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Metabolomics analysis of the saliva in patients with chronic hepatitis B using nuclear magnetic resonance: a pilot study

Kambiz Gilany [1, 2], Ashraf Mohamadkhani [3], Saeed Chashmniam [4], Parisa Shahnazari [1], Mehdi Amini [1], Babak Arjmand [5], Reza Malekzadeh [3], Bibi Fatemeh Nobakht Motlagh Ghoochani [6, 7]*

[1] Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
[2] Integrative Oncology Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran
[3] Digestive Diseases Research Center, Digestive Diseases Research Institute, Tehran University of Medical Sciences, Tehran, Iran
[4] Department of Chemistry, Sharif University of Technology, Tehran, Iran
[5] Cell Therapy and Regenerative Medicine Research Center, Endocrinology and Metabolism Molecular Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
[6] Noncommunicable Diseases Research Center, Neyshabur University of Medical Sciences, Neyshabur, Iran
[7] Department of Basic Medical Sciences, Neyshabur University of Medical Sciences, Neyshabur, Iran

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ABSTRACT

Objective(s): Hepatitis B virus infection causes chronic disease such as cirrhosis and hepatocellular carcinoma. The metabolomics investigations have been demonstrated to be related to pathophysiologic mechanisms in many disorders such as hepatitis B infection. The aim of this study was to investigate the saliva metabolic profile of patients with chronic hepatitis B infection and to identify underlying mechanisms as well as potential biomarkers associated with the disease.
Materials and Methods: Saliva from 16 healthy subjects and 20 patients with chronic hepatitis B virus were analyzed by nuclear magnetic resonance (NMR). Then, multivariate statistical analysis was performed to identify discriminative metabolites between two groups.
Results: A set of metabolites were detected, including propionic acid, putrescine, acetic acid, succinic acid, tyrosine, lactic acid, butyric acid, pyruvic acid, 4-pyridoxic acid and 4-hydroxybenzoic acid, which in combination with one another could accurately distinguish patients from healthy controls. Our results clearly demonstrated altered metabolites are involved in nine metabolic pathways.
Conclusion: Metabolomics has the potential to be considered as a novel clinical tool for hepatitis B diagnosis while contributing to a comprehensive understanding of disease mechanisms.

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Introduction

Saliva is a complex fluid, mostly composed of water (99%) and organic and inorganic compounds including a variety of electrolytes, metabolites, proteins, enzymes, immunoglobulins, antimicrobial factors, bacterial cells, mucosal glycoproteins, polypeptides and oligopeptides which are important in oral health (1, 2). Indeed, these compounds derived from the blood by passing through the transcellular or paracellular spaces, therefore saliva is comparable to serum in regard to showing the physiological state of the body (3). In comparison with other bio-fluids, such as urine and blood, saliva is a simple, cost-effective, accurate, and noninvasive diagnostic method. The saliva metabolome performance as biomarkers and monitoring diseases is an efficient and informative molecule. Metabolome is currently used in diagnosis of many diseases such as autoimmune diseases, infectious diseases, cardiovascular disease, and cancers (3-5).

Since the human fluid metabolome reveals enormous complexity, plenty of feasible analysis methods are used to demonstrate metabolites and develop metabolomics integration into the systems biology (6). Nuclear magnetic resonance (NMR) is a highly sensitive technology which is frequently applied in the metabolomics studies in human bio-fluids. NMR based metabolomics is a well-defined technique for describing the metabolic profile of biological fluids and investigating any alteration in response to biological or clinical imaging. In other words, NMR-based metabolomics demonstrates the ability of biomarkers to detect, diagnose and monitor the treatment. The development of this analytical technique provides a powerful method for clinicians to address clinical decisions and improve prescriptions in treatment strategies (6, 7).

