· Original article ·

Metabolomics analysis revealed acupuncture treatment target the upstream for dry eye disease

Jun Rao[1], Shuo-Chuan Liu[2*], Lin-Feng Zhang[3], Zhi Zheng[1], Yan Yu[1], Shang Lou[1], Ai-Hua Xiong[1], Lu-Hua Wu[4]*

引用:饶军,刘铄川,张林锋,等. 针灸治疗干眼的代谢组学分析. 国际眼科杂志 2021;21(3):389-397

Foundation items: National Natural Science Foundation of China (No.81960865); Fundamental Research Funds for the Central Universities (No.2017-JYB-JS-200); Health and Family Planning Commission of Jiangxi Province (No. 2017A271, 20203533); Jiangxi Provincial Department of Science (No.20171BBG70119, 20181BAB205047)

[1]Jiangxi Provincial Key Laboratory of Translational Medicine and Oncology, Jiangxi Cancer Hospital of Nanchang University, Jiangxi Cancer Center, Nanchang 330029, Jiangxi Province, China
[2]School of Medicine, Nanchang University, Nanchang 330029, Jiangxi Province, China
[3]Department of Oncology, the First People's Hospital of Fuzhou, Fuzhou 344000, Jiangxi Province, China
[4]Department of Ophthalmology, the Third Affiliated Hospital of Beijing University of Chinese Medicine, Beijing 100029, China
Co-first authors: Jun Rao and Shuo-Chuan Liu.
Correspondence to: Jun Rao. Jiangxi Provincial Key Laboratory of Translational Medicine and Oncology, Jiangxi Cancer Hospital of Nanchang University, Jiangxi Cancer Center, Nanchang 330029, Jiangxi Province, China. raojun1986@126.com

Received: 2020-01-30　　Accepted: 2020-09-29

针灸治疗干眼的代谢组学分析

饶军[1*],刘铄川[2*],张林锋[3],郑智[1],余妍[1],娄尚[1],熊爱华[1],吴鲁华[4]

基金项目:国家自然科学基金项目(No.81960865);中央高校基本科研业务项目(No.2017-JYB-JS-200);江西省卫健委项目(No. 2017A271, 20203533); 江西省科技厅项目(No. 20171BBG70119,20181BAB205047)

(作者单位:[1]330029 江西省南昌市,江西省肿瘤转化医学重点实验室 江西省肿瘤医院(南昌大学附属肿瘤医院) 江西省癌症中心;[2]330029 中国江西省南昌市,南昌大学医学院;[3]344000 中国江西省抚州市,抚州市第一人民医院肿瘤科;[4]100029 中国北京市,北京中医药大学第三附属医院眼科)
*:饶军与刘铄川对本文贡献一致。
作者简介:饶军,毕业于上海交通大学,博士研究生,副研究员,研究方向:基础医学;刘铄川,南昌大学医学院,在读硕士研究生,研究方向:基础医学。
通讯作者:饶军. raojun1986@126.com

摘要

目的:探讨干眼(DED)发病及其有效治疗的分子机制。

方法:采用超高效液相色谱-四极杆飞行时间质谱(UHPLC-Q-TOF-MS)非靶向技术对 18 例经药物或针灸治疗的 DED 患者的泪液样本进行代谢组学研究。

结果:在待测样品中共鉴定出 190 种代谢物,是迄今为止最广泛的泪液代谢组学研究。结果表明,所有患者的代谢组学特征明显不同,但药物或针灸治疗后的代谢组学差异非常细微。药物治疗后只有 6 种代谢物发生显著变化,其中肌苷、单胺四乙酸、尿酸盐、丙酰胆碱、烟酰胺等 5 种代谢物的含量均增加并参与炎症反应。针刺治疗后只有 4 种代谢物,其中丙氨酸、丝氨酸和高丝氨酸的含量有显著差异。对上述显著变化的代谢物的代谢途径进一步分析表明,针灸治疗的患者只有 1 种代谢途径,即氨酰 tRNA 生物合成受到显著影响,这与疾病的病因高度相关,表明针灸疗法可以解决干眼的病因,而不是症状,与药物治疗相比,在一定程度上显示出更好的疗效。

结论:代谢组学分析有助于明确有效治疗干眼的潜在机制中涉及到的关键调控因素或途径,并将有助于为 DED 治疗提供新的潜在靶点和策略。

关键词:人工泪液;针灸;干眼;代谢组学;妥布霉素地塞米松滴眼液

Abstract

• AIM: To find out the pathogenesis of dry eye disease (DED) and the possible mechanisms of available effective treatments.

• METHOD: Here we employed a non-targeted technology, ultra-high performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS), to investigate metabolic characterizations for tear samples from 18 patients with DED upon drug or acupuncture treatment.

• RESULTS: A total of 190 named metabolites were identified, which presented so far the broadest tear metabolome. Further analysis indicated a significantly distinct metabolomics profile among all patients, but very subtle metabolic differences upon drug or acupuncture treatment. On one hand, only six significantly changed metabolites were determined after drug treatment, five of which including inosine, monopalmitin, urate, propionylcarnitine, and nicotinamide were all increased and involved in inflammatory responses. On the other hand, merely four metabolites including alanine, serine, and homoserine were found to be significantly different. Further pathway analysis of those six and four significantly changed metabolites revealed that only one pathway, aminoacyl-tRNA biosynthesis was significantly influenced in acupuncture-treated patients, which were highly associated with the cause of the disease. The results here indicated acupuncture treatment may address the cause rather than the symptoms for dry eye disease, displaying partially better compared with drug treatment.

- CONCLUSION: Collectively, this work extended our understanding on the key regulatory elements or pathways involved in the potential mechanisms of available effective treatments, and would be useful for providing novel potential targets and therapeutic strategies for DED.

