RESEARCH

Metabolite analysis-aided diagnosis of papillary thyroid cancer

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Abstract

Thyroid cancer is the most frequent endocrine tumor with a growing incidence worldwide. However, common diagnostic strategy for thyroid cancer classification is hardly to make a proper diagnosis in some cases. To assist classical approach, this study used metabolomics to screen and validate biomarkers from serum and urinary for papillary thyroid cancer (PTC). Overall, 124 untreated PTC, 76 untreated benign thyroid nodule (BTN), and 116 healthy control (HC) were collected in this study. Thirty-six differential metabolites were screened from non-targeted metabolomics with a discovery sample set in comparison with HC and BTN. Serum β -hydroxybutyrate (BHB), docosahexaenoic acid (DHA), 1-methyladenosine (1-MedA), pregnanediol-3-glucuronide (PdG), urinary nicotinic acid mononucleotide (NAM) and xanthosine (Xan) were validated to be significantly differential by targeted metabolomics in validation set. The logistic regression model incorporating six biomarkers had excellent discrimination from receiver-operating characteristics (ROC) analysis, with area under the receiver-operating characteristic curve (AUC) of 0.943 (95% CI 0.902 to 0.983) and 0.952 (95% CI 0.921 to 0.983) for female alone and female + male samples, respectively. The prediction accuracy and false-negative rate in the real setting of one PTC to ten suspicious nodules were 84.7 and 17.7% with the threshold at probability of 0.5. Results of a double-blind study for PTC and BTN had true positive value of 100% and true negative value of 91.7%. To conclude, BHB, DHA, 1-MedA, PdG, NAM and Xan are suitable biomarkers for PTC, and logistic regression models with the six biomarkers can be potentially used as clinical diagnosis.

Key Words

- ▶ papillary thyroid carcinoma
- ▶ biomarker
- ▶ logistic regression model
- ▶ metabolomics
- ▶ double-blind

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Introduction

Thyroid cancer is a common endocrine malignancy with an increasing incidence worldwide (La *et al.* 2015). In particular, papillary thyroid cancer (PTC) accounts for almost 90% of all thyroid tumors. Although the overall

survival rate of thyroid cancer patients is high, it is worrying that the recurrence rate of 10 years is up to 30%, and the survival rate of these high-risk patients is about 50%. Ultrasound-guided fine-needle aspiration (FNA) biopsy,

as the best imaging method to diagnose high-risk cancer lesions, depends heavily on the technical advantages and experience of the operator (Yao *et al.* 2011, Ravetto *et al.* 2000), which has a sensitivity of 93% and specificity less than 75%. However, in 10–30% cases, a reliable diagnosis of malignant tumors could not be performed by cytological analysis of FNA samples, mainly because it is difficult to distinguish between benign and malignant follicular thyroid lesions (Wang *et al.* 2011). Although FNA cytological analysis is popular and useful, immunocytochemical and genetic tests still have some limitations (Lin *et al.* 2017, Wojtowicz *et al.* 2017, Coperchini *et al.* 2019). Furthermore, indolent thyroid cancer does not require FNA biopsy unless there is a lymph node metastasis, extrathyroidal invasion, or previous exposure to radiation or a family history of thyroid cancer, and the treatment of these over-diagnosed cancers may increase the risk of patient. To prevent patients from unnecessary injuries and improve diagnostic accuracy, a convenient, noninvasive and specific diagnosis method is urgently to be developed for thyroid cancer (Wojtowicz *et al.* 2017).

Measuring small-molecule metabolites is essentially a cornerstone of clinical chemistry and even clinical practice. Molecular markers, mainly found in blood/serum, urine, other body fluids, or tissues, are expected to have a significant impact on cancer prognostication (Haugen *et al.* 2016, Pan *et al.* 2016, Wang *et al.* 2017a). Metabolomics is a relatively new field for detecting biochemical changes associated with genetic differences, environmental impacts, diseases or drug interferences (Nicholson & Lindon 2008, Ceglarek *et al.* 2009, Dunn *et al.* 2011, Zafón Llopis *et al.* 2019), and may be useful for characterizing the pathogenicity of gene variants and their levels of aggressiveness (Veyrat-Durebex *et al.* 2019). Thyroid cancer showed obvious metabolic heterogeneity in tumor microenvironment, and there was metabolic coupling between tumor and stromal cells. The actual understanding of the metabolic phenotype of thyroid cancer cells will provide new prognostic biomarkers and therapeutic targets for aggressive tumor behavior (Wojakowska *et al.* 2015a). Metabolic biomarkers are hopefully to be incorporated into diagnostic and therapeutic practices to improve the outcomes of thyroid cancer, which may also be used for early detection or screening, differentiation of benign from malignant diseases, histologic identification, staging and response to treatment, diagnosis of recurrence and prognosis. These targets have generated considerable interest in identifying promising tumor markers (Bustamante *et al.* 1981, Wise & Thompson 2010, Locasale 2013, Goodwin *et al.* 2014).

