Original Article

Metabolomic characterization of congenital microtia: a possible analysis for early diagnosis

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Background: Although metabolic abnormalities have been deemed one of the essential risk factors for growth and development, the relationship between metabolic abnormalities and microtia is still unclear. In this study, we aimed to establish a cell model of microtia and the changes of serum metabolites in patients with microtia.

Methods: After constructing a cell model of microtia with low expression of BMP5, we performed integrative metabolomics analysis. For the altered metabolites, the content of glycerophosphocholine (PC), triacylglycerol (TG), and choline in the serum of 28 patients (15 patients with microtia and 13 controls) with microtia was verified by enzyme-linked immunosorbent assay (ELISA).

Results: Detailed metabolomic evaluation showed distinct clusters of metabolites between BMP5-low expressing cells and normal control (NC) cells. The cell model of microtia had significantly higher levels of TG, PC, glycerophosphoethanolamine (PE), sphingomyelin, sulfatide, glycerophosphoglycerol, diacylglycerol, and glycosphingolipid. The main abnormal metabolites were mainly concentrated in the glycerophospholipid metabolism pathway, and PC and choline were closely related. In the serum of patients with microtia, the contents of PC, TG, and choline were significantly increased.

Conclusions: The individual serum samples confirmed the different metabolites between patients with microtia and controls. In particular, we showed that a newly developed metabolic biomarker panel has a high sensitivity and specificity for separating patients with microtia from controls.

Keywords: Metabolomic characterization; congenital microtia; glycerophosphocholine; triacylglycerol; choline

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Introduction

Congenital microtia is one of the most common congenital craniofacial malformations in children, with an incidence of (0.83–17.40)/10,000 (1-6), which also has a significant familial clustering (7). There are significant regional differences in the incidence of congenital microtia worldwide (1-6). It has been reported that there is a correlation between the occurrence of congenital microtia and parental dietary habits (8,9), and some foods, such as those containing caffeine, have even been considered risk factors for the occurrence of congenital microtia (9). Meanwhile, metabolomic features can affect the development and outcome of a variety of diseases (10-15). Abnormal metabolomics can lead to congenital heart malformations (16-18), diaphragmatic hernia (19), and glaucoma (20). Some metabolomic abnormalities can even serve as biomarkers of disease (21). Normal metabolic pathways were an important guarantee for the normal development of cartilage (22,23), while abnormal metabolic pathways may lead to cartilage tumors or osteoarthritis (24-28). Although there was a study on metabolic mass spectrometry of microtia cartilage (29), it cannot be used for early diagnosis. Abnormal metabolites in the blood were more conducive to early diagnosis. Currently, the relationship between metabolomic abnormalities and blood of congenital microtia remains unclear.

Bone morphogenetic protein 5 (BMP5), as a member of the bone morphogenetic protein family, is involved in the morphogenesis of bone and cartilage, the attachment of soft tissue, and the development of limbs in humans (30). Several studies have found that BMP5 is related to the occurrence of congenital microtia (31-34). Our group has previously demonstrated the possible mechanism of congenital microtia, which may be caused by abnormal lipid metabolism due to the low expression of BMP5, changing mitochondrial function (34). The influence of specific metabolites and possible drug interventions have not been investigated. This study aimed to verify the metabolites with low BMP5 expression through *in vitro* experiments, and to explore the metabolomic characteristics of congenital microtia with low BMP5 expression, which may help to provide guidance for the prevention of congenital microtia during pregnancy.

In this study, we established cell and patient arrays, including 15 microtia patients and 13 controls, for metabolomic analysis in cell models and further identification in serum samples. We used metabolomic analysis to investigate contributions of BMP5-low expressing cells and normal control (NC) cells signatures and analyzed the metabonomic characteristics to diagnosis of microtia. The data presented here furnish a resource for future early diagnosis of microtia. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5614/rc).

Highlight box

Key findings

- Serum metabolic biomarkers could distinguish between microtia and controls.

What is known and what is new?

- Metabolic abnormalities are one of the essential risk factors for the abnormity of growth and development.
- The metabolites different between microtia and controls. Meanwhile, these metabolic biomarkers show high sensitivity and specificity for the diagnosis of microtia.

What is the implication, and what should change now?

- Metabolic biomarkers could be used for the early diagnosis of microtia.

