects were recorded followed by chemometric multivariate analysis. The proton NMR spectra were extracted from δ 0.00 to δ 10.00 and expanded to aliphatic region from δ 0.00-δ 4.70 as most of the metabolites in this study were found within this region. Pre-saturation (pre-sat) method was applied for the suppression of the water signal (δ 4.69 ppm-δ 4.97 ppm). The NMR spectra were binned to 0.04 ppm and scaled with Pareto scaling prior to chemometric analysis.

2.7. Chemometric analysis

2.7.1. Data reduction

The resulting spectra were manually phased and baseline corrected and reduced to ASCII file using Chenomx software (version 5.1, Alberta, Canada). For each spectrum, the spectral region δ 0.52–δ 10.00 was binned into regions of 0.04 ppm width giving a total of 238 integrated regions per NMR spectrum. The signals of δ 4.69–δ 4.97 were excised from the analysis, mainly to eliminate variation in water suppression efficiency peaks. The averaged signals of binned ^{1}H NMR data from each sub-sample group were subjected to PCA and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA). PCA and OPLS-DA were performed by SIMCA-P+ version 12.0.1.0 (Umetrics AB, Umeå, Sweden).

2.7.2. Pattern recognition (Multivariate analysis)

The datasets were arranged in such a way that the rows of each data matrix represent the subjects and the columns represent chemical shift (variable). The size of the data set for urine samples was 137 × 188. The NMR data was statistically justified using both unsupervised and supervised multivariate analysis *i.e.* PCA and OPLS-DA, respectively with SIMCA-P+ software (Version 12.0.1.0, Umetrics, Umeå, Sweden) using Pareto scaling method. OPLS-DA S-plot was used to propose potential multibiomarkers (SIMCA-P + Version 12.0.1.0). Different data preprocessing algorithms such as normalization and standard normal variate (SNV) were applied on dengue infected subjects versus healthy control and in dengue infected gender differences, respectively (Version 12.0.1.0, Umetrics, Umeå, Sweden).