Hepatitis B virus (HBV) is the main cause of chronic Hepatitis B infection (CHB), cirrhosis and hepatocellular carcinoma (HCC), with a high morbidity and mortality rate (8). Patients with CHB are different in levels of virus replication, disease progression and immune responses. HBV-induced liver inflammation and fibrosis can cause metabolic changes reflecting metabolic disorders of serum (9, 10). The main purpose of this study was the identification of the saliva metabolite in a case-control study. 1H-NMR spectroscopy was used to clarify the saliva metabolomics evaluations and to assess differences in CHB and healthy subjects.

**Corresponding author:* Bibi Fatemeh Nobakht Motlagh Ghouchani. Noncommunicable Diseases Research Center, Neyshabur University of Medical Sciences, Neyshabur, Iran; Department of Basic Medical Sciences, Neyshabur University of Medical Sciences, Neyshabur, Iran. Tel: +98-514263506; Fax: +98-5142627500; Email: nobakhtf1@nums.ac.ir

Materials and Methods

Participants

Unstimulated saliva samples were collected from 20 pre-selected chronic HBV patients and 16 healthy controls in fast conditions at Shariati Hospital, Tehran, Iran. The diagnosis of HBV was based on serum alanine aminotransferase (ALT) and HBV-DNA levels (11). To remove the effect of gender on the metabolomics profile, all participants were men with no alcohol consumption in the sample day. The specimens were collected at least 8 hr after the last meal, started approximately at the same time for each participant (9 am). Approximately 2 ml of the saliva was collected from each participate by requesting subjects to drop into a sterile plastic container. Saliva samples were free of blood in the visual evaluation. Samples were immediately frozen at -80 ˚C until assessment. This study was performed in accordance with the principles of clinical practice. The participant received written and informed consent. The study was reviewed and approved by the Institute for Ethical Research of Digestive Disease Research (DDRI).

NMR acquisition and data processing

NMR analysis was carried out according to our previous studies (12, 13). All NMR spectra were recorded with Bruker DRX500 MHz spectrometer operating at 500 MHz, equipped with 5 mm high-quality NMR tubes (Sigma Aldrich., RSA). After thawing the saliva in room temperature, the samples were centrifuged at 10,000 rpm for 10 min to remove particulate matter and the supernatants were used for NMR. Saliva (600 µl) and D_2O (100 µl) (deuterium oxide, 99.9% D, Aldrich Chemicals Company) were mixed and transferred to 5 mm NMR tube. ^1H NMR spectra of the saliva samples were acquired at 25 ˚C using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence, $\pi/2\text{-}t_D\text{-}\pi\text{-}t_D$, to reduce signals from high macromolecules such as polypeptides and proteins (14). The acquisition parameters included a 10.5 µs 90˚ pulse, a relaxation delay of 2 sec, a spectral width of 8389.26 Hz, an acquisition time of 1.95 sec, 32 k data points, 154 scans and line broadening 0.3 Hz.

The NMR spectra were referenced to solvent within XWINNMR. All spectra were manually phased and baseline corrected using the XWINNMR (version3.5, Bruker Spectrospin Ltd). The regions 0.2–10 ppm were divided into 0.02 ppm wide buckets by the ProMetab software (version prometab_ v3_3) (15) in MATLAB (version 6.5.1, The MathWorks, Cambridge, UK), excluding the region 4.2–5.5 ppm around the water peak. For all spectra baseline correction, normalization, and alignment were performed using ProMetab software in MATLAB. Then data were imported to SIMCA 14.0 (Umetrics, Umea, Sweden) for multivariate statistical analysis.

Statistical analysis

A multivariate analysis of the datasets obtained from ^1H-NMR spectra of the saliva samples using SIMCA 14.0 (Umetrics, Umeå, Sweden). First, principal component analysis (PCA), as an unsupervised statistical method, was used to detect outliers and find the structure of the data. Next, the orthogonal projection to latent structures-discriminant analysis (OPLS-DA) as a supervised statistical method, was performed to elucidate discriminant metabolites between two groups. The quality of the OPLS-DA model was evaluated by R^2X, R^2Y and Q^2, where R^2 and Q^2 are the goodness of fit and goodness of prediction, respectively. Moreover, a receiver operating characteristic (ROC) analysis obtained from the 7-fold cross-validation method using SIMCA for further validation of the performances.