Methods

Study population

Our study included 28 patients in total, who were grouped as follows: (I) Microtia group (n=15): patients with microtia; (II) Control group (n=13): patients without microtia. The diagnosis of microtia was based on our previous study (7). Cases of acquired auricle deformity was excluded. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All participants and their parents or legal guardians provided written informed consent, and all procedures were approved by the Ethics Committee of Sun Yat-sen Memorial Hospital of Sun Yat-sen University (No. 2019-KY-024).

Metabolite extraction

To extract metabolites from the cell model (siBMP5-2 and siBMP5-3), 2.5 mL of methyl tert-butyl ether (MTBE; Thermo Fisher Scientific, Waltham, MA, USA) was added to 1 mL of the cell extract after thawing at room temperature. Then, the mixture was thoroughly vortexed for 60 seconds, added to 600 µL of water (Merck Millipore, Burlington, MA, USA), shaken for 30 minutes at room

temperature, rested for 30 mins, and then centrifuged at 5,000 g for 15 minutes. After that, we transferred 1,000 µL of the lipid extract of the upper layer to a 1.5 mL EP tube, concentrated and dried it under reduced pressure (Labconco Corporation, Kansas City, MO, USA), and used 150 µL of acetonitrile-isopropanol mixture (Thermo Fisher Scientific, USA) for the lipid extract [containing the lipid extract standard PC(15:0/18:1(d7)), TG(15:0/18:1(d7)/15:0), SM(18:1(d9)), LPC(18:1(d7)), PG(15:0/18:1(d7)), ChE(18:1(d7)), LPE(18:1(d7)), and PE(15:0/18:1(d7)) (Avanti, Alabaster, AL, USA)] dissolution.

Ultra-high performance liquid chromatography-high resolution mass spectrometry analysis

For the untargeted metabolomics of the polar metabolites, the extracts were analyzed using ultra-high performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS; Thermo Fisher Scientific, USA). Lipid molecules were separated by chromatography on an Accucore C30 core-shell column (Thermo Fisher Scinetific, USA) at 50 ℃ and eluted with 60% acetonitrile in water (A) and 10% acetonitrile in isopropanol (B) (Thermo Fisher Scientific, USA). The separation gradient was set up as follows: 10% B at first, ramping up to 50% in 5 minutes, then increasing to 100% in 23 minutes, with the final 7 minutes being used for column washing and equilibration at a flow rate of 0.3 mL/min. The Quadrupole-Orbitrap mass spectrometer (Thermo Fisher, USA) was used in conjunction with a heated electrospray ionization source that had the following operating conditions: capillary temperature 320 ℃, heater temperature 355 ℃, sheath gas 45 arb, aux gas 10 arb, and S-Lens RF level 55%. The acquisition mass range was 250–2,000 m/z. For thorough lipid structural annotation, quality control (QC) samples were repeatedly injected to obtain the top 10 data-dependent MS2 spectra (full scan-ddMS2). For the whole tandem mass spectrometry (MS/MS) data capture, a resolution setting of 17,500 FWHM was chosen. The precursor isolation window was set at 1.0 Da; dynamic exclusion, isotope exclusion, and apex trigger were all activated. Utilizing ultra-pure nitrogen as the fragmentation gas, stepped normalized impact energy was used to cause the dissociation of a metabolite during a collision.

Data processing and analysis

MetaboAnalyst (version 5.0, http://www.metaboanalyst.ca/) (27,28), a free online software, was used to analyze the differences in metabolites and metabolite pathways between the siBMP5 and siNC groups. Principal component analysis (PCA) analysis among all QC samples and the other samples could evaluate the quality control throughout UPLC-HRMS analysis. Metabolites with analysis of variance (ANOVA) test P value <0.05 were considered for further investigation. The summit of the volcano plot showed data points with low ANOVA test P values (highly significant); and those with large fold change (FC) appeared toward the left or right of the center. The cutoff value was set at $|\log2(FC)| > 0.58$. Orthogonal partial least squares discriminant analysis (OPLS- DA) allowed selection of 15 metabolites with the highest variable influence on projection (VIP) scores (VIP >1.5) which were considered important for group discrimination. Visualizing the top 50 most important differential metabolites ranked by FC intuitively in a hierarchical clustering heatmap demonstrated the proportion of significantly altered metabolite components.

Pathway analysis

In order to identify differential metabolic pathways between the siBMP5 and siNC groups, pathway analysis was performed with MetaboAnalyst 5.0 (35,36). Over-representation was assessed using hypergeometric tests with Holm-Bonferroni-corrected P values. Differential metabolism was regarded to have significantly enhanced metabolic pathways when P<0.05. The pathway impact value threshold was set to >0.10 (37,38), and the pathway topological analysis was based on the relative betweenness centrality (39).