Metabolites related to lactic acid metabolism (Xu *et al.* 2015), one carbon metabolism (Ryoo *et al.* 2016), fatty acids metabolism (Tian *et al.* 2015) and lipid metabolism (Wojakowska *et al.* 2015b) were found to be significantly altered in thyroid cancer. Despite promising progress in serum and urine metabolomics of thyroid cancer, there are still some shortcomings, such as lack of validation or limited validation biomarkers of thyroid cancer early diagnosis.

To fill this gap, our most urgent interest is to develop a diagnostic strategy-based metabolomics study to help early diagnosis of PTC. Thirty-six metabolites were screened by non-targeted metabolomics in discovery set, and six differential metabolites were proved to be proper biomarkers for PTC by quantitation with validation set. A new biomarker panel based on logistic regression model with ROC analysis and bootstrapping validation was applied to estimate the probability of PTC from non-cancer group (healthy control (HC) and benign thyroid nodule (BTN)). Moreover, a prospective, randomized double-blinded study of 31 untreated patients with thyroid nodules was conducted to validate the performance of the logistic regression model.

Materials and methods

Clinical and pathologic characteristics of thyroid cancer

Samples of Han Chinese volunteers, including 124 serum/urine samples from untreated PTC, 76 serum/urine samples from untreated BTN, and 116 serum/urine samples from HC, were collected from the Tumor Hospital affiliated to Zhengzhou University and Zhengzhou Central Hospital affiliated to Zhengzhou University with ultrasound, FNA biopsy and histopathology. Tumor staging was performed according to the eighth edition of thyroid cancer tumor node metastasis staging by American Joint Committee (van Velsen *et al.* 2018). This study was approved by the Ethics Committee of School of Basic Medical Sciences, Zhengzhou University (approval number 18), and all participants received informed consent. HC were recruited and asked to undergo health checks to ensure everyone without cardiovascular, thyroid, liver, kidney or blood system dysfunction were excluded. The fasting serum sample was collected in the morning, and set at 4°C for 2h, and then centrifuged for 15 min (3000g, 4°C) (Dunn *et al.* 2011). Multiple aliquots were collected for each subject and stored at –80°C until further analysis. Histopathologic classification was performed according to the World Health Organization (WHO) Classification

of Tumours of Endocrine Organs 4th Edition by two pathologists (Lloyd *et al.* 2017). The baseline characteristics of enrolled patients and HC were listed in Table 1. Each discovery set has 58 subjects, including 29 female PTC stage I/II patients, 29 non-cancer subjects (15 HC and 14 female BTN patients), and the average ages of PTC stage I/II patients and non-cancer subjects were 43.7 and 43.7 years old, respectively. Quality control (QC) samples were mixture of female PTC, BTN and HC samples with same volume before preparation. An independent validation set with 166 female samples (62 PTC stage I/II, 4 stage III/IV, 64 HC and 36 BTN) was used to discover and verify the potential biomarkers (Table 1). In addition, male PTC test set was consisted of 28 PTC stage I/II, 2 stage III/IV,

37 HC and 2 BTN. Moreover, a double-blinded set was consisted of 31 untreated patients with thyroid nodules.

Development and validation of the untargeted metabolomics method

Apparatus

All chromatography separations were conducted with the Thermo Fisher Scientific Ultimate 3000 UHPLC system (including HPG-3400 SD pump, WPS-3000 SL auto-sampler and TCC-3000 SD column oven). The MS detection was performed on a Bruker microOTOF-Q II (Bruker Daltonics Corporation, Karlsruhe, Germany) using an electronic spray ion source.