Random Forest (RF) is another supervised statistical analysis suitable for "omics" data. It is based on decision trees. One third of samples were used as out-of-bag (OOB) samples and two-third of cases are called the training set. The OOB samples were used for error estimation. In the present study, the number of trees was selected 500. The RF analysis was performed using the RF package in MATLAB for statistical computing, similar to our previous study (13).

After performing two multivariate statistical analysis (OPLS-DA and RF), significant bins based on both models were reported.

Metabolite identification and pathway analysis

All of the identified metabolites were extracted from human metabolome database (HMDB), Kyoto Encyclopedia of Genes and Genomes(KEGG), literature and Bayesil software as a web system that automatically identifies and quantifies metabolites using 1D-H-NMR spectra.

MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) was used for metabolic pathways analysis (MPA) (16, 17). In this study, identified metabolites by ^1H-NMR which significantly altered in the saliva of CHB patients were entered, the Homo sapiens library was chosen and used the default hypergeometric test and relative-betweenness centrality algorithms as the options for the over-representation analysis and pathway topology analysis, respectively. The most relevant pathways were reported based on *P* values and false discovery rate (FDR) less than 0.05.

Results

Clinical patient characteristics

No significant difference in age differences exists between patients and healthy subjects in all male participants in this study (35.1(2.1) vs 32(2.5) P=0.1). In CHB patients, the mean (SD) of serum alanine transaminase (ALT) was 52.7(7) IU/l that was clearly higher than controls with 28.3(1.2) IU/l (P = 0.002). HBV DNA level of the serum in patients was 3.45 (0.26) log copies/ml which applied to characterize hepatitis B infection.

Discrimination between patients and healthy controls using multivariate analysis

CPMG spectra of the saliva samples from CHB patient is shown in Figure 1. For the analysis of untargeted metabolomics data, first, PCA was used to check general interrelation between the groups (healthy controls vs the CHB patients). The PCA score plot did not show a distinct clustering between two groups. In addition, three samples were not in the Hotelling's T2 99% confidence limit, therefore they were deleted. Consequently, final PCA was carried out after excluding