- KEYWORDS: artificial tears; acupuncture; dry eye; Metabolomics; Tobramycin dexamethasone eye drops

DOI:10.3980/j.issn.1672-5123.2021.3.02

Citation: Rao J, Liu SC, Zhang LF, *et al*. Metabolomics analysis revealed acupuncture treatment target the upstream for dry eye disease. *Guoji Yanke Zazhi*(*Int Eye Sci*) 2021;21(3):389-397

INTRODUCTION

Dry eye disease (DED), also called keratoconjunctivitis sicca, is a chronic multifactorial disorder of the tears and ocular surface resulting in symptoms of ocular discomfort and visual disturbance[1]. The most common symptoms behind DED include dryness, redness, burning/stinging, photophobia, ocular fatigue and blurred vision. Unfortunately, DED is increasing in prevalence affecting about 5%-30% of the worldwide population of different ages, which can significantly impair quality of life, as well as the patients' economic burden[2]. Moreover, the diagnosis of DED is supposed to be difficult due to poor correlations between reported symptoms and available clinical tests[3].

The treatment of patients with DED evolves continuously and quite a number of treatment strategies are now available, including artificial tears, alternative anti-inflammatory drugs, immunomodulators, oral supplements, hormonal therapy, and acupuncture[4]. Among them, tear replacement by artificial tears is currently the most widely used therapy for DED. As reported, artificial tears can lower tear osmolarity and dilute proinflammatory factors on the ocular surface, and also smooth the corneal surface to improved vision[5]. However, artificial tears may finally lose their efficacy over time since they do not target the underlying causes of DED. Anti-inflammatory drugs represents a necessary step during the chronic therapy of DED, and especially non-steroidal anti-inflammatory drugs (NSAIDs) are demonstrated as prospective therapy for DED[2]. Although these drugs target the underlying etiology and reduce inflammation and eye pain for patients with DED, they could reduce their eye sensitivities at the same time. Moreover, oral supplements of omega-3 (ω-3) fatty acids have been recommended in the treatment of moderate DED, which promote tear film stabilization and play a beneficial role in DED[6]. Additionally, acupuncture therapy is also determined to be effective for dry eye patients without serious side effects. Recent studies showed acupuncture could improve the signs and symptoms for patients with DED after 4wk of treatment[7].

Recently, the advent of the omics era (especially proteomics and metabolomics) has dramatically increased our understanding on the pathogenic mechanisms of complex diseases, as well as the potential mechanisms of improvement after various treatments[8-9]. For example, proteomics shows great potential application in large-scale clinical ophthalmology studies, which allows the simultaneous study of thousands of proteins and/or peptides in biological samples[10-11]. Proteomic analysis of 29 human aqueous humor samples from patients with cataract and glaucoma with and without the pseudoexfoliation syndrome revealed no significant difference between glaucoma and cataract AH proteomes, while apolipoprotein D was found to be a putative biomarker for pseudoexfoliation syndrome[10]. Moreover, Liu *et al*[12] employed two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry (2D nano-LC-MS/MS) to determine whether acupuncture is effective at treating dry eye disease and to find out the possible mechanisms. The results revealed that an acupuncture plus artificial tears (AC+AT) treatment improved clinical symptoms and increased protein synthesis and secretion. Metabolomics has emerged as a powerful tool, allowing simultaneous analysis of hundreds of metabolites in one biological sample. It has great potential to screen out biomarkers and functional pathways for various diseases including eye diseases, and thus lead to novel therapies[3,9,13]. We previously used gas chromatography coupled to time-of-flight mass spectrometer (GC/TOF MS) for metabolites profiling of 40 aqueous humor samples from patients with high myopia and the controls, which revealed that 29 significantly changed metabolites and their regulatory aspects of metabolic pathways might be key regulatory elements or pathways in the development of high myopia. Furthermore, Galbis-Estrada *et al*[14] applied nuclear magnetic resonance spectroscopy of hydrogen-1 nuclei (^{1}H NMR S) to investigate the role of the oral supplements with antioxidants and omega-3 fatty acids in relation to dry eye. The data suggested that the metabolic changes in dry eye syndrome could be modified with appropriate oral supplementation with antioxidants and essential polyunsaturated fatty acids.

Tear fluid is a valuable source for objective analysis on various eye diseases, whose components have various roles including protecting and maintaining the ocular surface[15]. It is composed of proteins, carbohydrates, lipids, electrolytes, and some small organic molecules, while defective quality and quantity of tear could lead to dry eye. In this study, we took advantage of a non-targeted technology, ultra-high performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS), to report the most comprehensive human tear metabolome from

Table 1 Information of human tear samples from dry eye patients

Groups	Sample name		Age, y	Gender
	Before	After		
Acupuncture treatment group	bAT1	aAT1	47	F
	bAT2	aAT2	81	F
	bAT3	aAT3	63	F
	bAT4	aAT4	42	M
	bAT5	aAT5	64	M
	bAT6	aAT6	76	F
	bAT7	aAT7	55	M
	bAT8	aAT8	45	M
	bAT9	aAT9	64	F
Drug treatment group	bDT1	aDT1	76	F
	bDT2	aDT2	78	F
	bDT3	aDT3	68	M
	bDT4	aDT4	48	M
	bDT5	aDT5	56	F
	bDT6	aDT6	60	M
	bDT7	aDT7	59	F
	bDT8	aDT8	74	M
	bDT9	aDT9	48	M

dry eye patients up to date. And more importantly, the roles of drug and acupuncture treatment for dry eye disease were further investigated, respectively.

SUBJECTS AND METHODS

Subjects Eighteen patients with DED were included in the present study (Table 1). The ophthalmic examination included subjective symptoms, visual acuity, tear schirmer I test (SⅠT) with anesthesia (basis secretory test, BST), and tear break-up time (BUT). Each patient was asked for subjective symptoms such as burning, itching, foreign body sensation, dryness, and photophobia. Patients were classified as having DED if they had the above-mentioned symptoms and abnormalities of test dynamics determined by a SⅠT score less than 10 mm/5min or a BUT score less than 10s. Notably, patients with neither Sjögren syndrome nor aqueous-deficient dry eye were excluded from this study. All participants provided written informed consent and the present study was approved by the Ethics Committee of the Third Affiliated Hospital of Beijing University of Traditional Chinese Medicine. Meanwhile, the study was performed in accordance with the Declaration of Helsinki. The tears were collected by capillary method. Each time we collected 5-15 μL tears by 25 μL capillary tube from the canthus, and immediately transferred them into the EP tube and stored at -80℃ refrigerator until further analysis.

