Circular RNA expression and association with the clinicopathological characteristics in papillary thyroid carcinoma

DAN GUO1*, FANGYUAN LI1*, XIAOXIAO ZHAO1*, BO LONG1, SUMEI ZHANG1, ANQI WANG1, DINGYAN CAO1, JIAN SUN2 and BINGLU LI3

1Medical Science Research Centre, Departments of 2Pathology and 3General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, P.R. China

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Abstract. Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer. Circular RNAs (circRNAs) are a novel class of RNAs, with higher stability and tissue specificity, which may be of value as novel clinical markers. High-throughput RNA sequencing was used to profile the expression of circRNAs in 5 pairs of cancer and normal tissues, and reverse transcription-quantitative PCR (RT-qPCR) analysis was employed to verify the results of the RNA sequencing in 45 cases of PTC. The dysregulated circRNA expression and clinicopathological characteristics were assessed and the potential roles of circRNAs in the cellular miRNA and mRNA network were predicted using bioinformatics analysis. The results demonstrated that, compared with normal tissues, a total of 53 circRNAs were dysregulated in tumour tissues, and 8 circRNAs were validated at the mRNA level (P<0.001 and P<0.01). Among those, the expression of chr5:161330882-161336769- (P=0.015), chr9:22046750-22097364+ (P=0.041) and chr8:18765448-18804898- (P=0.036) were obviously associated with the BRAF V600E mutation, chr12:129699809-129700698- was associated with capsular invasion (P=0.025) and chr:38523418-38530666- was associated with pT stage (P=0.037) and lymph node metastasis (P=0.002). Therefore, some dysregulated circRNAs were found to be associated with BRAFV600E mutation, capsular invasion, advanced pT stage and lymph node metastasis of PTC, indicating that circRNAs may be involved in tumourigenesis and cancer progression, and they may be putative biomarkers for the diagnosis and evaluation of progression of PTC.

Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, accounting for 75-85% of all primary thyroid carcinomas (1). The classical and follicular variants are the most common types of PTC, and they differ markedly regarding diagnosis, prognosis, treatment, recurrence, molecular alterations, genetic alterations and molecular biomarkers (2), such as with the preoperative diagnosis of the follicular variant of PTC being more challenging compared with that of classical PTC (3).

Several markers for the diagnosis or prognosis of PTC have been reported, including thyroglobulin for well-differentiated PTC (4,5), ATP5E for early diagnosis (6), RET/PTC, RAS and B-type Raf kinase (BRAF) mutations for diagnosis or prognosis (7). However, the mechanisms of action of those markers require further study and validation. Therefore, there is a clinical need for more reliable, sensitive and specific markers for PTC.

Most human transcripts are composed of non-coding RNA, and accumulating evidence supports the important regulatory role of non-coding RNAs, such as microRNAs (miRNAs) (8) and long non-coding RNAs (lncRNAs) (9), in several physiological and pathophysiological processes. Recently, circular RNAs (circRNAs) have attracted attention in the field of RNA research (10,11). CircRNAs are a novel class of RNAs that have a closed loop structure and are abundantly present in the eukaryotic transcriptome (12,13). The majority of circRNAs are composed of exon sequences, which are conserved across different species, and have expression specificity for different tissues and developmental stages (10). They are primarily considered to act as sponges that bind competitively with miRNAs to regulate the expression of target genes (10,14,15), and dysregulated expression of circRNAs has been found in several types of human cancer (16-19), such as gastric cancer (20,21), hepatocellular carcinoma (HCC) (17), colorectal cancer (18) and PTC (22-26). However, the detailed function and molecular mechanism of circRNAs in tumour diagnosis, prognosis and treatment have yet to be fully elucidated.

Therefore, in the present study, the circRNA profile in PTC tissues was screened with high-throughput RNA sequencing (RNA-Seq) and the dysregulated circRNAs were validated with reverse transcription-quantitative PCR (RT-qPCR)
analysis. Subsequently, the associations between circRNA expression and clinicopathological characteristics were analysed, and the potential roles of circRNAs were predicted with bioinformatics analysis, with the aim of determining their functions in the tumourigenesis, progression and diagnosis of PTC.

Materials and methods

Patient information. A total of 50 patients who underwent surgical treatment with a final diagnosis of PTC at Peking Union Medical College Hospital (Beijing, China) between January 2017 and December 2018 were enrolled in this study, and the clinicopathological characteristics of the patients are listed in Tables I and III. The recruited patients did not receive any adjunctive treatment prior to surgery, such as radiotherapy, chemotherapy or targeted therapy. Clinical information, including patient age, sex, multifocality, subtype (classical/follicular variant PTC), Ki67, vascular endothelial growth factor (VEGF), pT, pN, metastasis, BRAF mutation, capsular invasion and thyroiditis, was collected from the clinical records and stratified as characteristics according to the 8th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (27).