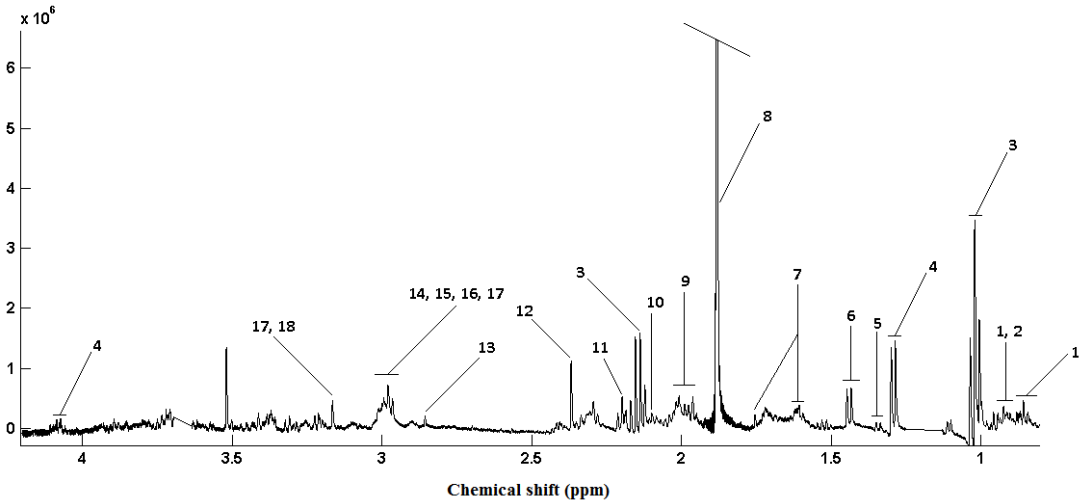


Figure 1. Representative 500 MHz 1H NMR spectra of human saliva from chronic Hepatitis B infection (CHB)patient. 1, isoleucine; 2, leucine; 3, propionate; 4, lactate; 5, 2-hydroxyisobutyrate; 6, alanine; 7, ornithine; 8, acetate; 9, proline; 10, butyrate; 11, 2-aminoadipate; 12, pyruvate; 13, trimethylamine; 14, creatine; 15, histamine; 16, 2-oxoglutarate; 17, anserine; 18, dimethyl sulfone

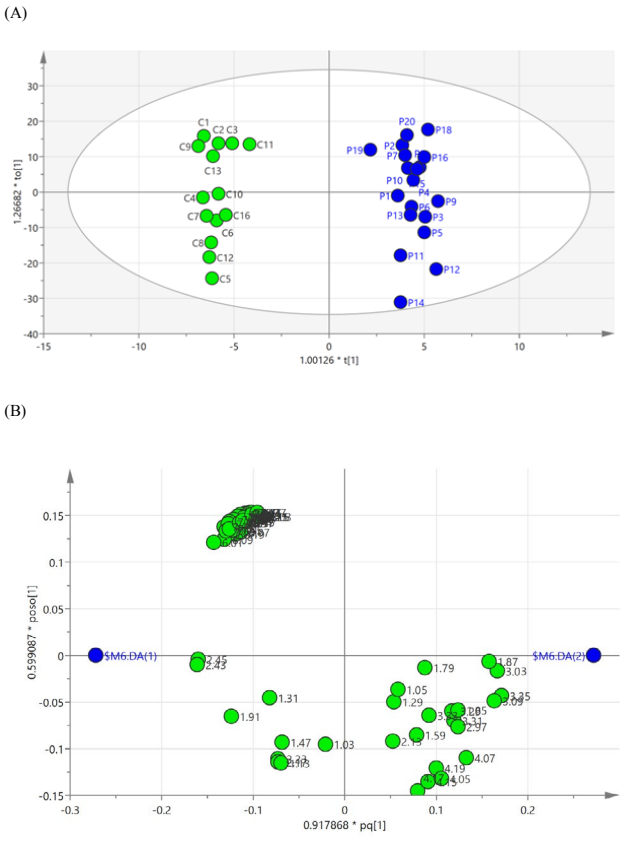


Figure 2. (A) The score plot of the OPLS-DA. The OPLS-DA score plot illustrating a clear discrimination between CHB patients (blue circle) and healthy controls (green circle), (B) The OPLS-DA loading plot displacing discriminant variables between CHB patients and controls

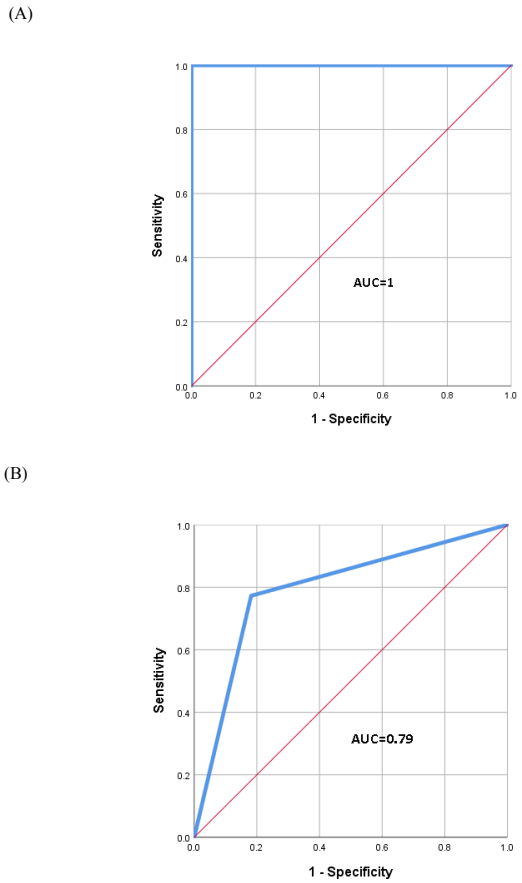


Figure 3. (A) ROC curve analysis for the OPLS-DA, (B) ROC curve analysis for the RF model

three outliers (R^2X=0.772, Q^2=0.614).