Treatment Procedure All participants were divided into either drug treatment group or acupuncture treatment group, while both lasted for 3wk. In the drug treatment group, each patient was offered with artificial tears and tobramycin dexamethasone eye drops. The two drugs were successively dropped into the conjunctival sac with a minimal interval of 10min apart, one drop each time and four times 1d. While the acupuncture treatment was taken by the same ophthalmologist, which was mainly consisted of the acupoints of Jingming, Chengqi, Shangming, Cuanzhu, Hegu. Another different acupoints were further allocated according to the differentiation of symptoms and signs: For type of fei-yin deficiency with Kongzui and Chize acupoints; for type of liver-yin and kidney-yin deficiency with Taixi and Sanyinjiao acupoints; for type of damp heat in the spleen and stomach with Quchi and Zusanli acupoints. Keep the needles for 30min after getting air. Both eyes of each patient were treated every other day and totally ten times were performed in one course lasting for 3wk. After treatment, their symptoms, SⅠT and BUT scores were recorded again, and tears were also collected. According to the paired *t*-test (SPSS 17.0 software), no significantly differences were identified in BUT & SⅠT before treatment between those two groups. Likewise, no significantly differences of IOPs between all enrolled patients before and after treatment were observed.

Metabolomics Analysis The metabolites profiling of all tear samples was conducted as our previous report[16]. Briefly, 300 μL methanol was firstly added into each tear sample (about 15 μL), which was then ultrasonicated for 20min and centrifuged at 12000×g in 4℃ for 10min. 250 μL of the supernatant was transferred into 1.5 mL centrifuge tube and completely dried in a vacuum concentrator completely. Secondly, 75 μL methanol were added and then centrifuged at 12000×g in 4℃ for 10min. 50 μL of the supernatant was finally transferred into a glass vial for metabolomic analysis. A volume of 3 μL was injected into the Aglient 1290 Infinity II LC TM system with an Agilent Eclipse-plus C18 column (150×3.0 mm i.d., 1.8 μm). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (100% acetonitrile), while the flow rate was 0.40 mL/min. Gradient elution conditions were set as follows: 0min, 98.0% A; 1.0min, 98% A; 5.0min, 60% A; 12.0min, 30% A; 15.0min, 5% A; 20.0min, 5% A. MS detection was conducted on the Agilent 6550 iFunnel/Q-TOF mass spectrometer with Agilent Jet-Stream source[16]. Metabolites annotation was finished by searching Personal Compound Database and Library (PCD/PCDL), Massbank database and literatures[14,17-19]. Moreover, the softwares of MassHunter Acquisition 6.0, MassHunter Qualitative 6.0 and Mass Profinder 6.0 were respectively used for data acquisition, data review and metabolite identification, peak area extraction.

Data Analysis Metabolomics data were firstly normalized as our previous reports, which registered the median level of each metabolites to equal to one (1.00) while missing values (if any) were imputed with the observed minimum ones[7]. Mev (Multiexperiment Viewer) 4.8 software was further used for hierarchical cluster analysis, and meanwhile SIMCA-P software (v13.0, Umetrics, Malmö, Sweden) was applied for both principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Moreover, paired

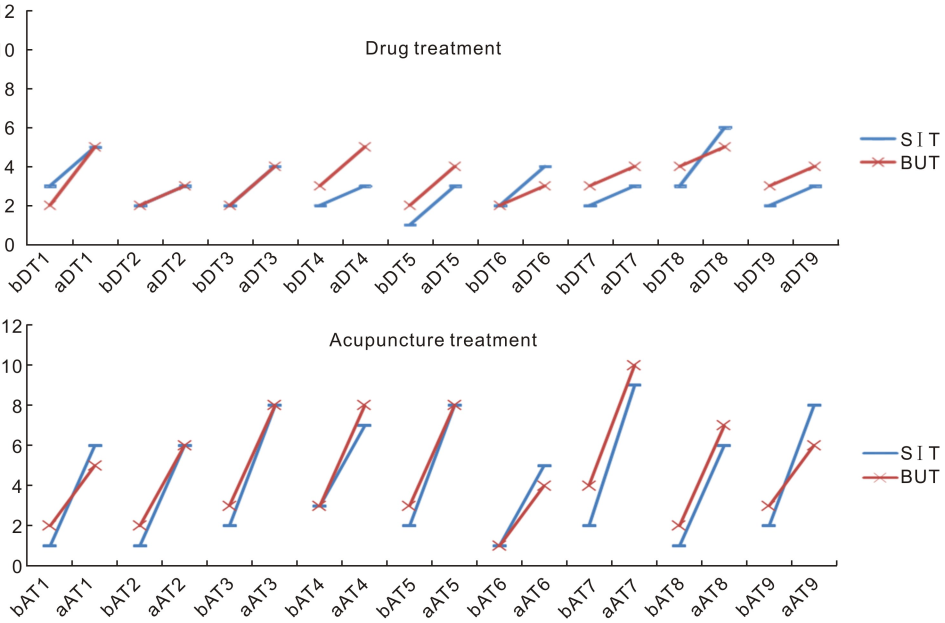

Figure 1　The information of the SⅠT and BUT scores record in both drug and acupuncture treatment for patients with DED.

t-test (SPSS 17.0 software) was used to identify metabolites which were significantly different between before and after treatment in patients with DED. Metabolites with both VIP (variable importance in the projection) values in PLS-DA model more than 1 and *P* values (in *t*-tests) less than 0.05 were considered significant. In addition, metabolic pathway analysis of significantly different metabolites was conducted by MetaboAnalyst web server (http://www.metaboanalyst.ca). Significant pathways were judged based on *P* threshold 0.05.

