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Original Article

Metabonomics analysis of aqueous humor samples from cataract patients with branch retinal vein occlusion

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Abstract

Cataract (CAT) has a very high incidence rate among the middle-aged and elderly, with most patients complicated by branch retinal vein occlusion (BRVO), a key cause of blindness. In this study, through metabolomic analysis of aqueous humor samples from CAT patients with BRVO, a total of 319 different metabolites were found, most of which belonged to the categories of carboxylic acids and derivatives, fatty acyls, and organooxygen compounds. The most typical metabolites were 3-methylhistidine and biliverdin, which were up-regulated, as well as the down-regulated beta-glycerophosphoric acid. Tricosanoic acid showed the most significant correlation with CAT+BRVO. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, the most commonly related keywords for differentially expressed metabolites were biosynthesis of unsaturated fatty acids and synaptic vesicle cycle. These results can not only help to further understand the pathogenesis of CAT complicated by BRVO in clinical practice, but also provide some new therapeutic research directions.

Keywords: Cataract, Branch retinal vein occlusion, Metabonomics analysis, Aqueous humor

1. Introduction

Cataract (CAT), a high-incidence visual disorder among middle-aged and elderly people, is caused by lens protein degeneration and opacity and is also the disease with the highest blinding rate at present [1]. According to statistics, the global incidence of CAT is as high as 20-21.5% in people over 60 years old and climbs to 40% in those aged over 70 [2]. Retinal vein occlusion (RVO) is an extremely important pathological process in the blinding process of CAT, with branch RVO (BRVO) being the most common, which is mainly caused by obstruction of ocular venous return due to blood hypercoagulability and dyslipidemia [3]. At present, approximately 40-60% of CAT patients are complicated by RVO, which not only accelerates the blinding process of patients, but also greatly increases the difficulty of clinical treatment [4]. Therefore, an in-depth understanding of the pathogenesis of CAT complicated by BRVO is also the key to ensuring the prognosis and vision health of patients.

In recent years, the study of pathogenic mechanisms from the molecular perspective has gradually become a hotspot in clinical research [5]. Metabolomics, proposed by Professor Jeremy Nicholson of Imperial College London, UK at the end of last century, is a science that studies the types, quantities, and changing rules of metabolites (endogenous metabolites) with molecular weight less than 1500Da caused by external stimuli, pathophysiological changes, and gene mutation of living organisms [6]. Metabolomics is a new "omics" after genomics, transcriptomics, and proteomics. As an extension of transcriptomics and proteomics, metabolomics can directly and accurately reflect the physiological state of organisms and is an important part of systems biology [7]. Metabolomics analysis may reveal the potential pathogenesis of diseases by exploring the unique metabolites and comprehensive metabolic status, which is considered to be a new direction in the future medical field [8]. In CAT, metabolomics analysis has also been carried out, and its products have been confirmed to be closely related to the occurrence and development of CAT [9]. However, there are currently still few metabolomics analyses of CAT complicated by BRVO.

Therefore, this study conducts metabolomics analysis of aqueous humor (AH) samples from patients with CAT complicated by BRVO, to further understand the pathogenesis of CAT+BRVO to better help the clinical diagnosis and treatment of the disease.

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2. Materials and methods

2.1. Study subjects

Thirty patients with CAT admitted to our hospital from January 2023 to August 2023 were included, 15 of which had concurrent BRVO and the other 15 had simple CAT. The hospital's ethics committee approved this study, and informed consent was obtained from all subjects.

2.2. Eligibility and exclusion criteria

The inclusion criteria were the presence of CAT clinical manifestations [10], confirmed diagnosis of CAT by slit-lamp microscopy (those complicated by BRVO were further confirmed by angiography), age 18 to 70 years, and complete medical records. The exclusion criteria were the presence of other ocular diseases (diabetic retinopathy, pterygium, etc.), history of eye surgery or trauma, corneal dysfunction, pre-admission treatment with non-steroidal anti-inflammatory drugs and steroid drugs, organic diseases, and malignant tumors.

2.3. Sample collection

In BRVO patients, the atrial fluid was taken prior to the injection of medication into the vitreous cavity, and in CAT patients, the atrial fluid was taken prior to intraocular lens implantation. Paracentesis of the anterior chamber at the 3- or 9-o'clock position was performed to collect about 0.1 mL of AH into a sterile tube and stored in a refrigerator at -20°C.