Table 1 Baseline clinical and pathological features of enrolled patients and controls.

(1) Baseline clinical features of enrolled patients and controls of discovery set (female)				
	HC	BTN	Stage I	P value (ANOVA)
Cases	15	14	29	
Age (average, range)	43.7, 24–57	43.8, 24–54	43.7, 23–55	$P = 0.864$
Tumor stage				
T1/2			29	
T3/4			0	
Lymph node metastases				
N0			26	
N1			3	
Distant metastases				
M0			29	
M1			0	
(2) Baseline clinical features of enrolled patients and controls of external validation set (female)				
	HC	BTN	Stage I/II	Stage III/IV
Cases	64	36	62	4
Age (average, range)	41.8, 26–68	46.8, 24–58	43.8, 20–74	66.5, 63–69
Tumor stage				
T1/2			53	0
T3/4			9	4
Lymph node metastases				
N0			45	1
N1			17	3
Distant metastases				
M0			60	1
M1			2	3
(3) Baseline clinical features of enrolled patients and controls of external test set (male)				
	HC	BTN	Stage I/II	Stage III/IV
Cases	37	2	28	2
Age (average, range)	34.2, 23–62	41.0, 31–51	42.4, 21–66	61.5, 59–64
Tumor stage				
T1/2			26	0
T3/4			2	2
Lymph node metastases				
N0			17	0
N1			11	2
Distant metastases				
M0			27	0
M1			1	2

Chromatographic conditions

An ACQUITY UPLC® BEH column (2.1 × 100 mm, 1.7 μm) (Waters Corporation) was used. RP-UHPLC mobile phases were consisted of A (0.1% formic acid) and B (acetonitrile with 0.1% formic acid). HILIC-UHPLC mobile phases were consisted of A (0.1% formic acid and 10 mM ammonium acetate in water) and B (0.1% formic acid and 10 mM ammonium acetate in acetonitrile). Injection volume and column temperature were 5 μL and 50°C, respectively. Gradient elution programs of RP-UHPLC and HILIC-UHPLC in positive and negative mode were displayed in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article).

ESI-QTOF/MS conditions

The MS detection was obtained on a Bruker microQTOF-Q II (Bruker Daltonics Corporation, Karlsruhe, Germany) equipped with an electronic spray ion source operating in negative ion mode. Argon was used as collision gas. The parameters were set as follows: Temperature of source, 180°C; spray voltage, 3500 V; flow rate of dry gas, 6 L/min; nebulizer gas, 1.5 bar; transmission time, 60 μs; collision energy, 7 eV; mass range, 50–1000 Da. We used sodium formate to correct the mass accuracy.

ESI-QTOF/MS conditions

The MS detection was obtained on a Bruker microQTOF-Q II (Bruker Daltonics Corporation) equipped with an electronic spray ion source operating in positive ion mode. Argon was used as collision gas. The settings are as follows: Temperature of source, 180°C; spray voltage, 3500 V; flow rate of dry gas, 4 L/min; atomizer gas, 0.04 MPa; transmission time, 100 μs; collision energy, 7 eV; mass range, 50–1000 Da. Mass accuracy was also corrected by sodium formate.

GC-MS conditions

GC-MS analysis was performed with Shimadzu GC-MS QP2010 coupled with a DB-5MS capillary column (60 m × 250 μm i.d., 0.25 μm film thickness; Agilent Technologies). Solvent delay was set for 3.5 min. The measurements were conducted with electron impact ionization (70 eV) under the full scan mode (m/z 45–600). The oven temperature was initially held at 100°C for 4 min. Then raised the temperature with a gradient of 4°C/min to 320°C and held for 8.0 min. The injection temperature, the interface temperature and the ion source temperature were set to 230°C, 280°C and 200°C, respectively. The flow through the column was constant with 2.2 mL He/min.

The compound libraries, Smartdatabase (OA_TMS_DB5_67min_V3.lib) and National Institute of Standards and Technology (NIST) and reference compounds available, were commercially available to identify metabolites.