RESULTS

Clinical Characteristics of Dry Eye Patients　In the present study, we recruited 18 patients with DED and their clinical characteristics were listed in Table 1. Nine patients were selected into drug treatment group, while the rest 9 patients were enrolled into acupuncture treatment group. The median age of the patients in drug treatment group was 63-year-old (range, 48-78 years), 4/9 of which were female. On the other hand, the average age of the patients treated by acupuncture treatment group was 59.7-year-old (range: 42-81 years), and meanwhile 5/9 of these patients were female. The statistical analysis showed that there were no significantly difference of between the two groups for both sex and age. Moreover, both scores of SⅠT and BUT for these 18 patients before and after treatment were also recorded (Figure 1). Obviously, scores of SⅠT and BUT of these 18 patients were both low at the beginning but significantly improved after treatment. Notably, the changes of SⅠT and BUT scores for patients in acupuncture treatment group were larger than those in drug treatment group. The results here indicated that both acupuncture and drug treatments were effective for patients with DED, and especially acupuncture treatment seemed to be partially better compared with drug treatment.

Metabolic Characterization of Tears from Dry Eye Patients　In order to comprehensively uncover the human tear metabolome, we here applied UHPLC-Q-TOF-MS to metabolic profiling of 36 tears samples from 18 dry eye patients. A total of 190 named metabolites were identified in the tested tear samples. Those identified 190 metabolites were categorized into 13 groups according to the database from Kyoto Encyclopedia of Genes and Genomes (KEGG). The largest group contained 36.8% of the total number of detected metabolites, which were 70 amino acids and derivatives. The second largest group (22.1%) was 42 lipids, followed by 24 (12.6%) nucleotides, and 20 (9.5%) carbohydrates.

An un-supervised approach, hierarchical cluster analysis was firstly performed to investigate metabolic variations among the 18 tear samples from dry eye patients before treatment (Figure 2A). The heat map indicated remarkable diversities in metabolite abundance across these dry eyepatients. Several metabolites such as 3-(pyrazol-1-yl)-alanine (Figure 2B), cytidine, cytosine, cyclic AMP, and guanosine 2′3′-cyclic phosphate displayed low abundance in all the samples, while the enrichment of certain metabolites appeared to be individual-specific. For example, pyridoxine was highly abundant only in sample bAT7 when compared to that in other 17 tear samples (Figure 2C). Moreover, the major of the detected metabolites in several tear samples including sample bDT4 and bAT3 seemed to have lower abundance, while most of 190 metabolites displayed highly enriched in sample bAT5.

To further provide a snapshot of the information hidden in the metabolomic data, we conducted another unsupervised approach, PCA, for all 36 tear samples from patients with DED before and after treatment. As shown in Figure 3, PCA could not distinguish tear samples with different treatment groups (acupuncture and drug treatment groups), and different groups (untreated and treated groups). However except bAT5 and aAT5 & bDT1 and aDT1, every two tear samples from the same individual displayed close in the PCA score plot. The results confirmed that patients with DED had remarkable metabolic variation, and more importantly either

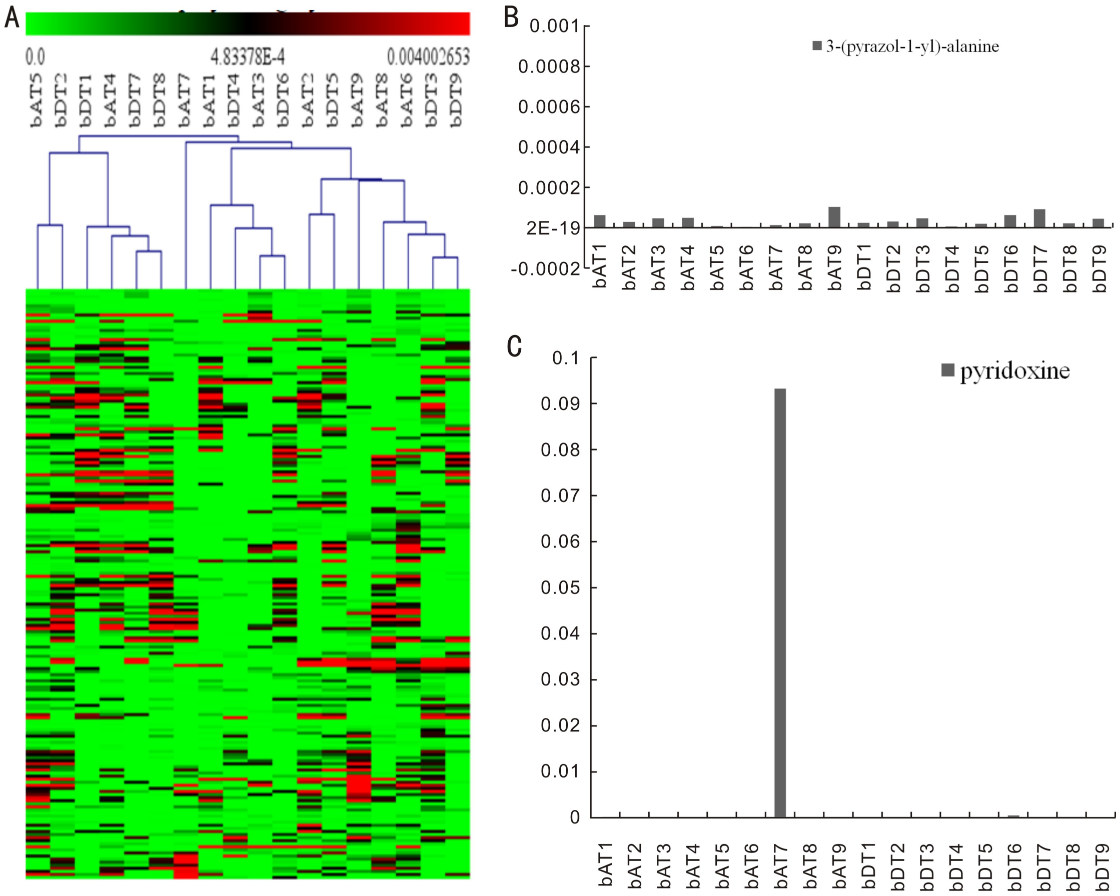

Figure 2　Metabolic variations among the 18 tear samples from dry eye patients before treatment　A: Heat map representation of the 190 identified metabolites across all tears samples from 18 dry eye patients before treatment. Each line represented one metabolite. The deeper the red color, the higher its level in the tear samples; while the deeper the green color, the lower its level in the tear samples; B: The levels of 3-(pyrazol-1-yl)-alanine across all tears samples from 18 dry eye patients were shown in greater detail; C: The levels of pyridoxine across all tears samples from 18 dry eye patients were shown in greater detail.