2.4. Sample extraction

After the sample was slowly thawed at 4°C, a proper amount of it was added into precooled methanol/acetonitrile/aqueous solution (2:2:1, v/v) for vortex mixing. After ultrasonic treatment at a low temperature for 30min and standing at -20°C for 10 min, the sample was centrifuged for 20 min (14000 g, 4°C), and the supernatant was taken for vacuum drying. During mass spectrometry, 100μL of acetonitrile aqueous solution (acetonitrile:water=1:1, v/v) was added for redissolution, vortex mixing, and the subsequent centrifugation at 4°C for 15 min, and the supernatant was collected for analysis.

2.5. Metabolomics analysis

Metabolomics analysis was carried out using an AbsoluteIDQ p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria) and an AB 6500$^+$ QTRAP mass spectrometer (AB SCIEX). The kit can quantify 188 different endogenous molecules, such as free carnitine (C0), 39 kinds of acylcarnitines (C), hexoses (H1), 21 kinds of amino acids, 21 kinds of biogenic amines, and 105 kinds of lipids. A complete list of individual metabolites can be obtained in an open database (http://www.biocrates.com/products/research-products/absoluteidq-p180-ki). Electron spray ionization (ESI) condition: source temperature: 580°C, ion source gas 1 (GS1): 45, ion source gas 2 (GS2): 60, curtain gas (CUR): 35, ion spray voltage (IS): +4500 V or -4500 V in positive or negative modes, respectively. The multiple reaction monitoring (MRM) mode was used.

2.6. Statistical analyses

Univariate analysis of clinical data was performed using a two-tailed Student's t-test, with $P<0.05$ as the threshold of statistical significance. Prior to statistical analysis, raw metabolomics data were examined to exclude metabolites with concentrations below the lower limit of quantification (LLOQ) or above the upper limit of quantification (ULOQ) by 20%. The MRM raw data were extracted using MultiQuant to obtain the ratio of the peak area of each substance to the target peak area, and the content was calculated according to the standard curve.

3. Results

3.1. Principal component analysis (PCA)

The PCA plot of the metabolomics data did not indicate any grouping according to POAG or control groups, nor did it show any strong outliers in the first principal plot (PC1 vs. PC2). The high intra-group aggregation and significant inter-group separation indicated the reliability of the model and significant inter-group differences. After being corrected by the partial least squares discrimination analysis (PLS-DA) or the orthogonal partial least squares discriminant analysis (OPLS-DA), the Q^2 of the model was within the range of $0.3<Q^2\leq0.5$, suggesting high reliability. Meanwhile, according to the permutation test graph of the OPLS-DA model, the R^2 and Q^2 of the random model reduced gradually with the gradual decrease of permutation retention, indicating the absence of over-fitting of the original model and the robustness of the model (Figure 1).

3.2. Metabolomics analysis

AH samples from patients were tested and analyzed and 319 metabolites were identified (supplementary Tab. 1), which were then classified and counted according to their chemical taxonomy attribution information. The quantities of various metabolites are shown in Figure 2. Among them, a great number of metabolites belonged to the categories of carboxylic acids and derivatives, fatty acyls, and organooxygen compounds.

