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The differential metabolite profiles of acute lymphoblastic leukaemic patients treated with 6-mercaptopurine using untargeted metabolomics approach

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ABSTRACT

Background: Acute lymphoblastic leukaemia (ALL) has posed challenges to the clinician due to variable patients' responses and late diagnosis. With the advance in metabolomics, early detection and personalised treatment are possible.

Methods: Metabolomic profile of 21 ALL patients treated with 6-mercaptopurine and 10 healthy volunteers were analysed using liquid chromatography/mass spectrometry quadrupole-time of flight (LC/MS Q-TOF). Principal components analysis (PCA), recursive analysis, clustering and pathway analysis were performed using MassHunter Qualitative and Mass Profiler Professional (MPP) software.

Results: Several metabolites were found to be expressed differently in patients treated with 6-mercaptopurine. Interestingly, 13 metabolites were significantly differently expressed [p-value <0.01 (unpaired t-test) and 2-fold change] in 19% of the patients who had relapses in their treatment. Down-regulated metabolites in relapsed patients were 1-tetrahexanoyl-2-(8-[3]-ladderane-octanyl)-sn-GPEtn, GPEtn (18:1(9Z)/0:0), GPCho(O-6:0/O-6:0), GPCho(O-2:0/O-1:0), methyl 8-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]-octanoate and plasma free amino acids (PFAA). Characterizing the subjects according to their *ITPA 94C > A* genotypes reveal differential expression of metabolites.

Conclusions: Our research contributes to identification of metabolites that could be used to monitor disease progress of patients and allow targeted therapy for ALL at different stages, especially in preventing complication of relapse.

Introduction

Acute lymphoblastic leukaemia is a malignant proliferation of lymphoid cells that affects children and adults. It is more prevalent among those age between 2 and 5 years. However, the exact pathogenetic factors that lead to this disorder are unclear. It has been reported that only few cases (<5%) are associated with inherited, predisposing genetic syndromes [1]. With the advances of new molecular technologies, understanding the pathophysiological mechanisms of acute lymphoblastic leukaemia has increased. Concerted effort from the researchers in unraveling the mechanistic events that cause acute lymphoblastic leukaemia and to identify better treatment regimens will enhance the clinical outcomes, and enhance the generally low cure rates. This will lead to personalized treatment that revolutionize management of ALL patients.

Metabolism of living systems could be influenced by endogenous factors such as genetics, as well as exogenous factors including diet, environment and medication. However, the identities, concentrations and fluxes of these small metabolites are controlled by synergy of gene and protein expression in response to the environment [2,3]. Moreover, perturbation in the metabolism of patients with different disease states is shown as different metabolic profiles that could be useful signatures in monitoring treatment outcomes and disease progress. Several metabolic signatures have been reported for motor neuron diseases,

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cardiovascular diseases, type 2 diabetes, liver cancer, ovarian cancer and breast cancer [2]. Solid tumours can cause metabolic alterations with high rate of glycolysis and increase glucose consumption [4]. Similarly, Boag et al. (2006) reported that there was alteration of the glucose metabolism in children suffering from acute leukaemia. They observed higher rates of glycolysis and lactate which strongly suggest that changes in the metabolic profile of ALL are similar to that of solid tumours. However, reasons for the altered metabolism in leukaemia cells are still unknown. Therefore, this study provides further understanding on the differential expression of metabolites in ALL patients using metabolomics approach. We also attempted to study the impact of polymorphism of inosine triphosphatase enzymes on the differential metabolite profiles of patients.

Materials and methods

Subjects

The study protocol was approved by local Research Ethics Committee to investigate factors that affect patient's disease progression and therapy outcomes using both genomic and metabolomics approaches. Written informed consents were obtained from all subjects. Venous blood (3 mL) was obtained from 21 patients who were diagnosed with acute lymphoblastic leukaemia (ALL) and 10 age-matched healthy volunteers.

Clinical data and genotyping

Information about the occurrence of adverse events and interruptions of chemotherapy in all patients were obtained from medical records. All patients' samples were screened to determine their *ITPA 94C > A* genotypes using a previously published method [5].

Sample processing for metabolomics

Extraction method was modified from Sana et al. [6] for the processing of whole blood. Firstly, 250 μl thawed blood were added with 75 μl ice-cold deionized water (pH 7.0) and vortexed thoroughly. The tube was plunged into liquid nitrogen for 1 min then immediately immersed into a water bath at 37 °C and left for 1 min. Next, 300 μl of −20 °C methanol was added to the mixture and vortexed. Two hundred and twenty five (225) μl of ice-cold chloroform was added to the mixture and the tube was vortexed. Then, 75 μl of ice-cold sterile water (pH 7.0) was added followed by vortexing. Later, the tube was centrifuged at 10,000 ×*g* for 1 min at 4 °C before overnight incubation (about 16 h) at −20 °C.