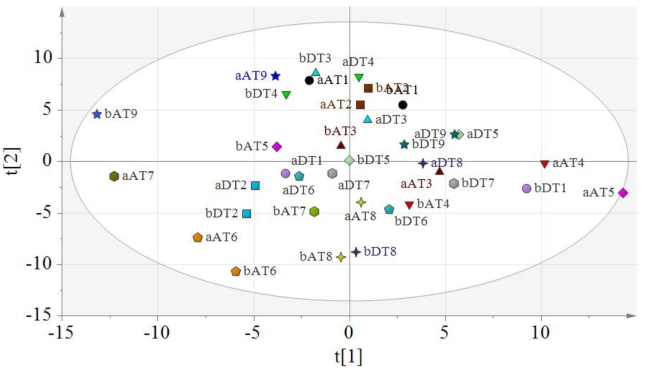

Figure 3　PCA scores plot generated from the 190 identified metabolites across all 36 tears samples from 18 dry eye patients before and after (drug/acupuncture) treatment.

acupuncture or drug treatment had little effect on metabolomics profile.

Metabolic Changes in Dry Eye Patients After Drug Treatment　To exactly determine the metabolic differences between patients with DED before and after drug treatment, the supervised statistical method PLS-DA was then employed. Obviously, the tear samples before treatment clustered together, as did those after treatment (Figure 4A). Furthermore, six metabolites (Table 2 and Figure 4B) were determined to be significantly changed (VIP $\geqslant 1$ and $P \leqslant 0.05$), playing important roles for the separation. Among them, the levels of 5 compounds were increased, while only glycerate showed lower level after treatment. These metabolites were related to five classes such as carbohydrates, organic acids and derivatives, lipids, nucleotides, and cofactors. In addition, further metabolic pathway analysis of these six significantly different metabolites revealed six relevant pathways, among which purine metabolism, and nicotinate & nicotinamide metabolism were significantly influenced ($P < 0.05$, Figure 5A).

Metabolic Changes in Dry Eye Patients After Acupuncture Treatment　Likewise, we also conducted pls-da and paired *t*-tests to find out the metabolic changes resulted from acupuncture treatment for patients with DED. As shown in Figure 4C, tear samples from patients in the untreated group were clearly separated from those in the treated group. Together with paired *t*-tests, the results showed that only 4 metabolites (Table 3 and Figure 4D) were significantly changed (VIP $\geqslant 1$ and $P \leqslant 0.05$), responsible for the separation. Interestingly, all four metabolites displayed lower levels in patients with DED after acupuncture treatment, whose ratios ranged from 0.50 to 0.79. Meanwhile, three of four metabolites belonged to class of amino acids and derivatives, while only one compound cytidine was grouped

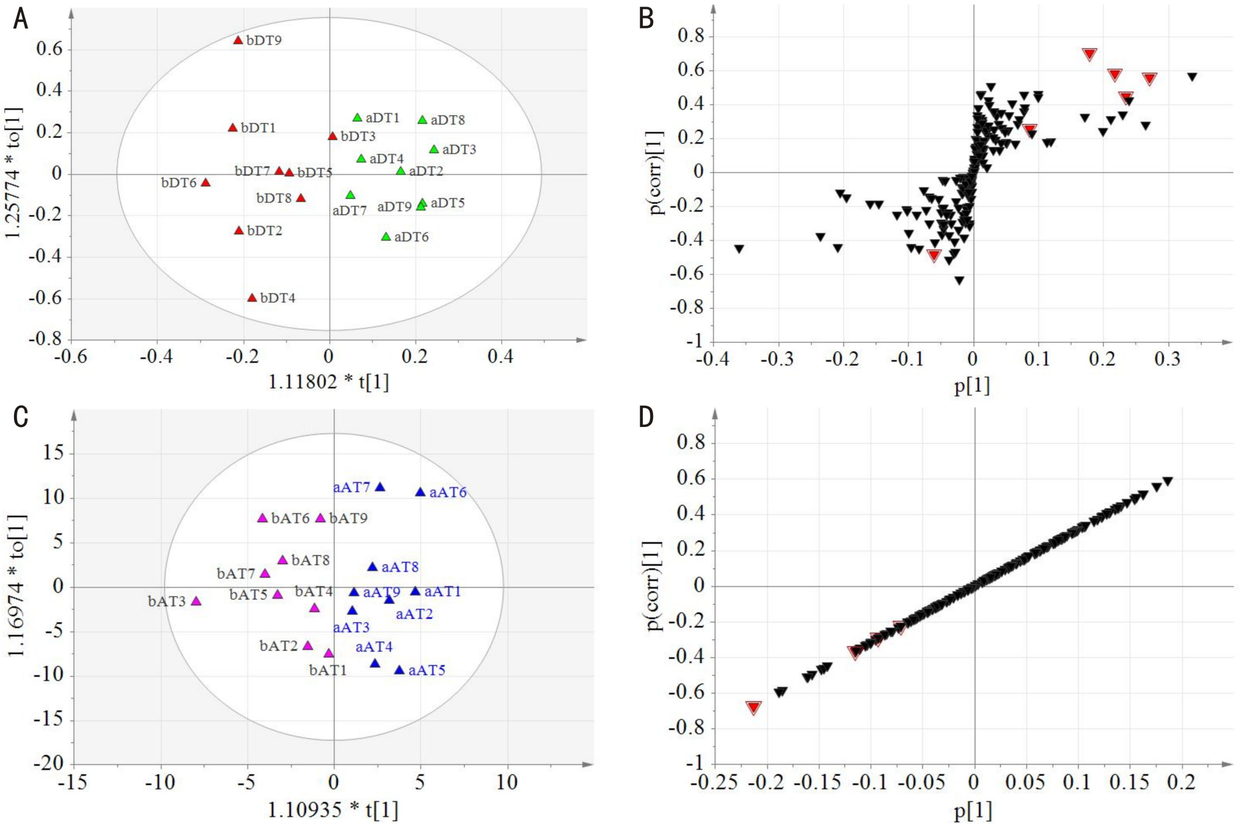

Figure 4　PLS-DA models for dry eye patients before and after treatment　A: Score plot in PLS-DA model for dry eye patients before and after drug treatment; B: S plot in PLS-DA model for dry eye patients before and after drug treatment; C: Score plot in PLS-DA model for dry eye patients before and after acupuncture treatment; D: S plot in PLS-DA model for dry eye patients before and after acupuncture treatment. Significantly changed metabolites were labeled with red triangle.