Data preprocessing

Raw MS data for each sample was uploaded to XCMS online, and then extracted and overlaid variables (the integral results of peaks) for all the samples according to retention time or accurate mass data. In order to process the data as a whole, multivariate analysis was used to observe the differences between groups. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) models by SIMCA software were outlined to overview the differences in metabolic profiling between the PTC and non-cancer. The results were shown in [Fig. 1](#), where X is the numerical value of the targeted steroids, and Y is the class assignment. It was revealed that the PTC patients were obviously separated from the non-cancer. The models were validated using a permutation test with 200 cycles. $P < 0.05$ (CV-ANOVA) indicated that the OPLS-DA models were not over fitting.

The sequential processing of the peak table used the raw data output (including RT and characteristic ions) after the operation of the quantization table and the peak area was normalized to the total peak area. Principal component analysis (PCA) and OPLS-DA models were performed using SIMCA 14.1 (Umetrics, Sweden). Nonparametric tests (Mann–Whitney U test) were performed with SPSS 22.0 software to screen for the metabolites of $P < 0.05$. Changes in differential metabolite levels were observed in MultiExperiment Viewer (<http://www.tm4.org/mev.html>). The altered pathways were identified by the Human Metabolome Database (HMDB), MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca>), and KEGG PATHWAY Database

Targeted analyses: identification and quantitation of marker compounds and creatinine

From the standpoint of application, an additional validation sample was also acquired. The validated differential metabolites were further screened for the discovery of potential biomarkers for PTC diagnosis. To confirm whether potential biomarkers screen for PTC diagnosis, six validated differential metabolites were quantified in female external validation set subjects, both alone and combined with male external test set subjects;

Figure 1

Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) models (green for control and red for PTC): (A1) serum HPLC-ESI(+)-QTOF-MS, (A2) urinary HPLC-ESI(+)-QTOF-MS, (B1) serum HPLC-ESI(-)-QTOF-MS, (B2) urinary HPLC-ESI(-)-QTOF-MS, (C1) serum HILIC-ESI(+)-QTOF-MS, (C2) urinary HILIC-ESI(+)-QTOF-MS, (D1) serum HILIC-ESI(-)-QTOF-MS, (D2) urinary HILIC-ESI(-)-QTOF-MS, (E1) serum GC-MS. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0344>.

the significant changes among groups were verified by SPSS 22.0 software. Representative ESI product ion mass spectra of the precursor ions of markers and total ion chromatogram (TIC) were shown in Supplementary Fig. 1. Detection of creatinine in urine was pretreated and detected according to previous method (Jones *et al.* 2018).

Risk prediction model

Logistic regression models were widely used in medicine to predict patient outcome (prognosis) and construct diagnostic tests (diagnosis). Multivariable logistic regression models built with Matlab were constructed to estimate the probability. The performance of the multivariable model was quantified by assessing its discrimination and calibration. Model calibration was

evaluated using Hosmer–Lemeshow goodness-of-fit test, together with the deviance and residual test. The overall model performance was evaluated using the Brier score. Bootstrap resampling (1000 cycles) was used for internal validation of the risk model, which was created in each cycle and corrected for optimism to evaluate how much the model performance decrease when applied in new data sets. ROC curve is a plot of all the (false-positive rate (FPR), true-positive rate (TPR)) pairs by varying the decision threshold over the whole range of the bootstrap sampling distributions, providing a more comprehensive evaluation on the model. Sensitivity and specificity at a probability threshold of 0.5 were calculated through ROC analysis.

As permutation analysis might overestimate the accuracy, we repeated 1000 cycles to extract one PTC

to ten suspicious nodules as the prediction set, and calculated the predicted accuracy and false-negative rate with logistic regression model built on the leaving samples. Besides that, a prospective, randomized double-blinded study of 31 untreated patients with thyroid nodules was conducted to assess the performance of the logistic regression model. The results of the laboratory metabolic markers will be unknown to the clinicians who will make the diagnosis, while the results of the clinical diagnosis will not be known to the laboratory technician who will determine these metabolic markers.

Results

Differentiation between PTC patients and non-cancer group based on metabolomic profiles

OPLS-DA models were outlined to overview the differences in metabolism profile between PTC and non-cancer (HC+BTN) (Fig. 1). The results revealed that PTCs were obviously separated from non-cancer. $P < 0.05$ (CV-ANOVA) indicates that the OPLS-DA models

were not overfitting. The samples of non-cancer group and PTC group were successfully separated in Fig. 1, demonstrating that specific metabolites are different between two groups. Based on the above models, differential metabolites passing VIP threshold ($VIP > 1$) and Welch's t -test ($P < 0.05$) were selected. The differential metabolites were identified by searching databases or compared with the spectra of known standard compounds. Thirty-six potential differential metabolites identified by high-throughput screening were included in serum or urine (Fig. 2).