The OPLS-DA was performed to identify metabolic pattern differences between control and CHB groups. The Score plot of the OPLS-DA illustrated that two groups were effectively separated from each other (R^2X=0.834, R^2Y=0.979, Q^2=0.715; Figure 2A). The loading plot of the OPLS-DA model has been shown in Figure 2B. This loading plot demonstrated the chemical shifts of the NMR spectra that are responsible for clustering in the OPLS-DA score plot (Figure 2B).

The accuracy of the OPLS-DA and RF model was calculated by receiver operating characteristic (ROC) curve which compares sensitivity versus specificity across a range of values for the ability to predict a dichotomous outcome (Figure 3A and B), the area under the ROC curve (AUC) value was 1 for the OPLS-DA and

Table 1. Confusion matrix of the random forest model for the training and test sets

	Observed	Predicted	
		CHB class	Healthy class
Training set	CHB class	13	1
	Healthy class	4	6
Test set	CHB class	4	1
	Healthy class	1	3

Table 2. The classification parameters of training and test sets

	Error rate	Non-error rate	Specificity	Sensitivity	Accuracy
Training set	0.20	0.80	0.60	0.92	0.80
Test set	0.22	0.78	0.75	0.80	0.78

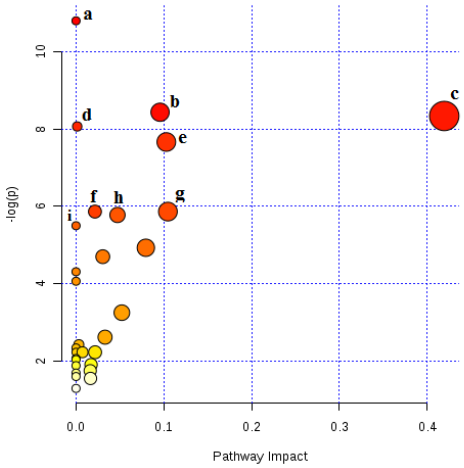
0.79 for the RF model. Table 1 and 2 demonstrated the confusion matrix and the classification parameters of the RF model for the training and test sets.

Metabolite identification and pathway analysis

A list of metabolites responsible for the discrimination between two groups of study (with fold change > 1.5) was shown in Table 3. Propionic acid, putrescine, acetic acid, succinic acid and tyrosine were clearly identified in the saliva of CHB patients. However, a remarkable reduction in concentrations was observed for L-lactic acid, butyric acid, pyruvate, 4-pyridoxic acid and 4-hydroxybenzoic acid.

Figure 4A and B showed altered metabolic pathways in the saliva from CHB patients using MetaboAnalyst tool. This method could identify nine metabolic pathways indicating alterations in CHB patients. These pathways were phenylalanine metabolism, glycolysis or gluconeogenesis, pyruvate metabolism, propanoate metabolism, butanoate metabolism, taurine and hypotaurine metabolism, citrate cycle, tyrosine metabolism, and alanine, aspartate, and glutamate metabolism.