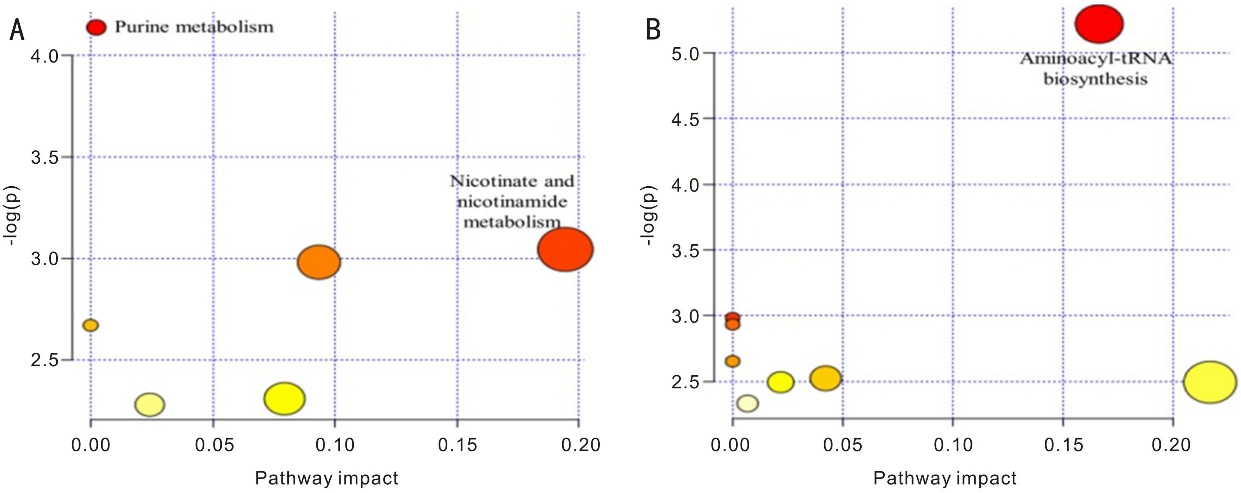

Figure 5　Metabolic pathway analysis　A: Metabolic pathway analysis of 6 significantly different metabolites among dry eye patients before and after drug treatment by using MetaboAnalyst web server; B: Metabolic pathway analysis of 4 significantly different metabolites between dry eye patients before and after acupuncture treatment by using MetaboAnalyst web server.

Table 2　List of six significantly different metabolites between dry eye patients before and after drug treatment

Superclass	Compound name	HMDB number	KEGG number	Fold change (after/before)	*P*
Carbohydrates	glycerate	HMDB00139	C00258	0.60	0.03
Organic acids and derivatives	urate	HMDB00289	C00366	1.48	0.02
Lipids	monopalmitin	HMDB31074	-	2.61	0.00
	propionylcarnitine	HMDB00824	C03017	1.31	0.02
Nucleotides	inosine	HMDB00195	C00294	1.39	0.04
Cofactors	nicotinamide	HMDB01406	C00153	1.40	0.01

into class of nucleotides. Additionally, metabolic pathway analysis of these 4 significantly changed metabolites revealed that only one pathway, aminoacyl-tRNA biosynthesis was significantly influenced ($P<0.05$, Figure 5B).

Table 3 List of four significantly different metabolites between dry eye patients before and after acupuncture treatment

Superclass	Compound name	HMDB number	KEGG number	Fold change (after/before)	*P*
Amino acids and derivatives	alanine	HMDB00161	C00041	0.70	0.02
	serine	HMDB00187	C00065	0.59	0.03
	homoserine	HMDB00719	C00263	0.79	0.04
Nucleotides	cytidine	HMDB00089	C00475	0.50	0.04

DISCUSSION

DED is considered to be one of the top three most prevalent chronic eye diseases, which significantly interferes with patients' well-being and increases their economic burden of healthcare costs[4]. However, it is difficult to diagnose and treat due to the lack of correlation between the signs and symptoms of DED[20]. As a result, it is very important to extend our understanding on the pathogenesis of DED and the possible mechanisms of available effective treatments. Here we used the non-targeted technology, UHPLC-Q-TOF-MS to investigate the metabolic differences between dry eye patients before and after (drug/acupuncture) treatment. This study may not only provide new insight into the metabolomics features in patients with DED, but present potential targets therapy and therapeutic strategies as well.

Generally, the main analytical platforms for metabolomics studies are NMR, GC-MS, and LC-MS. Each analytical platform has its own unique advantages and disadvantages, for instance, NMR is efficient, highly reproducible, and non-destructive, but it has relatively lower sensitivity[9]. GC-MS is the most widespread tandem technique and has high sensitivity and specificity, but especially targeting for volatile non-polar compounds. LC-MS is also an important metabolomic technology, suitable for analyzing large highly polar metabolites. UHPLC, evolved from LC, has greater analytical power, which allows high throughput sample handling and down scaling of sample volume[21]. Since tear is mainly composed of non-volatile compounds including amino acid and lipids, we thus employed UHPLC-Q-TOF-MS global metabolomics analysis on 38 tear samples from patients with DED. Totally 190 named metabolites were identified in the tear, most of which were amino acids & derivatives, and lipids. These identified metabolites were grouped into 13 classes, covering most of the central metabolism pathways. It is believed that our study presented so far the broadest tear metabolome especially for dry eye, corroborating the power of the non-targeted and high-throughput technology in uncovering their metabolic characteristics[14,17-19].