Pooled QC samples were used in the study to make sure that there is no or minimal loss of metabolic information. The analysis sequence consists of QC samples and subject samples. At the beginning of each analysis batch, six QC samples were injected, and then QC samples were analyzed every eighth times. In order to process the data as a whole, multivariate analysis was used to observe the differences between groups. Score scatter plots of PCA based on 36 differential metabolites ($RSD < 20\%$) revealed that the QC samples were closely assembled in the center (Fig. 3). The results showed that there was no drift during metabolomics profiling, and the stability and reproducibility were acceptable.

Figure 2

Heat map of identified differential metabolites screened by untargeted metabolomics in discovery set samples. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0344>.

Figure 3

Score scatter plots of principal component analysis (PCA) based on 36 differential metabolites: QC samples were closely assembled in the center. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0344>.

Dynamics of metabolite alterations in PTC

Six differential metabolites were selected as candidate markers for further testing as they represented different metabolic pathways associated with cancer and had more

significant differences. Serum β -hydroxybutyrate (BHB), docosahexaenoic acid (DHA) and 1-methyladenosine (1-MedA) were significantly increased in PTC. On the contrary, serum pregnanediol-3-glucuronide (PDG), urinary nicotinic acid mononucleotide (NAM) and xanthosine (Xan) significantly decreased (Fig. 2), which were rarely used as biomarkers for PTC, and they were mainly involved in fatty acid biosynthesis (Rodrigues *et al.* 2017), purine metabolism (Stoychev *et al.* 2010, Ishiwa *et al.* 1995), and nicotinate and nicotinamide metabolism (Wang *et al.* 2017b). As the differential metabolites were found mainly based on the difference of peak area of the compounds from a small sample experimental result, it is necessary to carry out the verification experiment by quantitative LC-MS/MS method (Supplementary Fig. 1) and correct with creatinine (Cre) in validation set (Table 1) (Levey *et al.* 1988). Compared to previously untargeted results (Fig. 2), these metabolites were proved to be potential biomarkers for PTC (Fig. 4). Serum BHB, DHA and 1-MedA were upregulated in PTC compared with non-cancer, while serum PDG, urinary NAM/Cre and Xan/Cre were downregulated

Figure 4

Differential biomarker in serum or urine of validation set samples: (A) serum BHB levels in female PTC patients, BTN patients and Healthy; (B) serum DHA levels in female PTC patients, BTN patients and Healthy; (C) serum 1-MedA levels in female PTC patients, BTN patients and Healthy; (D) serum PdG levels in female PTC patients, BTN patients and Healthy; (E) urine NAM/Cre levels in female PTC patients, BTN patients and Healthy; (F) urine xanthosine levels in female PTC patients, BTN patients and Healthy; (G) serum BHB levels in whole PTC patients, BTN patients and Healthy; (H) serum DHA levels in whole PTC patients, BTN patients and Healthy; (I) serum 1-MedA levels in whole PTC patients, BTN patients and Healthy; (J) serum PdG levels in whole PTC patients, BTN patients and Healthy; (K) urine NAM/Cre levels in whole PTC patients, BTN patients and Healthy; (L) urine xanthosine levels in whole PTC patients, BTN patients and Healthy; (M) Spearman's rank correlation of BHB with thyroid status; (N) Spearman's rank correlation of DHA with thyroid status; (O) Spearman's rank correlation of 1-MedA with thyroid status; (P) Spearman's rank correlation of PdG with thyroid status; (Q) Spearman's rank correlation of NAM with thyroid status; (R) Spearman's rank correlation of Xan with thyroid status; (thyroid status: HC, BTN and PTC were defined as 1, 2 and 3, respectively.) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0344>.