(A)



(B)

Pathway Name	Match Status	p	-log(p)	Holm p	FDR
Phenylalanine metabolism	4/45	2.0653E-5	10.788	0.0016523	0.0016523
Glycolysis or Gluconeogenesis	3/31	2.1854E-4	8.4286	0.017264	0.0063083
Pyruvate metabolism	3/32	2.4061E-4	8.3323	0.018768	0.0063083
Propanoate metabolism	3/35	3.1542E-4	8.0616	0.024287	0.0063083
Butanoate metabolism	3/40	4.7093E-4	7.6608	0.035791	0.0075348
Taurine and hypotaurine metabolism	2/20	0.002837	5.865	0.21278	0.030948
Citrate cycle (TCA cycle)	2/20	0.002837	5.865	0.21278	0.030948
Tyrosine metabolism	3/76	0.0030948	5.778	0.22592	0.030948
Alanine, aspartate and glutamate metabolism	2/24	0.0040847	5.5005	0.2941	0.036309

Figure 4. Metabolic pathway analysis using MetaboAnalyst 3. (A) Altered metabolic pathways in the saliva from CHB patients. (B) Statistics for pathways with major change based on FDR and *P-values* less than 0.05: (a) phenylalanine metabolism, (b) glycolysis or gluconeogenesis, (c) pyruvate metabolism, (d) propanoate metabolism, (e) butanoate metabolism, (f) taurine and hypotaurine metabolism, (g) citrate cycle, (h) tyrosine metabolism, and (i) alanine, aspartate, and glutamate metabolism

Discussion

Metabolomics has a few advantages over genomics on the diagnosis of complex chronic disease. In this study, ten different metabolites were observed in CHB patients in comparison with healthy subjects using non-targeted metabolomics analysis. 1_H NMR spectra of the saliva clearly indicated up-regulated levels of propionic acid, putrescine, acetic acid, succinic

Table 3. The saliva metabolites that play an important role in discrimination of patients with chronic hepatitis B infection (CHB) and healthy controls. The arrow directions indicate the up-regulation and down-regulation of metabolites in CHB patients as compared with control group

No.	Metabolite name	HMDB ID	Chemical shift (ppm) [a]	Fold change (CHB/control)	Direction of fold change	*P*-value [b]	VIP [c]
1	L-Lactic acid	HMDB00190	1.31	6.92	↓	<0.05	1.08
2	Butyric acid	HMDB00039	1.47,2.17	2.47	↓	<0.05	1.35
3	Pyruvate	HMDB00243	2.47	5.22	↓	<0.05	0.83
4	4-pyridoxic acid	HMDB00017	7.53	1.78	↓	<0.01	2.05
5	4-hydroxybenzoic acid	HMDB00500	7.79, 7.81	1.6	↓	<0.05	1.73
6	Propionic acid	HMDB00237	1.05	3.05	↑	<0.05	2.37
7	Putrescine	HMDB01414	1.79	1.73	↑	<0.05	1.24
8	Acetic acid	HMDB00042	1.87	22.77	↑	<0.001	2.21
9	Succinic acid	HMDB00254	2.43	2.04	↑	<0.01	2.03
10	Tyrosine	HMDB00158	3.03	1.55	↑	<0.05	1.22

[a] Chemical shift of signal used for quantification; [b] P-value calculated by independent t-test (for variables with a normal distribution) and Mann-Whitney U test (for variables with a non-normal distribution); [c] Variable importance in the projection

acid and tyrosine metabolite. However, a significant reduction was detected in L-lactic acid, butyric acid, pyruvate, 4-pyridoxic acid and 4-hydroxybenzoic acid. As shown in Figure 4, nine metabolic pathways found to be significantly changed between the two groups. These pathways were phenylalanine metabolism, glycolysis or gluconeogenesis, pyruvate metabolism, propanoate metabolism, butanoate metabolism, taurine and hypotaurine metabolism, citrate cycle, tyrosine metabolism, and alanine, aspartate, and glutamate metabolism. To the best of our knowledge, this is the first time that metabolic profiling using NMR was investigated in the saliva from CHB patients.

In this study, the level of acetate, the end product of lipid metabolism, increased. This finding supports the increase of lipid metabolism in the response to the liver injury which is related to the CHB patients. This finding is in agreement with previous investigations that reported an increase in serum acetate levels in other liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) (18, 19).