Further analysis indicated a significantly distinct metabolomics profile among all patients with DED, but very subtle metabolic differences upon acupuncture or drug treatment for each individual patient. In the drug treatment group, dry eye patients were offered with artificial tears and tobramycin dexamethasone eye drops. Tobramycin dexamethasone eye drops have been used in ophthalmology since 1983 and are well-accepted ophthalmic solutions effectively used to prevent infection and control inflammation[22]. Obviously, the improvement of both SⅠT and TBUT scores suggested that artificial tears and tobramycin dexamethasone eye drops had an effective and beneficial effect on DED as previously reported[23]. Notably, metabolomic study showed that there were only six significantly changed metabolites. Of particular interest, all the five increased compounds including inosine, monopalmitin, urate, and propionylcarnitine exert a wide variety of anti-inflammatory responses[24-27]. For example, inosine was reported to suppress immune responses and protect mice from experimental autoimmune encephalomyelitis, while monopalmitin may be associated with the cellular toxicity in common across various types of autoimmune inflammatory disorders of the central nervous system[26,28]. Moreover, urate is a powerful antioxidant, which was associated with inflammation, endothelial and renal dysfunction, and cardiovascular disease[24]. Especially, nicotinamide has been proposed as a therapeutic alternative for the prevention of diseases with metabolic dysfunctions, playing critical roles in regulating the inflammatory response and the expression of genes related to oxidative stress[29].

Acupuncture has been used as one of the major therapeutic modalities to treat various diseases for several thousands of years in eastern Asian countries[7]. Recent ophthalmologic studies have shown that acupuncture therapy is an effective treatment for several eye diseases including amblyopia and glaucoma. Especially, the efficacy and effectiveness of acupuncture for DED has also been identified, showing partially better when compared to artificial tear treatment[30]. The results in our study confirmed that acupuncture treatment was likely better compared with drug treatment according to the scores of SⅠT and TBUT. Meanwhile, further metabolomic analysis revealed that merely 4 metabolites were significantly changed in patients after acupuncture treatment, three of which including alanine, serine, and homoserine belonged to amino acids and derivatives. Metabolic pathway analysis of these 4 significantly changed metabolites indicated that only one pathway, aminoacyl-tRNA biosynthesis was significantly influenced. Interestingly, our previous study revealed that the activity of amino acids metabolism was increased in tear samples from patients with DED when compared with those from normal individuals[31]. As a matter of fact, amino acids metabolism plays important roles in multiple aspects of cell biology, which have been identified in various eye diseases including high myopia and cataract[9,32]. Additionally, further metabolic pathway analysis of those 34 significantly changed metabolites in our previous study determined that four pathways including aminoacyl-tRNA biosynthesis were significantly enriched, which was also found in dry eye patients treated by acupuncture[31]. The above changed amino acid associated metabolites and pathways (especially aminoacyl-tRNA biosynthesis) may be involve in protein synthesis and secretion and thus play significant roles on the effective mechanisms of acupuncture therapy[12].

In conclusion, we for the first time comprehensively uncovered metabolic characteristics on dry eye and observed a remarkable metabolic variation among all patients with DED. Moreover, either drug or acupuncture treatment had little effect on metabolomics profile. Importantly, acupuncture may have a distinctive mechanism of improvement on dry eye, which displayed to be partially better compared with drug treatment. The results here enabled us to extend our understanding on the key regulatory elements or pathways involved in the underlying mechanisms of DED treatment, and may further provide novel potential targets and therapeutic strategies. Since the number and types of samples were limited, extensive validation of our findings would be further performed in a larger scale study.

REFERENCES

1 Inomata T, Iwagami M, Hiratsuka Y, Fujimoto K, Okumura Y, Shiang T, Murakami A. Maximum blink interval is associated with tear film breakup time: a new simple, screening test for dry eye disease. *Sci Rep* 2018;8(1):13443

2 Nebbioso M, Fameli V, Gharbiya M, Sacchetti M, Zicari AM, Lambiase A. Investigational drugs in dry eye disease. *Expert Opin Investig Drugs* 2016;25(12):1437-1446

3 Srivastava N, Kollipara RK, Singh DK, Sudderth J, Hu ZP, Nguyen H, Wang S, Humphries CG, Carstens R, Huffman KE, DeBerardinis RJ, Kittler R. Inhibition of cancer cell proliferation by PPARγ is mediated by a metabolic switch that increases reactive oxygen species levels. *Cell Metab* 2014;20(4):650-661

4 Gadaria-Rathod N, Lee KI, Asbell PA. Emerging drugs for the treatment of dry eye disease. *Expert Opin Emerg Drugs* 2013;18(2):121-136

5 Liu Z, Pflugfelder SC. Corneal surface regularity and the effect of artificial tears in aqueous tear deficiency. *Ophthalmology* 1999;106(5):939-943

6 Wojtowicz JC, Butovich I, Uchiyama E, Aronowicz J, Agee S, McCulley JP. Pilot, prospective, randomized, double-masked, placebo-controlled clinical trial of an Omega-3 supplement for dry eye. *Cornea* 2011;30(3):308-314

7 Shin MS, Kim JI, Lee MS, Kim KH, Choi JY, Kang KW, Jung SY, Kim AR, Kim TH. Acupuncture for treating dry eye: a randomized placebo-controlled trial. *Acta Ophthalmol* 2010;88(8):e328-e333

8 Fan X, Rao J, Zhang Z, Li D, Cui W, Zhang J, Wang H, Tou F, Zheng Z, Shen Q. Macranthoidin B modulates key metabolic pathways to enhance ROS generation and induce cytotoxicity and apoptosis in colorectal cancer. *Cell Physiol Biochem* 2018;46(4):1317-1330

9 Ji Y, Rao J, Rong X, Lou S, Zheng Z, Lu Y. Metabolic characterization of human aqueous humor in relation to high myopia. *Exp Eye Res* 2017;159:147-155

10 Kliuchnikova AA, Samokhina NI, Ilina IY, Karpov DS, Pyatnitskiy MA, Kuznetsova KG, Toropygin IY, Kochergin SA, Alekseev IB, Zgoda VG, Archakov AI, Moshkovskii SA. Human aqueous humor proteome in cataract, glaucoma, and pseudoexfoliation syndrome. *Proteomics* 2016;16(13):1938-1946