After incubation, the supernatant was transferred to another tube. An equal amount of ice cold acetonitrile (ACN) was added and followed by incubation for 30 min at 4 °C. After that, the tube was centrifuged at 10,000 ×*g* at 4 °C for 15 min. The supernatant was removed to a fresh tube and dried under vacuum. The samples were then reconstituted with 50 μl of 1% formic acid and ACN at a ratio of 95:5 prior to LC/MS analysis. In order to track the uniformity of each extraction, an aliquot from the pooled blood were extracted along with the samples. This serves as a control for the extraction step as any aberration in the profile of the pooled blood indicates an error in the extraction process.

LC/MS Q-TOF analysis for metabolomics

A 1200 Rapid Resolution system (Agilent Technologies, CA, USA) complete with a binary pump and degasser, well-plate autosampler with thermostat, temperature controlled column compartment and an Agilent 6520 Q-TOF mass spectrometer equipped with an ESI source was used to analyse the samples. Column Zorbax Eclipse Plus C18 (1.8 μm particle size, 2.1 × 100 mm column dimension) was used for chromatographic separation and maintained at 40 °C during the run. Samples were run in positive mode. LC parameters: solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in ACN. The flow rate was 0.25 mL/min and the injection volume was 2 μl. A linear gradient was developed over 36 min from 5% to 95% of mobile phase (B). Total run time was 48 min for each analysis. ESI source settings were: V Cap 4000 V, skimmer 65 V and fragmentor 125 V. The nebulizer was set at 45 psig and the nitrogen drying gas was set at a flow rate of 12 L/min. Drying gas temperature was maintained at 350 ºC. Data was acquired at a rate of 2.5 spectra/s with a stored mass range of m/z 50–1000. Autocalibration was performed before each batch of analysis and reference mass correction was enabled throughout the run. The mass spectrometer was tuned to allow detection of compounds at accuracy of ± 2 ppm before the analysis. Two reference masses (121.050873 and 922.009798) were selected for correction of low and high masses.

Data processing

Data were collected using Agilent MassHunter Workstation Data Acquisition software and processed by Agilent MassHunter Qualitative Analysis software (Agilent Technologies, CA, USA). These steps include molecular feature extraction, background subtraction, data filtering, statistical analysis by ANOVA and PCA, followed by database search and alignment. Compound exchange format file (.CEF) was created for each sample and further analysed using Mass Profile Professional (MPP) (Agilent Technologies, CA, USA). Identification of endogenous and exogenous metabolites was done by using metabolite identification software, METLIN Personal Metabolite Database and Mass Profiler Professional (MPP) (Agilent Technologies, Santa Clara, CA, USA).

Data analysis

Data mining was done using the Molecular Feature Extractor (MFE) algorithm in the MassHunter workstation software. Noise was removed by using absolute height parameter which was set at 200. All entities that presented with less than this abundance were considered as noise. The settings were applied for data processing method and used to process all generated data files in a batch mode.

The first filter (frequency analysis) determined the compounds (entities) presented at 100% of the time in at least one studied group. The second filter of frequency selected entities that present in at least 50% of samples. Filtering by Analysis of Variance (ANOVA) was the next step in selecting entities that are of significantly different of 2 experimental groups. In order to identify metabolites with differential differences in abundance between the experimental groups, fold change of 2 and above was used to eliminate possible discriminating compounds.

The following step was data recursion which permits the re-examination of data to ensure that each entity is real. The software automatically re-extracted the final group of metabolites from the data to generate extracted ion chromatograms (EICs). To eliminate false positive and false negative, peaks inspection of the resulted EICs was done. The confirmed metabolites then were statistically analysed.

Statistical analysis and visualization

Statistical analysis and visualization of metabolite profiles were performed using MPP software (Agilent Technologies, Santa Clara, CA, USA). Un-paired t-test and Analysis of Variance ANOVA with Benjamini-Hochberg multiples testing correction were used to determine significant differences in the abundance of compounds between two or more groups respectively. Segregation of patients was done according to several parameters that include: (1) Healthy controls versus patients (2) Relapsed patients versus non-relapsed patients and (3) *ITPA94C > A* genotypes in patients.

Results

Twenty one (21) ALL patients (mean age ± SD; 13.4 ± 4.09 years old) were recruited; nine of them were boys. The medical history recorded six cases of hepatotoxicity and four cases of relapse among the