Determination of PC, TG, and choline contents

Serum samples of all the participants were collected and stored at –80 ℃ for later analysis. We used enzyme-linked immunosorbent assay (ELISA) kits (Meimian, Nanjing, China) to quantify the concentration (PC, TG, and choline) of the samples for confirmation of the differential metabolite changes in cell model, following the kit manufacturer's instructions. On a microplate reader (SpectraMax® absorbance readers, Molecular Devices, Shanghai, China), the optical density was read at 450 nm. All the samples were analyzed in a single step and in duplicate.

Statistical analysis

The data from serum samples were analyzed using SPSS

Table 1 Summary of participants' clinical characteristics

Variables	Microtia group (n=15)	Control group (n=13)
Age (years)	10.47±2.53	15.75±11.23
Gender		
Male	13 (86.67)	8 (61.54)
Female	2 (13.33)	5 (38.46)
Affected side		
Left side	5 (33.33)	0
Right side	10 (66.67)	0
Both sides	0	0
Grade of hearing loss		
Normal hearing	1 (6.67)	11 (84.61)
Mild hearing loss	1 (6.67)	2 (15.38)
Moderately severe hearing loss	6 (40.00)	0
Severe hearing loss	7 (46.66)	0

Data are expressed as mean ± standard deviation or n (%).

23.0 software (IBM Corp., Armonk, NY, USA), which were expressed as a mean ± standard deviation via the *t*-test between two groups. Statistical significance was considered when P<0.05. Meanwhile, receiver operating characteristic (ROC) analysis illustrated the performance of the newly established metabolic biomarker panel by considering sensitivity and specificity of model through SPSS 23.0.

Results

Clinical characteristics

To confirm the metabolomics changes in the cell model, we profiled serum metabolites of 28 participants, which were collected consecutively at random time period, including 15 patients with microtia and 13 controls. The clinical characteristics of these participants are displayed in *Table 1*.

Analyses of characteristic metabolites to distinguish between microtia patients with low BMP5 expression and control groups