11 Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, Li XR, Beuerman RW. In-depth analysis of the human tear proteome. *J Proteomics* 2012;75(13):3877-3885

12 Liu Q, Liu J, Ren C, Cai W, Wei Q, Song Y, Yu J. Proteomic analysis of tears following acupuncture treatment for menopausal dry eye disease by two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry. *Int J Nanomedicine* 2017;12:1663-1671

13 Mayordomo-Febrer A, López-Murcia M, Morales-Tatay JM, Monleón-Salvado D, Pinazo-Durán MD. Metabolomics of the aqueous humor in the rat glaucoma model induced by a series of intracamerular sodium hyaluronate injection. *Exp Eye Res* 2015;131:84-92

14 Galbis-Estrada C, Martinez-Castillo S, Morales JM, Vivar-Llopis B, Monleón D, Díaz-Llopis M, Pinazo-Durán MD. Differential effects of dry eye disorders on metabolomic profile by 1H nuclear magnetic resonance spectroscopy. *Biomed Res Int* 2014;2014:542549

15 Li N, Wang N, Zheng J, Liu XM, Lever OW, Erickson PM, Li L. Characterization of human tear proteome using multiple proteomic analysis techniques. *J Proteome Res* 2005;4(6):2052-2061

16 Hu C, Tohge T, Chan SA, Song Y, Rao J, Cui B, Lin H, Wang L, Fernie AR, Zhang D, Shi J. Identification of conserved and diverse metabolic shifts during rice grain development. *Sci Rep* 2016;6:20942

17 Galbis-Estrada C, Pinazo-Durán MD, Martínez-Castillo S, Morales JM, Monleón D, Zanon-Moreno V. A metabolomic approach to dry eye disorders. The role of oral supplements with antioxidants and Omega 3 fatty acids. *Mol Vis* 2015;21:555-567

18 Karamichos D, Zieske JD, Sejersen H, Sarker-Nag A, Asara JM, Hjortdal J. Tear metabolite changes in keratoconus. *Exp Eye Res* 2015;132:1-8

19 Nishtala K, Pahuja N, Shetty R, Nuijts RMMA, Ghosh A. Tear biomarkers for keratoconus. *Eye Vis* 2016;3(1):1-7

20 Nichols KK, Nichols JJ, Mitchell GL. The lack of association between signs and symptoms in patients with dry eye disease. *Cornea* 2004;23(8):762-770

21 Yang B, Li H, Ruan Q, Tong Y, Liu Z, Xuan S, Jin J, Zhao Z. Rapid profiling and pharmacokinetic studies of multiple potential bioactive triterpenoids in rat plasma using UPLC/Q-TOF-MS/MS after oral administration of Ilicis Rotundae Cortex extract. *Fitoterapia* 2018;129:210-219

22 Chen PQ, Han XM, Zhu YN, Xu J. Comparison of the anti-inflammatory effects of fluorometholone 0.1% combined with levofloxacin 0.5% and tobramycin/dexamethasone eye drops after cataract surgery. *Int J Ophthalmol* 2016;9(11):1619-1623

23 Xu YM, Li WM. Analysis of the efficacy of meibomian gland dysfunction associated dry eye treated with integrated strategy. *Modern Medicine Journal of China* 2015;17(11):28-31

24 Carvalho LAC, Truzzi DR, Fallani TS, Alves SV, Toledo JC, Augusto O, Netto LES, Meotti FC. Urate hydroperoxide oxidizes human peroxiredoxin 1 and peroxiredoxin 2. *J Biol Chem* 2017;292(21):8705-8715

25 Haskó G, Kuhel DG, Németh ZH, Mabley JG, Stachlewitz RF, Virág L, Lohinai Z, Southan GJ, Salzman AL, Szabó C. Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. *J Immunol* 2000;164(2):1013-1019

26 Park SJ, Jeong IH, Kong BS, Lee JE, Kim KH, Lee DY, Kim HJ.

Disease type - and status - specific alteration of CSF metabolome coordinated with clinical parameters in inflammatory demyelinating diseases of CNS. *PLoS One* 2016;11(11):e0166277

27 Waagsbø B, Svardal A, Ueland T, Landrø L, Øktedalen O, Berge RK, Flo TH, Aukrust P, Damås JK. Low levels of short-and medium-chain acylcarnitines in HIV-infected patients. *Eur J Clin Invest* 2016;46(5):408-417

28 Junqueira SC, Santos Coelho I, Lieberknecht V, Cunha MP, Calixto JB, Rodrigues ALS, Santos ARS, Dutra RC. Inosine, an endogenous purine nucleoside, suppresses immune responses and protects mice from experimental autoimmune encephalomyelitis: a role for A2A adenosine receptor. *Mol Neurobiol* 2017;54(5):3271-3285

29 Mejía SÁ, Gutman LAB, Camarillo CO, Navarro RM, Becerra MCS, Santana LD, Cruz M, Pérez EH, Flores MD. Nicotinamide prevents sweet beverage-induced hepatic steatosis in rats by regulating the G6PD, NADPH/NADP$^+$ and GSH/GSSG ratios and reducing oxidative and inflammatory stress. *Eur J Pharmacol* 2018;818:499-507

30 Yang L, Yang Z, Yu H, Song H. Acupuncture therapy is more effective than artificial tears for dry eye syndrome: evidence based on a Meta-analysis. *Evid Based Complement Alternat Med* 2015;2015:143858

31 Chen X, Rao J, Zheng Z, Yu Y, Lou S, Liu L, He Q, Wu L, Sun X. Integrated tear proteome and metabolome reveal panels of inflammatory-related molecules *via* key regulatory pathways in dry eye syndrome. *J Proteome Res* 2019;18(5):2321-2330

32 Ling CA, Weiter JJ, Buzney SM, Lashkari K. Competing theories of cataractogenesis after pars Plana vitrectomy and the nutrient theory of cataractogenesis: a function of altered aqueous fluid dynamics. *Int Ophthalmol Clin* 2005;45(4):173-198