A total of 50 paired specimens (tumour and normal tissues) were obtained from the same patients during surgery. Following gross examination, the tissues were resected and washed with PBS (pH 7.2). One part of each specimen was immediately snap-frozen using liquid nitrogen within 30 min and cryopreserved in liquid nitrogen (−196˚C) until RNA sequencing and gene expression analysis. The remaining part of the specimen was fixed in formalin at room temperature for 48 h and embedded in paraffin, and then 4-μm sections were cut from each block and stained with haematoxylin and eosin (H&E). The morphology of H&E slides were reviewed by 2 experienced pathologists to confirm the final diagnosis (28).

The present study was approved by the Institutional Review Board of Peking Union Medical College Hospital, and written informed consent was obtained from all patients regarding the use of their tissues for research purposes.

Sample preparation and circRNA sequencing. A total amount of 5 μg RNA per sample was used as input material for RNA sample preparation. First, ribosomal RNAs were depleted using a Ribo-Zero™ rRNA Removal Kit (EPIcentre) to obtain rRNA-depleted RNAs. The rRNA-depleted RNAs were further treated with RNase R (EPIcentre) and were then subjected to TRIzol extraction (Thermo Fisher Scientific, Inc.) and random primers at 70˚C for 10 min followed by a 60-min incubation at 37˚C. Then the cDNA was analysed by qPCR with Fast SYBR® Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.; cat. no. 4385612) and a 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification conditions were 95˚C (20 sec), 40 cycles of 95˚C (3 sec) and 60˚C (30 sec) according to the manufacturer's instructions. GAPDH was used as the internal control. The divergent primers used for circRNA validation are listed in Table SI, the specificity of primers was assessed in NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the backsplice junction of circRNA in the PCR product was verified with Sanger sequencing. The relative expression level of each circRNA with GAPDH was calculated using the 2−ΔΔCt method (24,33).

Immunohistochemistry (IHC). IHC staining was performed on 4-μm sections using the DAKO Autostainer Link 48 (Dako; Agilent Technologies, Inc.). The tissue epitopes were repaired using the automated water bath heating process in Dako PT Link (Dako; Agilent Technologies, Inc.); the sections were incubated in Tris-EDTA retrieval solution (10 mM Tris, 1 mM EDTA; pH 9.0) at 98˚C for 20 min. The sections were subsequently incubated for 50 min at room temperature with the primary anti-Ki67 (cat. no. MIB1; Dako; Agilent Technologies, Inc.) antibodies, diluted at 1:200 and 1:100, respectively, in Dako Envision™ Flex antibody diluents, followed by anti-rabbit immuno-peroxidase polymer (Envision FLEX/HRP) for 20 min at room temperature according to the manufacturer's instructions, and developed with freshly prepared 0.05% 3,3’-diaminobenzidine tetrahydrochloride. Finally, the slides were counterstained with haematoxylin, dehydrated, and mounted.

BRAFV600E mutation detection. Genomic DNA was extracted from fresh-frozen tissues using the TIAmp Genomic DNA Kit (Tiangen Biotech Co., Ltd.; cat. no. DP304). Subsequently, the genomic DNA was used to detect the V600E mutation in the BRAF oncogene through the AmoyDx BRAFV600E Mutations Detection Kit (AmoyDx; cat. no. ADx-BR04).
**Results**

**CircRNA expression profiles in PTC tissues relative to adjacent tissues.** The clinicopathological characteristics of the 5 patients are listed in Table I. The majority were male (4 cases, 75%), 3 cases were classical PTCs, 1 case was follicular PTC and 1 case was mixed classical and follicular PTC, with different tumour-node-metastasis (TNM) stages. Two cases were multifocal and all displayed capsular invasion. The histomorphology of tissues are shown in Fig. 1. Among them, Fig. 1A and C shows the histomorphology of normal tissues and with lymphocyte infiltration. Fig. 1B and represent the classical PTC and follicular variant of PTC, respectively.

Subsequently, RNA-seq demonstrated that the circRNA expression profile of the 5 paired normal and PTC tissue samples exhibited significant differences. The hierarchical clustering (Fig. 2A) and volcano plots (Fig. 2B) revealed that there were 53 circRNAs differentially expressed in carcinoma (Fig. 3) were in accordance with the RNA-seq data (Fig. 2D).