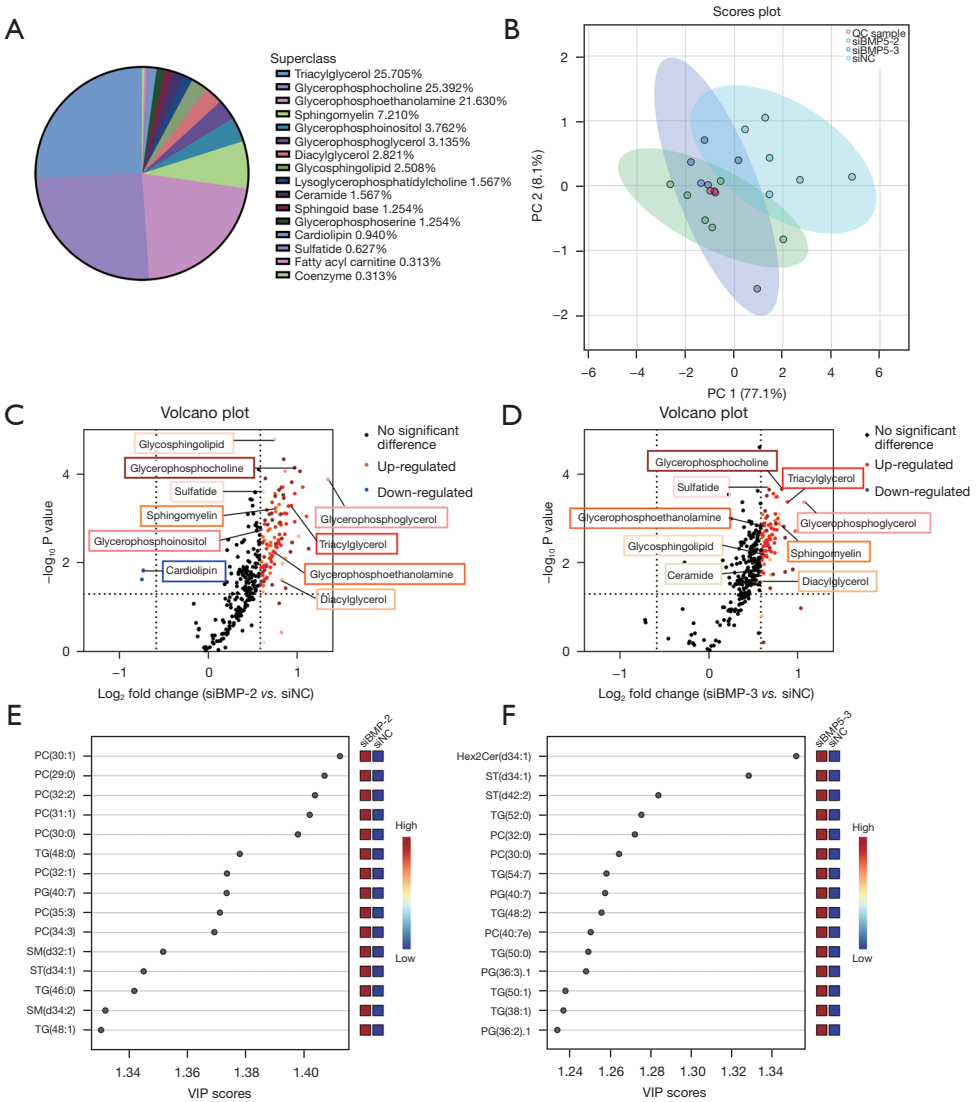
Through detection of non-targeted metabolite profiles on transfected 293T cell samples, we found 319 metabolites in total, in which TGs (25.705%), PCs (25.392%) and glycerophosphoethanolamines (PEs) (21.630%) accounted for the largest proportion (*Figure 1A*). According to the PCA analysis (*Figure 1B*), the QC samples were closely clustered together, confirming that the analytical data was reliable. We identified 190 differential metabolites between siBMP5-2 and siNC control groups, and 255 between siBMP5-3 and siNC groups (ANOVA test P<0.05). Discriminant analysis of the OPLS-DA model revealed distinctive clustering for siBMP5 and siNC groups (Figure S1A,S1B). The performance of OPLS-DA showed no overfitting (Q2 =0.71, R2Y =0.872 in siBMP5-2 *vs.* siNC groups, and Q2 =0.79, R2Y =0.995 in siBMP5-3 *vs.* siNC) (Figure S1C,S1D), evaluated by means of cross-validation and permutation test. These findings suggested that metabolites altered distinctly in microtia patients with low BMP5 expression compared to controls.

Comparison of characteristic metabolites between groups

We then found the visibly altered metabolites to discriminate between microtia patients and controls. There were 117 (with 115 enriched and 2 depleted metabolites) and 95 (all were enriched) significantly differential metabolites altogether in siBMP5-2 and siBMP5-3 versus the siNC group respectively (adjusted P<0.05 and |log2(FC)| >0.58), which was annotated according to metabolite chemical classes (*Figure 1C,1D*). These included 8 significantly upregulated metabolites of TG, PC, PE, sphingomyelin, glycerophosphoglycerol, diacylglycerol, glycosphingolipid, and sulfatide. The OPLS-DA model also provided the VIP score (*Figure 1E,1F*), showing the top 15 metabolites with the highest VIP scores and respective concentrations for each comparison, including TG, PC, PE, sphingomyelin, glycerophosphoglycerol, and glycosphingolipid. Hierarchical clustering heatmaps were constructed based on the differential metabolites correspondingly (P<0.05) (*Figure 1G,1H*). These results indicated that 8 of these metabolites may play a role in distinguishing between microtia patients with low BMP5 expression and controls.

Pathway analysis

To evaluate potentially altered metabolic pathways, we found that these differential metabolites were enriched in 6 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in both groups in comparison, but only glycerophospholipid metabolism showed an important number of effective metabolites matching to this pathway