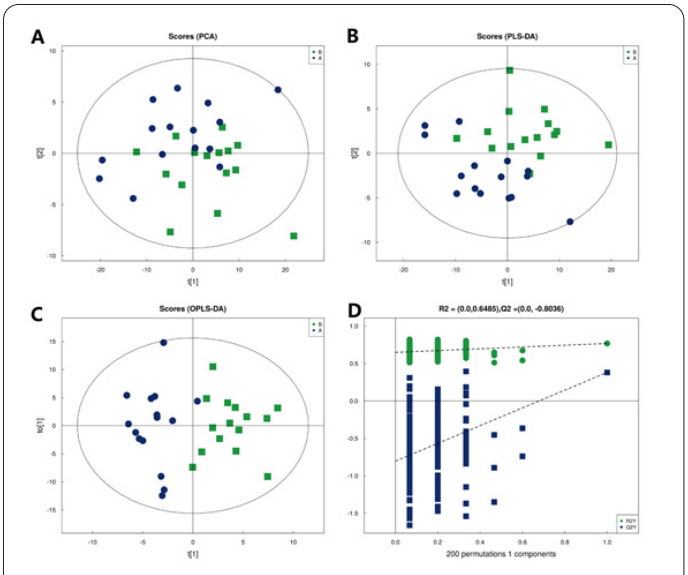

Fig. 1. Principal component analysis (PAC) results. (A) PCA score. (B) PLS-DA score. (C) OPLS-DA score. The abscissa t[1] represents principal component 1, the ordinate t[2] represents principal component 2, and the ellipse represents the 95% confidence interval (95%CI). The dots of the same color represent various biological replicates within the group, and the distribution of the dots reflects the inter- and intra-group differences. (D) OPLS-DA permutation test. The abscissa represents the permutation retention, and the ordinate represents the values of R^2 and Q^2. The green dot represents R^2, the blue dot represents Q^2, and the two dashed lines represent the regression lines of R^2 and Q^2, respectively.

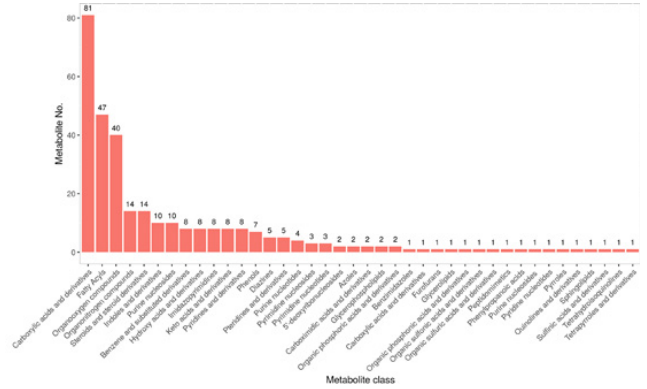

Fig. 2. Quantity statistics of identified metabolites by chemical taxonomy.

3.3. Analysis of inter-group differences

P<0.0 was used as the screening criterion for differentially expressed metabolites. The volcano plot (Figure 3A) was used for visualization. 3-methylhistidine and biliverdin, which were up-regulated, as well as the down-regulated beta-glycerophosphoric acid, were the most typical metabolites (Figure 3).

3.4. Bioinformatics analysis of differentially expressed metabolites

Cluster analysis results, shown in Figure 4A, indicate that all metabolites have similar expression patterns. Most metabolites were positively correlated with AH in patients with CAT or CAT+BRVO, with tricosanoic acid showing the most significant correlation (r=0.58).

3.5. Enrichment analysis

According to KEGG pathway enrichment analysis, the keyword with the highest involvement in the differentially expressed metabolites was biosynthesis of unsaturated fatty acids, followed by synaptic vesicle cycle (Figure 5).

4. Discussion

Metabolites, as intermediates of biological reactions and activities, play an important role in connecting various cellular pathways and communication between different systems [11]. In layman's terms, genes determine what is likely to happen, and metabolites represent what is happening [12]. Therefore, the level of metabolites represents a comprehensive picture of the phenotype of a cell or tissue in response to genetic or environmental changes, and is the result of the interaction between an organism's internal activities (e.g., gene expression, protein activity, cell metabolism) and external factors (diet, health status, lifestyle, gut microbes, drugs, etc.) [13]. Metabolomics identifies metabolites and metabolic pathways that are closer to phenotype than many other omics techniques, as it detects the end product of gene expression, so it is considered a more sensitive method for measuring biological phenotype and can more accurately reflect changes in the biological behavior of organs, cells, and tissues [14].