in female PTC (Fig. 4A, B, C, D, E and F) and female+male PTC (Fig. 4G, H, I, J, K and L). The differential metabolites level and *P* value in Mann–Whitney *U* test were as follows: BHB (nmol), $P=8.85\times 10^{-11}$; DHA (nmol), 7.88×10^{-5} ; 1-MeDA (nmol), $P=0.02$; PDG (nmol), $P=1.15\times 10^{-4}$; NAM/Cre ($\mu\text{mol}/\text{mmol}$), $P=1.12\times 10^{-7}$; Xan/Cre ($\mu\text{mol}/\text{mmol}$), $P=3.09\times 10^{-7}$. As the characters of six biomarkers' values appeared to be non-normal distributions, Box-Cox transformation method was carried out to make them close to Gaussian by Matlab. The relationships of serum/urine biomarkers levels with age or thyroid status were also analyzed in the Han Chinese volunteers aging from 24 to 74 years. No correlation was found between six biomarkers and age. Furthermore, spearman's rank correlation was carried out to explore the relationship between marker levels and thyroid status. HC, BTN, PTC were scored with 1, 2 and 3, respectively. As shown in Fig. 4M, N, O, P, Q and R, six biomarkers generally have medium correlations with thyroid status ($0.17<|R|<0.56$, $P<0.01$) except NAM/Cre ($R=-0.06$, $P>0.05$).

Biomarker panel of logistic regression model

Multivariable logistic regression model was constructed to estimate the probability of incidence for female PTC patients. Regression coefficients (β -coefficients), odds ratio (OR, 95% CI) and standard error (S.E.) for covariates in the logistic regression model for the probability of female PTC patients were presented in Table 2. The Hosmer–Lemeshow goodness-of-fit test showed a good calibration $\chi^2(8)$ 2.917, $P=0.939$. The overall model performance

evaluated using the Brier score gave a *P* value of 0.0895. For a perfect classifier, the AUC is close to 1.00 when the curve with 100% sensitivity and 100% specificity, whereas for a poor classifier, the ROC curve is close to the 1:1 diagonal. Biomarker panel achieved a promising prediction rate with an AUC of 0.943 (95% CI 0.902 to 0.983) for female samples alone (Fig. 5A, B and C), demonstrating that the model established in our study represents a viable tool for PTC diagnosis. $P>0.5$ indicates the prospective incidence of the PTC, whereas $P<0.5$ indicates grade non-cancer. For female logistic regression model, the sensitivity and specificity were 81.8 and 90.0%, respectively. The false-negative rate in the real setting of one female PTC to ten female suspicious nodules was 17.9%, the FPR was 16.8%, and the prediction accuracy was 83.1%.

For female+male PTC test set (both male and female), the risk model was rebuilt to estimate the probability of incidence for female and male PTC. Biomarker panel achieved a promising prediction rate with an AUC of 0.952 (95% CI 0.921 to 0.983) (Fig. 5D, E and F). For female+male PTC test, the sensitivity and specificity were 81.3 and 90.7%, respectively. Hosmer–Lemeshow goodness-of-fit test demonstrated an excellent calibration $\chi^2(8)$ 8.508, $P=0.386$. Regression coefficients, odds ratio (95% CI) and S.E. for covariates in the logistic regression model for the probability of female+male PTC patients were presented in Table 2. The overall model performance evaluated using the Brier score gave a *P* value of 0.0857. The false-negative rate in the real setting of 1 PTC to ten suspicious nodules was 17.7%, the FPR was 15.1%, and the prediction accuracy was 84.7%.

Table 2 Regression coefficients (β -coefficients), odds ratio (OR, 95% CI) and standard error (S.E.) for covariates in the final model for the probability of PTC.

Covariates	β -coefficients	S.E.	OR (95% CI)	<i>P</i> value
PTC (female)				
BHB	4.010	0.834	55.156 (10.747 to 283.077)	<0.0001
DHA	1.283	0.302	3.608 (1.996 to 6.521)	<0.0001
MedA	2.545	0.768	12.748 (2.830 to 57.427)	0.001
PdG	−1.042	0.371	0.353 (0.171 to 0.729)	0.005
NAM	0.286	0.777	1.331 (0.290 to 6.102)	0.713
Xan	−2.246	0.806	0.106 (0.022 to 0.513)	0.005
Intercept	−2.322	0.316	–	0.098
PTC (female + male)				
BHB	3.276	0.556	26.461 (8.903 to 78.644)	<0.0001
DHA	0.774	0.175	2.168 (1.538 to 3.057)	<0.0001
MedA	1.868	0.499	6.478 (2.436 to 17.228)	0.0002
PdG	−0.956	0.282	0.384 (0.221 to 0.668)	0.001
NAM	1.329	0.392	3.778 (1.751 to 8.153)	0.001
Xan	−2.600	0.474	0.074 (0.029 to 0.188)	<0.0001
Intercept	−2.951	2.849	–	0.300