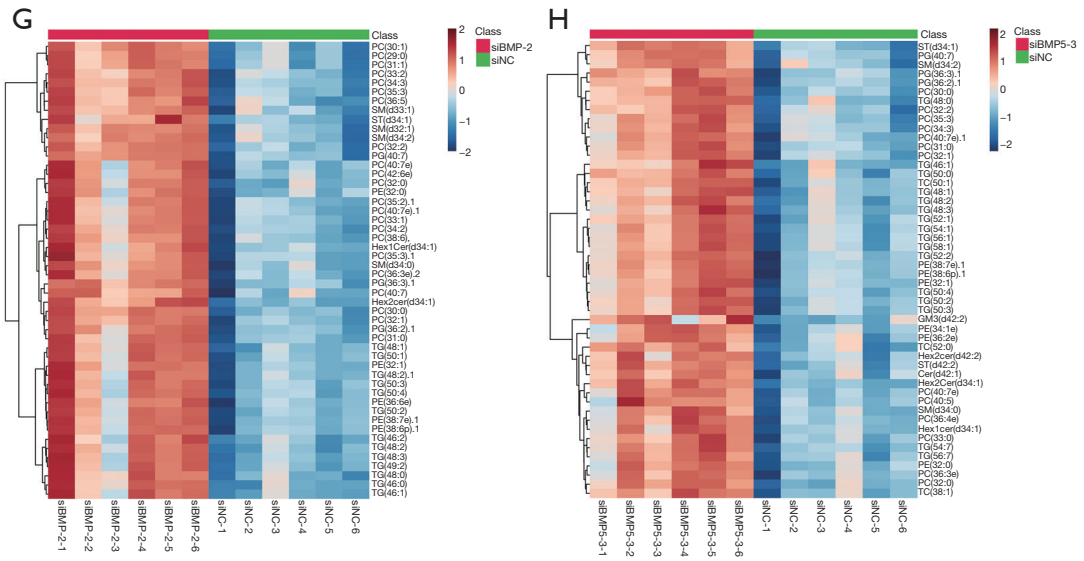


Figure 1 Metabolites profiling for siBMP5 *vs.* siNC. (A) Proportion of identified metabolites in each chemical classification. (B) Overview of PCA score plot obtained from all siBMP5-2, siBMP5-3, siNC, and QC samples. Volcano map of metabolites for siBMP5-2 *vs.* siNC groups (C), siBMP5-3 *vs.* siNC groups (D). Colors indicate up- or down-regulation of metabolites according to the cut-off adjusted P value <0.05 and |log2(FC)|>0.58. The top 15 VIP metabolites of siBMP5-2 *vs.* siNC (E), siBMP5-3 *vs.* siNC (F). The colored boxes on the right indicate the relative amount of the corresponding metabolite in each group. Hierarchical clustering heatmap of the top 50 most important differential metabolites. For class name, red represents siBMP5-2 (G) and siBMP-3 groups (H); green represents siNC groups. For the expression level of each lipid, red represents high and green represents low. PCA, principal component analysis; QC, quality control; VIP, variable importance in projection.

(*Tables 2,3* and *Figure 2*) via over-representation analysis. It displayed the greatest impact value (0.26 in *Table 2*, 0.20 in *Table 3*) through topology analysis.

Patients with microtia have multiple metabolite abnormalities

In order to verify the abnormal metabolite changes found in the previous metabolomics, we used ELISA to analyze the contents of PC, TG, and choline in the serum of microtia and control groups. We detected PC (microtia: 41.20±18,06, NC: 25.23±6.922, P=0.0059, P<0.01), TG (microtia: 29.51±12.25, NC: 16.35±9.779, P=0.0045, P<0.01), choline (microtia: 234.1±33.26, NC: 202.3±43.64, P=0.0378, P<0.05) in the serum of participants, as shown in *Figure 3A-3C*. Then, we used these data from 28 participants to assess the diagnostic performance of 2 biomarkers (PC and TG) in a cell model. Significantly, the biomarker panel was able to distinguish patients with microtia from controls with an area under the curve (AUC) of 89.74% [95% confidence interval (CI): 78.29–100.00%], sensitivity of 93.33%, and specificity of 76.92% (*Figure 3D*), which confirmed that this biomarker panel was an efficient diagnostic approach of microtia.

Discussion

Although microtia does not affect the patient's survival, it can cause inconvenience to the patient's life and psychology (40-43). Microtia is currently mostly diagnosed after birth. According to reports, although the ears of some patients with microtia can be restored to their normal shape after birth, most patients require surgical treatment (44-47), which is a hugely harmful intervention for patients. Microtia-based diagnostic assays, however, continue to face challenges in their development and clinical application. Commercially available molecular testing is still lacking. Although certain tests have demonstrated decent performance when analyzed on a single institutional cohort, variations in the performance of the majority of these tests have been noted when evaluated in multiple independent cohorts (48-51). Furthermore, these tests are

Table 2 Metabolic pathway analysis for the siBMP5-2 group compared with the siNC group