The circRNA relative expression level of GAPDH was used to analyse the association between circRNA expression and clinical characteristics. The clinical characteristics of 45 PTC patients are summarized in Table III. The median age of the patients at diagnosis was 45 years (range, 17-64 years), while the majority of the patients were female (n=33, 73.3%); 32 cases (71.1%) were classical PTC, 12 cases (26.7%) were the follicular variant of PTC and only 1 case (2%) was a mixture of the classical and follicular variant. Moreover, 31 cases (68.9%) were unifocal, and 14 cases (31.1%) were multifocal. In regards to pT stage, 29 cases (64.4%) had a diameter <2 cm or localized, 4 (8.9%) ranged between 2 and 4 cm, and 11 (24.4%) were >4 cm or invading the soft tissues beyond the thyroid. Only one-quarter of the patients had regional lymph node metastasis. None of the patients had metastasis. The Ki67 index of 33.3% patients was very high (≥3), and VEGF was positive for 91.1% patients. BRAFV600E mutation, capsular invasion and thyroiditis with diffuse lymphocytic infiltration were observed in 80, 75.6 and 20% of the cases, respectively.

Subsequently, we analysed the association between the mean ± standard deviation of circRNA expression in the tumour and the clinical characteristics in 45 PTC patients, including age, sex, multifocality, Ki67, capsular invasion, VEGF, BRAFV600E mutation, TNM stage and thyroiditis (Table III). Among the 8 dysregulated circRNAs, chr5:161330882-161336769- with the highest AUC (0.878) and chr12:19699809-19700698- was associated with the BRAF V600E mutation, TNM stage and thyroiditis (Table III). Among the 3 dysregulated circRNAs, chr5:161330882-161336769- was associated with capsular invasion (P=0.025). Additionally, chr5:38523418-38530666- was associated with pN mutation (P=0.002) stage. This association between circRNA expression and clinicopathological characteristics indicated that those circRNAs may participate in tumourigenesis and progression of PTC. The other three dysregulated circRNAs exhibited no
statistically significant association with clinicopathological characteristics.

Furthermore, the association between the mRNA expression of these 5 circRNAs and the clinical characteristics was assessed with ROC curve analysis (Fig. 4B), revealing that the AUCs of all circRNAs were >0.7 (P<0.05), with chr5:38523418-38530666-(associated with pN stage) having the highest AUC (0.8209, P<0.01; Table IV). This result was consistent with the data mentioned above, and indicated that those 5 circRNAs associated with BRAF\textsuperscript{V600E} mutation, capsular invasion, advanced pT stage and lymph node metastasis may be putative biomarkers for the diagnosis and evaluation of the progression of PTC.

**Gene ontology (GO) analysis and pathway analysis of circRNA genes.** GO enrichment analysis for the host genes of the identified differentially expressed circRNAs was performed, and the significantly enriched GO terms in the biological process, cellular component and molecular function categories, e.g., carbohydrate derivative catabolic process (GO: 1901136, \( P=0.00021557 \)), membrane (GO: 0016020, \( P=0.0012195 \)) and GTPase regulator activity (GO: 0030695, \( P=0.00021632 \)), are presented in Fig. 5A. A total of 28 host genes of the differentially expressed circRNAs were associated with the membrane GO term. The results of the Kyoto Encyclopaedia of Genes and Genomes pathway analysis of the 20 highly related circRNA genes are presented in Fig. 5B. Those circRNA genes were mainly associated with important pathways (Rap1, VEGF and Ras signalling pathways), tumourigenesis (transcriptional misregulation and proteoglycans in cancer), cell signal transduction (axon guidance, retrograde endocannabinoid signalling, cytokine-cytokine receptor interaction), infection and immune defence (bacterial invasion of epithelial cells, chemokine signalling pathway). Furthermore, the Rap1 and Ras signalling pathway contained more related circRNA genes, which suggested that circRNAs may play a key role in PTC tumourigenesis and progression.

**Prediction for the circRNA-miRNA network.** circRNAs have been shown to contain miRNA-binding sites and negatively regulate the inhibitory effects of miRNAs on their target mRNAs, thereby essentially acting as miRNA sponges (14). To evaluate the potential functions of the identified differentially expressed circRNAs, miRNAs that were potentially associated with those circRNAs were investigated using the RegRNA2 platform and Circular RNA Interactome (42,43). The first 5 predicted miRNAs for the dysregulated circRNAs are listed in Table V. For example, circRNA chr5:161330882-161336769 was predicted to harbour has-miR-1273g-3p, has-miR-498, has-miR-1268a, has-miR-363-5p and has-miR-566.