Another altered metabolite is succinate, a key substrate of the tricarboxylic acid (TCA) cycle, which increased in CHB patients as compared with healthy controls. In HepG2.2.15 cells, highly used in studies on HBV, some enzymes that regulate TCA were up-regulated and also several TCA cycle intermediates such as succinate were increased that could indicate the increase in TCA cycle (20). Enhanced TCA along with mitochondrial dysfunction (21, 22) can result in the production of oxidative stress (23) in hepatitis patients.

Our results showed reduced levels of pyruvate and lactate, produced by glycolytic pathway (24), in the saliva from CHB patients compared with the control group. This finding is consistent with the study of Gonçalves *et al.* (25) who investigated the salivary proteomes of patients infected by HBV by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and showed that glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved in glycolysis pathway, is down-regulated in patients infected by HBV in comparison with healthy controls. Besides, lactate can be metabolized to acetate and propionate by oral bacterium *Veillonella* (26). Furthermore, lactic acid and butyric acid are the by-product of bacterial metabolism and catalyzed by lactate dehydrogenase (LDH) which derived from *Streptococcus*, *Lactobacillus*, and *Actinomyces* (27). One more metabolite of interest, 4-pyridoxic acid, decreased in the saliva of CHB patients as compared with control group. 4-pyridoxic acid is the catabolic product of vitamin B_6 which is formed by the action of aldehyde oxidase (endogenous enzyme) and pyridoxal 4-dehydrogenase (microbial enzymes). Presumably reduced levels of 4-pyridoxic acid might be due to impaired vitamin B_6 metabolism, altered levels of oral microbiota (28) or decreased concentrations of vitamin B_6 in these patients (29). Since B vitamins have antioxidant and anti-inflammatory effects (30), the host can use these vitamins to diminish hepatic inflammatory stress. Besides, several reports have demonstrated that oral dysbiosis, an imbalance in oral microbiota, could be involved in liver diseases such as HBV-induced chronic liver disease and cirrhosis (28, 31). Since various metabolites including short-chain organic acids, amines and phenyl compounds are produced by oral microbiota (28); thus oral dysbiosis could be related to the alterations in these small molecules in the saliva of CHB patients.

An increased level of serum tyrosine has been observed in non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH) (32) and HCC patients (19). These observations are in line with our findings and suggested that liver diseases may promote similar molecular events which are involved in tyrosine metabolism. One reason for this result may be explained by aromatic amino acid (AAA) transporters. SLC16A10, a uniporter that transports of AAA into the liver from the blood, was down-regulated in NASH patients that might lead to a reduction in AAA metabolism in the liver and an accumulation of these amino acids in the blood (33, 34) and subsequently in the saliva.

In the present study, a potential of biomarkers for detecting CHB patients was established. It indicates that diagnosis based on the saliva metabolomics not only may discern CHB compared with healthy controls, but also can be a promising approach to clarify underling mechanisms of disease.

Conclusion

The successful establishment of a metabolic profiling in the saliva revealed that utilizing metabolomics is a promising approach to find characteristic metabolites in the saliva from HBV patients. Our research is useful to understand the origin of salivary metabolites in CHB patients that is more related to the oral microbiota. The relation between the saliva metabolites which extracted from MetaboAnalyst software and KEGG pathway Database shows alterations in metabolism several important pathways. Metabolites showing potential biomarkers between CHB patients and healthy controls are related to lipid metabolism, amino acid metabolism, and vitamin metabolism. One metabolite of interest was 4-pyridoxic acid that reduced in patients with CHB. Decreased levels of this metabolite might be because of changed levels of oral microbiota, reduced concentrations of vitamin B_6 or impaired metabolism of vitamin B_6. The characteristic of down-regulated metabolites identified in Vitamin B metabolism pathway would provide reliable evidence supporting the clinically use of vitamin B in CHB patients. Additionally, the quantity of 4-pyridoxic acid in the saliva in association with CHB outcome could be valuable. Future research should focus on these characteristic metabolites to confirm the clinical value.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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