Pathway	Total[a]	Hits[b]	Raw P value[c]	Holm adjust P value[d]	Impact value[e]
Glycerophospholipid metabolism	36	4	<0.001	<0.001	0.26
Linoleic acid metabolism	5	1	0.022	1.00	<0.01
alpha-Linolenic acid metabolism	13	1	0.057	1.00	<0.01
GPI-anchor biosynthesis	14	1	0.062	1.00	<0.01
Glycerolipid metabolism	16	1	0.070	1.00	0.01
Arachidonic acid metabolism	36	1	0.152	1.00	<0.01

[a], total is the total numbers of compounds in the pathway. [b], hits represents the number of metabolites that matched with the metabolites of the HMDB based on the accurate mass of each MF that differed significantly (adjusted P<0.05 and |log2(FC)| >0.58) in siBMP5 *vs.* siNC groups. [c], the original P value calculated from the over-representation analysis. [d], the P value adjusted by Holm-Bonferroni method. [e], the impact value is calculated from the pathway topology analysis. GPI, glycosylphosphatidylinositol; HMDB, Human Metabolome Database; MF, metabolomic features.

Table 3 Metabolic pathway analysis for the siBMP5-3 group compared with the siNC group

Pathway	Total[a]	Hits[b]	Raw P value[c]	Holm adjust P value[d]	Impact value[e]
Glycerophospholipid metabolism	36	2	0.003	0.26	0.20
Linoleic acid metabolism	5	1	0.013	1.00	<0.01
alpha-Linolenic acid metabolism	13	1	0.033	1.00	<0.01
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	14	1	0.036	1.00	<0.01
Glycerolipid metabolism	16	1	0.041	1.00	0.01
Arachidonic acid metabolism	36	1	0.090	1.00	<0.01

[a], total is the total numbers of compounds in the pathway. [b], hits represents the number of metabolites that matched with the metabolites of the HMDB based on the accurate mass of each MF that differed significantly (adjusted P<0.05 and |log2(FC)| >0.58) in siBMP5 *vs.* siNC groups. [c], the original P value calculated from the over-representation analysis. [d], the P value adjusted by Holm-Bonferroni method. [e], the impact value is calculated from the pathway topology analysis. GPI, glycosylphosphatidylinositol; HMDB, Human Metabolome Database; MF, metabolomic features.

very expensive and require tissue samples to be taken during surgery, which presents problems for both the economy and clinical practice. A recent study illustrated the importance of bioactive lipids in mesoderm differentiation (52). It can even be determined that metabolic abnormalities will affect the development of mesoderm (53,54). Since auricular cells originate from mesoderm parotid arch cells, the metabolic state caused by mesodermal cell changes can alter cell development in the form of metabolic reprogramming derivatives or secreted molecules (53,54). At the same time, the early detection of metabolic abnormalities is crucial to preventing microtia since metabolites are the products of biochemical reactions in the organ and the molecules that are closest to the phenotype. In our study, we discovered lots of different metabolites and 6 enriched pathways of metabolites through metabolomic analysis of cell samples. In addition, the corresponding abnormal metabolites were verified in the serum of patients with microtia and controls. We discovered that the combination of PC and TG provide an AUC of 89.74%, sensitivity of 93.33%, and specificity of 76.92%, that could distinguish microtia patients from controls. These findings may indicate that metabolites may be endogenously generated in microtia and may serve as biomarkers for identifying the underlying cause of microtia as well as for early detection and prevention of the condition during mesoderm development. The limitations of this study include the relatively small number of discovery samples, the lack of validation cohorts, and the temporary absence of metabolomic signatures during embryonic development. Further investigations are required to confirm the origin of circulating metabolites and to identify whether they have a crucial contribution in the development of microtia.