In this study, metabolomics analysis was carried out on AH samples from patients with CAT and those with CAT+BRVO. It was found that most of the 319 metabolites identified fell into the categories of carboxylic acids and derivatives and fatty acyls. The PCA was highly reliable after verification, indicating that the metabolites belonging to carboxylic acids and derivatives and fatty acyls are the key factors affecting BRVO in CAT patients. Among them, the compounds produced by the substitution of hydroxyl groups in carboxylic acid molecules by other atoms or atomic groups such as halogens and amino groups are called carboxylic acids and derivatives, including acid halides, anhydrides, esters, amides, etc. [15]. As is well known, glutamate amino acids are related to vision in the tricarboxylic acid cycle. The tricarboxylic acid cycle is a ubiquitous metabolic pathway in aerobic organisms, distributed in the cytoplasm in prokaryotes and mitochondria in eukaryotes, with organic acids containing three carboxyl groups being the main intermediate metabolites in this cycle [16]. Therefore, the relationship between carboxylic acids and derivatives and BRVO+CAT can also

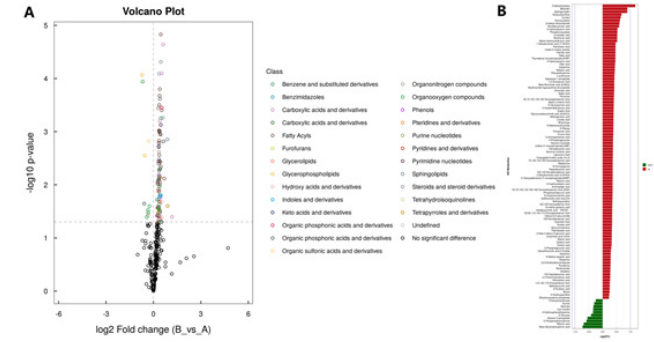

Fig. 3. Analysis of inter-group differences. (A) Volcano plot. The non-significantly different metabolites are black. (B) Fold change analysis of differentially expressed metabolites. Red indicates up-regulation and green indicates down-regulation of the differentially expressed metabolites.

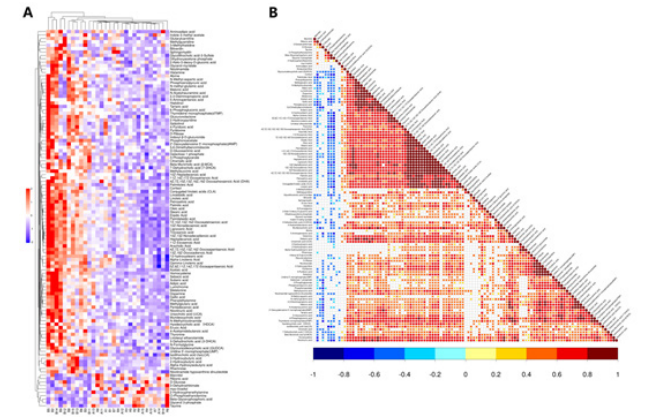

Fig. 4. Bioinformatics analysis of differentially expressed metabolites. (A) Cluster analysis results of differentially expressed metabolites. (B) Correlation analysis.

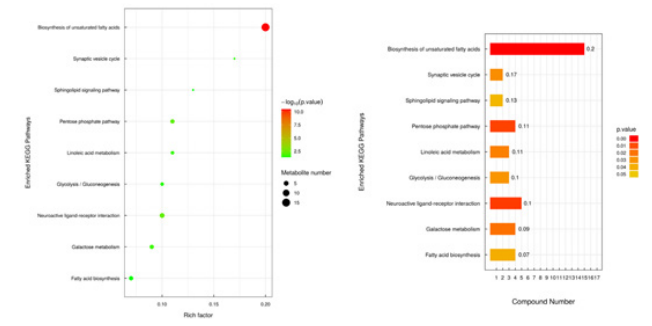

Fig. 5. KEGG pathway enrichment analysis.

be expected. Fatty acyls, widely found in organisms, are the major component of biofilms and participate in various cellular metabolism processes. In living organisms, fatty acid acyls can combine with glycerol to form triacylglycerol, which is stored in fat cells as energy reserves [17]. Clinically, BRVO formation is believed to be primarily caused by increased blood viscosity, elevated platelet count, and increased platelet agglutination [18]. Therefore, we speculated that the high proportion of fatty acyls may be caused by the massive accumulation of lipid cells in AH. In addition, organooxygen compounds are also one of the non-negligible metabolites in CAT+BRVO, accounting for the second largest proportion after fatty acyls, but showing significant differences with organonitrogen compounds, which account for the fourth largest proportion. Oxidative damage and stress reaction following BRVO are the main causes of visual dysfunction in patients and the key factors that promote the intensification of inflammation. The relationship between oxidative stress injury and BRVO has been repeatedly verified clinically [19, 20], so this paper will not elaborate too much. Therefore, the increase in the proportion of organooxygen compounds in the AH of patients with CAT+BRVO is in line with the pathological manifestations. In the previous full-spectrum metabolomics analysis of CAT, glutamate accounts for the highest proportion and is considered to be the most important metabolite [21]. In this study, however, the proportion of glutamic acid in AH of CAT+BRVO patients was not significant, which may be due to the fact that glutamic acid is mainly related to the metabolic capacity of the lens [22], while the occurrence of BRVO has no significant relationship with the metabolism of the lens.