Figure 5

(A) Risk score and probability for female of non-cancer with logistic regression model; (B) Risk score and probability for female PTC with logistic regression model; (C) ROC curve plotted with false-positive rate (FPR) and true-positive rate (TPR) of logistic regression probability for female PTC classification. (D) Risk score and probability for non-cancer in female + male group with logistic regression model; (E) risk score and probability for whole PTC in logistic regression model; (F) ROC curve plotted with FPR and TPR of logistic regression probability for female + male PTC classification. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0344>.

Double-blind study

As the sample information and the results of the clinical diagnosis were unknown to the laboratory technician, the risk model based on female+male PTC test set was used to predict the blind samples. As shown in Table 3, the misclassification rate, calculated as undiagnosed divided by the total sample size, was 6.5%. The true-positive and true-negative values were 100 and 91.7%, respectively. Double-blind study revealed that the logistic regression model built in this research performed well, and the method could be used as an auxiliary tool for clinical diagnosis.

Discussion

Veyrat-Durebex *et al.* conveyed a metabolomic study on metabolomics signatures with a subset of *RET* variants according to their oncogenic risk level and demonstrated the efficacy of metabolomics in testing *RET* mutations' pathogenicity (Veyrat-Durebex *et al.* 2019). A serum-plasma-matched untargeted metabolomics study was conducted to reveal the significant metabolic pattern differences of BTN and PTC, and no significant difference was found in circulating metabolic signatures between

BTN and PTC (Huang *et al.* 2019). For differential diagnosis of HC versus BTN and PTC, a panel of six metabolic markers, namely myo-inositol, N-phenylacetyl-L-glutamine, proline betaine, L-glutamic acid, LysoPC (18:0) and LysoPC (18:1) provided AUC of 97.68% in discovery phase and predictive accuracies of 84.78–98.18% (Huang *et al.* 2019). However, there are still some deficiencies, such as lacking validation or limited validation objects of early diagnosis of PTC biomarkers from BTN and HC.

In this article, differential metabolites were screened from non-targeted metabolomics with a discovery sample set, and then validated by targeted metabolomics and corrected with creatinine in another validation set. Thirty-six potential differential metabolites identified by high-throughput screening were included in serum or urine. Six candidate markers were selected for further testing as they represented different metabolic pathways associated with cancer. 1-MeA, one of the modified nucleosides involved in the purine metabolism, was found downregulated in serum, and the levels were disordered in urine of patients with malignant tumors (Ishiwata *et al.* 1995). Ketone bodies played both metabolic and epigenetic roles in cancer, while β -hydroxybutyrate (BHB) was involved in the synthesis and degradation of ketone bodies. Some studies proposed that ketone bodies could inhibit histone deacetylases from achieving anticancer effect (Seyfried *et al.* 2003, Poff *et al.* 2014, Shukla *et al.* 2014), while other studies noted faster tumor growth (Bonuccelli *et al.* 2010, Whitakermenezes *et al.* 2011, Martinezoutschoorn *et al.* 2012, Rodrigues *et al.* 2017, Wojtowicz *et al.* 2017). Rodrigues found that BHB administration accelerated the rate of tumor growth rather than inhibited it (Rodrigues *et al.* 2017). Hence, upregulation of BHB seems to be an important element related to PTC. DHA is a member of

Table 3 Double-blind study result based on logistic regression model placed on a risk score for real setting of PTC to suspicious nodules.