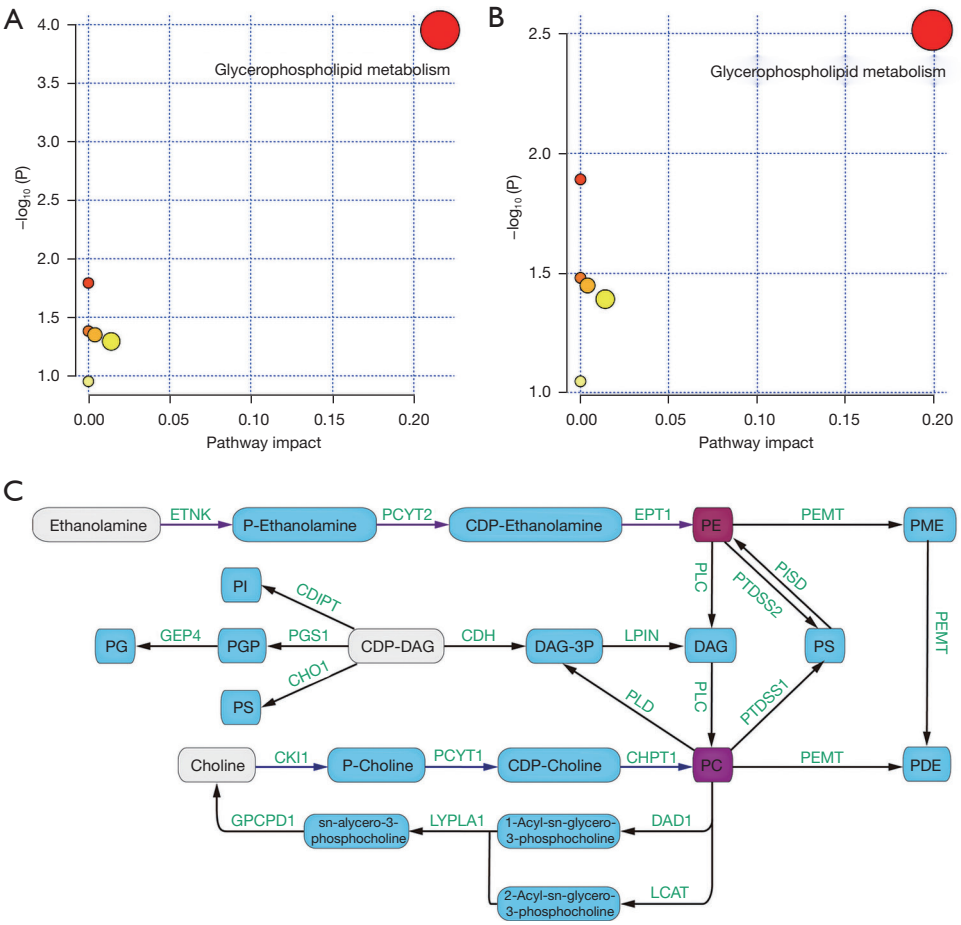


Figure 2 Pathway analysis in siBNP5-2 (A) and siBMP5-3 groups (B). (C) Glycerophospholipid metabolism. Metabolites are indicated in squares: PC, glycerophosphocholine; PE, glycerophosphoethanolamine; PME, monomethyl phosphatidylethanolamine; PDE, dimethyl phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; PS, phosphatidylserine; DAG, diacylglycerol. Enzymes in green: CKI1, choline kinase; PCYT1, choline-phosphate cytidylyltransferase; CHPT1, diacylglycerol cholinephosphotransferase; PEMT, phosphatidylethanolamine methyltransferase; ETNK, ethanolamine kinase; PCYT2, ethanolamine-phosphate cytidylyltransferase; EPT1, ethanolaminephosphotransferase; PLD, phospholipase D; PLC, phospholipase C; PTDSS1, phosphatidylserine synthase 1; PTDSS2, phosphatidylserine synthase 2; PISD, phosphatidylserine decarboxylase; LPIN, phosphatidate phosphatase; CDH, CDP-diacylglycerol pyrophosphatase; CDIPT, phosphatidylserine decarboxylase; PGS1, glycerol-3-phosphate 3-phosphatidyltransferase; CHO1, serine O-phosphatidyltransferase; GEP4, phosphatidylglycerophosphatase; DAD1, phospholipase A1; LCAT, lecithin-cholesterol acyltransferase; LYPLA1, lysophospholipase I; GPCPD1, glycerophosphodiester phosphodiesterase.

Protein, microRNA (miRNA) and other types of circulating diagnostic indicators such as messenger RNA (mRNA) are also suggested for distinguishing between microtia and healthy people (55-61). Studies have been conducted on the differences between the metabolites of auricle cartilage, but the changes in the metabolites of the auricle cannot indicate the occurrence of microtia (29). Since metabolites are the products of metabolic activities in the body, changes in the transcriptome (mRNA, miRNA), and proteome (proteins) due to pathophysiological modifications, might be reflected in the metabolome. Metabolites are the cornerstone of cell function. However, the field of metabolomics is still not mature, and it is easy to be interfered by upstream organisms (62,63). Although the diagnosis of congenital diseases such as microtia has obvious advantages, there are also shortcomings such as unstable results and difficult to obtain samples. This study has some limitations, such as small sample size and lack of functional