Through further analysis, we found that 3-methylhistidine and biliverdin were up-regulated in AH in patients with CAT+BRVO, while beta-glycerophosphoric acid was down-regulated. Of them, 3-methylhistidine is a non-arginine residue, which belongs to histidine derivatives and is an indicator of muscle decomposition. 3-methylhistidine is released when muscle contraction leads to the decomposition of skeletal muscle contractile proteins, and elevated 3-methylhistidine content usually reflects the abnormal secretion of contractile proteins because there is no corresponding enzyme for decomposition and reuse in muscle cells [23]. Hemoglobin forms biliverdin under the action of hemoglobin oxygenase of microsomes in mononuclear phagocytes, and its elevated level is usually considered to be associated with biliary obstruction or intravascular hemolytic disease [24]. Reduced levels of beta-glycerophosphoric acid, a major component of the normal synthesis of triglycerides, also confirm the increased viscosity in AH and the increased risk of obstruction [25]. The expression of the above compounds also basically accords with the pathological changes of BRVO. Meanwhile, we found similar results in the metabolomics analysis of patients with BRVO complicated by macular edema by Xiong X et al. [26], suggesting that intervening in the levels of 3-methylhistidine, Biliverdin, and beta-glycerophosphoric acid may be expected to develop new treatment regimens for CAT complicated by BRVO. Tricosanoic acid, known to form water-insoluble macromolecular complexes with various compounds in the human body and deposit them in the organs and tissues to which they belong [27], is also considered to be one of the most critical metabolites in the formation of obstruction in diseases such as kidney stones and myocardial infarction [28, 29]. In this paper, the strong correlation between tricosanoic acid and AH in patients with CAT+BRVO also shows the involvement of tricosanoic acid in vascular obstructions of the eye fundus, providing a new idea for the future treatment of BRVO, namely, improving tricosanoic acid metabolism.

Finally, through KEGG pathway enrichment analysis, we found that the keywords most frequently involved in the differentially expressed metabolites were biosynthesis of unsaturated fatty acids and synaptic vesicle cycle. Trans-fatty acids are known to be the root cause of vascular occlusion, so the importance of biosynthesis of unnatural fatty acids is self-evident [30]. Currently, the relationship between synaptic vesicle cycle and BROV has not been clarified, but we speculate that this may be related to the postsynaptic retinal ganglion cells in the dendritic region. When synaptic vesicles fuse with the plasma membrane, the neurotransmitters stored in the vesicles are released in different ways, which has an impact on the activities of postsynaptic cells and even neural networks at multiple levels, maintaining the normal operation of retinal cells and ensuring correct visual function. To test this hypothesis, more in-depth experiments are needed for analysis and validation.

Of course, there are still many limitations to be addressed in this study. For example, the AH samples analyzed were collected during surgery, and the stress injury caused by surgery may have some influence on the basal metabolism of AH, which may affect the accuracy of experimental results to a certain extent. In addition, due to regional restrictions, the research participants are limited to the yellow race in China, and whether there are differences among different races is still uncertain. Moreover, more experiments are still needed to verify the involvement of metabolomics in the process of CAT+BRVO to provide more reliable reference and guidance for clinical practice.

5. Conclusion

Through the metabolomics analysis of AH samples from patients with CAT complicated by BRVO, this study found that metabolites such as 3-methylhistidine, biliverdin, and beta-glycerophosphoric acid play an important role in the onset and progression of CAT+BRVO. The findings not only help us to further understand the pathogenesis of CAT complicated by BRVO, but also suggest a new direction for future treatment of the disease, which has important clinical implications.

Conflicts of Interest

The authors report no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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