Untargeted Metabolomics - Materials and Methods

1. Metabolites Extraction

Tissues (100 mg) were individually grounded with liquid nitrogen and the homogenate was resuspended with prechilled 80% methanol and 0.1% formic acid by well vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15000 rpm, 4°C for 5 min. A some of supernatant was diluted to final concentration containing 53% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then were centrifuged at 15000 g, 4°C for 10 min. Finally, the supernatant was injected into the LC-MS/MS system analysis.

PS: Liquid sample (100 μL) and prechilled methanol (400 μL) were mixed by well vortexing.

Cell sample (50 μL) and prechilled 80% methanol (200 μL) were mixed by well vortexing, and then sonicated for 6 min. Repeating this step once again and then operating the same steps as above.

2. UHPLC-MS/MS Analysis

LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher).Samples were injected onto an Hyperil Gold column (100×2.1 mm, 1.9μm) using a 16-min linear gradient at a flow rate of 0.2mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol).The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol).The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min；100-2% B, 14.1 min；2% B, 17 min. Q Exactive series mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 35 arb and aux gas flow rate of 10 arb.

3. Database search

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 minutes; actual mass tolerance, 5ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, 100000. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud (https://www.mzcloud.org/)、mzVault and MassList database to obtained the accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS release 6.6), When data were not normally distributed, normal transformations were attempted using of area normalization method.

4. Data Analysis

These metabolites were annotated using the KEGG database (http://www.genome.jp/kegg/) 、HMDB database(http://www.hmdb.ca/) and Lipidmaps database (http://www.lipidmaps.org/). Principal components analysis(PCA) and Partial least squares discriminant analysis(PLS‐DA) were performed at metaX (a flexible and comprehensive software for processing metabolomics data). We applied univariate analysis (t-test) to calculate the statistical significance (P-value). The metabolites with VIP > 1 and P-value< 0.05 and fold change≥ 2 or FC≤ 0.5 were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest which based on Log2(FC) and -log10(P-value) of metabolites. For clustering heat maps, the data were normalized using z-scores of the intensity areas of differential metabolites and were ploted by Pheatmap package in R language. The correlation between differential metabolites were analyzed by cor () in

R language (method = pearson). Statistically significant of correlation between differential metabolites were calculated by cor.mtest () in in R language. P-value < 0.05 was considered as statistically significant and correlation plots were ploted by corrplot package in R language. The functions of these metabolites and metabolic pathways were studied using the KEGG database. The metabolic pathway enrichment of differential metabolites were performed, when ratio were satisfied by x/n > y/N, metabolic pathway were considered as enrichment, when P-value of metabolic pathway < 0.05, metabolic pathway were considered as statistically significant enrichment.

Immunofluorescence – Methods

1. Deparaffinize and rehydrate: incubate sections in 2 changes of xylene, 15 min each. Dehydrate in 2 changes of pure ethanol for 5 min, followed by dehydrate in gradient ethanol of 85% and 75% ethanol, respectively, 5 min each. Wash in distilled water.

2. Antigen retrieval: immerse the slides in EDTA antigen retrieval buffer (pH 8.0) and maintain at a sub-boiling temperature for 8 min, standing for 8 min and then followed by another sub-boiling temperature for 7 min. Be sure to prevent buffer solution evaporate. Let air cooling. Wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. Use the right antigen retrieval buffer and heat extent according to tissue characteristics.

3. Circle, Block endogenous peroxidase: wash three times with PBS (pH 7.4) in a Rocker device, 5 min each eliminate obvious liquid, mark the objective tissue with liquid blocker pen. Immerse in 3% H2O2 and incubate at room temperature for 15 min, kept in dark place. Then wash again three times with PBS (pH 7.4) in a Rocker device, 5 min each.

4. Block with serum: eliminate obvious liquid, mark the objective tissue with liquid blocker pen.

Cover objective tissues with 10% donkey serum (for the case of primary antibody originated from goat) or 3% BSA (for the case of primary antibody originated from others) at room temperature for 30 min.

5. First primary antibody: throw away the blocking solution slightly. Incubate slides with the first primary antibody (diluted with PBS appropriately) overnight at 4 ℃, placed in a wet box containing a little water.

6. Corresponding secondary antibody marked with HRP:wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then throw away liquid slightly. Cover objective tissue with secondary antibody (appropriately respond to first primary antibody in species), incubate at room temperature for 50 min in dark condition.

7. CY3-TSA solution:wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each.Incubate slides with CY3-TSA solution(diluted with TBST appropriately) for 10 min in dark condition.After that,wash slides three times with TBST in a Rocker device, 5 min each.

8. Microwave treatment:immerse the slides in EDTA antigen retrieval buffer (pH 8.0) and maintain at a sub-boiling temperature for 8 min, standing for 8 min and then followed by another sub-boiling temperature for 7 min,to remove the primary antibodies and Secondary antibodies combined with tissue.Be sure to prevent buffer solution evaporate.

9. Second primary antibody: Incubate slides with primary antibody (diluted with PBS appropriately) overnight at 4 ℃, placed in a wet box containing a little water.

10. Second corresponding secondary antibody marked with HRP: wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then throw away liquid slightly. Cover objective tissue with secondary antibody (appropriately respond to second primary antibody in species), incubate at

room temperature for 50 min in dark condition.

11. FITC-TSA solution:wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each.Incubate slides with FITC-TSA solution(diluted with TBST appropriately) for 10 min in dark condition.After that,wash slides three times with TBST in a Rocker device, 5 min each.

12. Microwave treatment:immerse the slides in EDTA antigen retrieval buffer (pH 8.0) and maintain at a sub-boiling temperature for 8 min, standing for 8 min and then followed by another sub-boiling temperature for 7 min,to remove the primary antibodies and Secondary antibodies combined with tissue.Be sure to prevent buffer solution evaporate.

13 Incubate the third primary antibody :Incubate slides with primary antibody (diluted with PBS appropriately) overnight at 4 ℃, placed in a wet box containing a little water.

14. The third corresponding secondary antibody marked with CY5: wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then throw away liquid slightly. Cover objective tissue with secondary antibody (appropriately respond to second primary antibody in species), incubate at room temperature for 50 min in dark condition.

15. DAPI counterstain in nucleus:incubate with DAPI solution at room temperature for 10 min, kept in dark place.

16. Spontaneouss fluorescence quenching: wash slides three times with PBS (pH 7.4) in a Rocker device, 5 min each eliminate obvious liquid,incubate slides with spontaneouss fluorescence quenching reagent for 5 min,then wash slides under flowing water for 10 min.

17. Mount: wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. Throw away liquid slightly, then coverslip with anti-fade mounting medium.

18. Detection and collect images by slice scanner: DAPI glows blue by UV excitation wavelength

330-380 nm and emission wavelength 420 nm; FITC glows green by excitation wavelength 465-495 nm and emission wavelength 515-555 nm; CY3 glows red by excitation wavelength 510-560 nm and emission wavelength 590 nm. CY5 glows pink by excitation wavelength 608-648nm and emission wavelength 672-712nm. (CY5 was originally red, in order to distinguish it from CY3, we set it to pink light.)