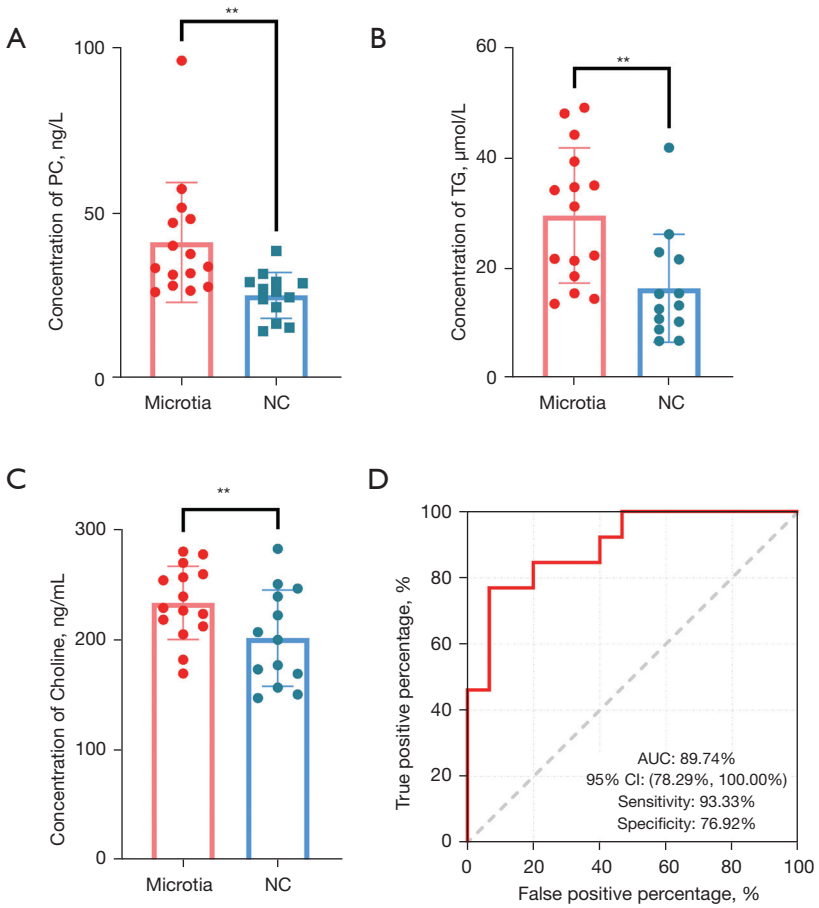


Figure 3 ELISA experiments confirmed the metabolomic characteristics of patients with microtia. Serum levels of (A) total glycerophosphocholine, (B) triacylglycerol, and (C) choline in patients with microtia. (D) ROC analysis for distinguishing patients with microtia from controls with 2 biomarkers (PC and TG) in cell model. **, P<0.01. PC, glycerophosphocholine; TG, triacylglycerol; NC, normal control; AUC, area under curve; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic.

students of key metadata. We will further increase the sample size and supplement the research of functional students of key metadata in the future. In addition, our metabolic biomarker panel demonstrated higher diagnostic performance in terms of both sensitivity and specificity, even before the auricle is produced, as compared to other types of circulating biomarkers.

Conclusions

In conclusion, by metabolomics profiling, we were able to determine which metabolites were significantly altered between patients with microtia and controls. We specifically showed that a newly developed panel of serum metabolic biomarkers could distinguish between patients with microtia and controls with high sensitivity and specificity, which was superior to other circulating biomarkers in the early diagnosis of microtia. Therefore, this study could provide a basis for a new metabolic biomarker panel for early diagnosis of microtia.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5614/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5614/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5614/coif). All authors report that the study was supported by grants from the National Natural Science Foundation of China (Nos. 82171160, 81801132, and 81971190); the National Natural Science Foundation of Guangdong (No. 2019A1515010902); the Science and Technology Program of Guangzhou (No. 201903010075); the National Key R&D Program of China (No. 2020YFB020003); and GD Frontier & Key Tech Innovation Program (Nos. 2018B030337001 and 2019B020228001). The authors have no other conflicts of interest to declare

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent was provided by all the participants and their parents or legal guardians for participation in and publication of this study. All procedures were approved by the Ethics Committee of Sun Yat-sen Memorial Hospital of Sun Yat-sen University (No. 2019-KY-024).

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