Outcome	The result			
	True	False	Total	
PTC	7	0	7	True positive = 7/7 (100%)
BTN	22	2	24	True negative = 22/24 (91.7%)
Total	29	2	31	

long-chain fatty acids, which can be obtained from seafood consumption. Previous study proposed that intake of salt water fish, fresh water fish, cruciferous vegetables, fruit, meat, and grains were not related with the risk of thyroid cancer (Liu & Lin 2014). However, DHA may also be synthesized endogenously from linolenic acid. Serum DHA concentration was found significantly upregulated in PTC in our study, which might be affected by abnormal citric acid metabolism. PdG was a natural metabolite generated by UDP glucuonyltransferase and pregnanediol, whose upregulation in urine was known as a biomarker to confirm ovulation (Souza *et al.* 1998; Ecochard *et al.* 2013). Previous studies found that the urine estrone-glucuronide/PdG ratio was significantly increased in the women with breast cancer, in spite of normal pregnanediol-3-glucuronide excretion (Magini *et al.* 1990). NaMN was an intermediate in the metabolism of nicotinate and nicotinamide, and its acetylation was based on the synthetic pathway of deamination from NAD (Wang *et al.* 2017b). Inhibition of nicotinamide phosphate transferase has become a therapeutic strategy in cancer (Santidrian *et al.* 2013, Sampath *et al.* 2015). Xan, a purine nucleoside, is an intermediate in purine metabolism (Stoychev *et al.* 2002, López-Cruz *et al.* 2014), while it is an important metabolic process of cell proliferation (Zhou *et al.* 2017). Notably, untargeted results revealed that serum BHB, DHA and 1-MedA were dramatically upregulated in PTC compared with non-cancer group, and serum PdG, urinary NAM and Xan were significantly downregulated in PTCs. The six metabolic biomarkers were upregulated/downregulated by targeting metabolomics.

We firstly studied the relationship between the six metabolic markers and age as well as thyroid status. For the population aging from 20 to 74 years: there was no correlation between six biomarkers and age. Although presented results of spearman's rank correlation indicated that six markers except NAM were significantly related to thyroid status, a larger sample size is needed for further validation and PTC stage classification developments. Staging and prognosis require independent sample analysis, while the number of patients with advanced disease and recurrence is too small to draw a final conclusion.

For the application perspective, our research aims to develop a predictive assay that can help early diagnosis of PTC. Since a single metabolite cannot be a universal marker, six metabolic biomarkers were combined as a metabolic biomarker panel and applied with logistic regression model to evaluate the potential diagnostic performance, which provided a satisfactory prediction accuracy of

84.7% (threshold at probability of 0.5) through training and validation for identifying PTC from HC as well as BTN. The result is comparable to the predicted value of available clinical markers reported so far: mutation testing panel (BRAF, RAS mutations, and PAX8-PPARG, RET/PTC1,3) (81–94%) (Nikiforov *et al.* 2011), HMGA gene (91.2%) (Vandergrift *et al.* 2018), Kronus/RSR TgAb (89%), Roche Elecsys TgAb (85%) (Spencer *et al.* 2014), and metabolic tumor volume (Δ MTV%, 87.2%) and total lesion glycolysis (Δ TLG%, 89.5%) in PET/CT (Shi *et al.* 2018), and F-18 fluorodeoxyglucose (F-18 FDG, 0.84) in PET/CT (Kim *et al.* 2018). Specifically, low-dose CT screening is expensive, characterized by high sensitivity but only 68.6% specificity (Shi *et al.* 2018). Although diagnostic value can be improved combining with measurable PTC markers, the sensitivity and specificity of these markers are limited, which cannot be widely used in clinical practice (Saleh *et al.* 2010, Alexander *et al.* 2012, Ohori *et al.* 2013, Xing *et al.* 2013). The results presented in this article would help to compare existing and new developments in the field objectively. The additional validation should include a larger scale of subjects, and these subjects' age and gender should be carefully matched, including individuals with various benign thyroid disease and high-risk groups. Advanced methods will be built and tested with the provided data for further study.

Conclusions

In summary, serum BHB, DHA, 1-MedA, PdG, and urinary NAM/Cre and Xan/Cre were screened to be differential metabolites by non-targeted metabolomics with a discovery sample set, and then validated to be potential markers by targeted metabolomics in another validation set. The six biomarkers had significant correlations with thyroid status except NAM. For the application in clinical analysis, the PTC prediction accuracy was assayed with a novel diagnosis strategy based on logistic regression model with biomarker panel. The risk model was proved to be highly sensitive, specific, and well matched after bootstrapping and double-blind validation. In addition, the model also might be developed for auxiliary diagnosis of PTC patients with further validation.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0344>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethics approval and consent to participate

This study was approved by the ethics committee of School of Basic Medical Sciences, Zhengzhou University (Approval number 18).

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