### Table I. Clinicopathologic characteristics of the 5 patients for circRNA sequencing.

| Patient no. | Age (years) | Sex | TNM   | Subtype                        | Capsular invasion | Multicentricity |
|-------------|-------------|-----|-------|---------------------------------|-------------------|-----------------|
| 1           | 46          | Male| T3N0M0| Classical+follicular variant    | Yes               | Yes             |
| 2           | 78          | Male| T3N0M0| Follicular variant              | Yes               | Yes             |
| 3           | 38          | Female| T1N1M0| Classical                       | Yes               | No              |
| 4           | 36          | Male| T1N0M0| Classical                       | Yes               | No              |
| 5           | 44          | Male| T1N1M0| Classical                       | Yes               | No              |

circRNA, circular RNAs; TNM, Tumour, Node, Metastasis.
Figure 2. Overview of the circRNA profiles (N, normal thyroid tissues; T, tumour thyroid tissues; chr, chromosome). (A) Hierarchical cluster analysis of differentially expressed circRNAs. The red and blue colours indicate high and low expression, respectively. (B) Volcano plot of differentially expressed circRNAs in PTC. The dotted line represents P=0.05. The red and green points represent circRNAs that were significantly upregulated or downregulated, respectively. (C) Chromosomal distributions of differentially expressed circRNAs. (D) RNA sequencing data of 8 circRNAs. PTC, papillary thyroid carcinoma; circRNA, circular RNA.
To further explore which miRNAs may be cancer-related, the starBase v2.0 (44) and miRPathDB v1.1 (45) platforms were used, and a network map of circRNA-miRNA interactions was constructed to identify the dysregulated circRNAs in...
PTC that potentially interacted with cancer-related miRNAs. From the initial list of 40 potential miRNAs identified by the RegRNA2 and circRNA Interactome analysis, several cancer-related miRNAs were identified following a literature research, including hsa-miR-498 (46), hsa-miR-766-3p (47,48) and hsa-miR-661 (49,50), which are associated with cell proliferation in several cancers. The results indicated that those dysregulated circRNAs may act as miRNA sponges, leading to the progression of PTC, but the specific underlying mechanism requires further investigation.

**Discussion**

The present study profiled the circRNA expression in papillary thyroid carcinoma (PTC) using RNA-seq, and a total of 45 upregulated and 8 downregulated circRNAs were identified in PTC tumours. Among the 53 dysregulated circRNAs, chr3:64594257-64596991-(hsa_circ_0066444) has been reported to be significantly differentially expressed in hepatocellular carcinoma (HCC) tissues by RNA-seq, but it exhibited no significant difference on PCR verification (34).
| Clinicopathologic parameters | No. of cases (%) | chr5:161330882-161336769 | chr12:129699009-129700698 | chr9:22046750-22097364+ | chr20:17456347-17465553+ | chr7:116699070-116700284+ | chr8:18765448-18804898- | chr7:22308338-22318037- | chr5:38523418-38530666- |
|-----------------------------|------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Age (years)                 |                  |                           |                           |                           |                           |                           |                           |                           |                           |
| <45                         | 28 (62.2)        | 1.21E-02±0.797            | 2.26E-03±0.574            | 4.34E-04±0.386            | 1.52E-04±0.708            | 2.39E-02±0.761            | 2.13E-02±0.374            | 8.81E-03±0.963            | 8.12E-04±0.673           |
| ≥45                         | 17 (37.8)        | 1.25E-02±0.006            | 2.12E-03±0.001            | 4.45E-04±0.001            | 1.54E-04±0.000            | 2.32E-02±0.038            | 2.21E-02±0.018            | 8.51E-03±0.011            | 8.01E-04±0.001           |
| Sex                         |                  |                           |                           |                           |                           |                           |                           |                           |                           |
| Female                      | 33 (73.3)        | 1.29E-02±0.209            | 2.06E-03±0.259            | 4.47E-04±0.472            | 1.57E-04±0.590            | 2.37E-02±0.505            | 2.26E-02±0.228            | 8.61E-03±0.878            | 7.99E-04±0.342           |
| Male                        | 12 (26.7)        | 1.12E-02±0.005            | 2.26E-03±0.001            | 4.33E-04±0.000            | 1.56E-04±0.000            | 2.47E-02±0.040            | 2.08E-02±0.018            | 9.05E-03±0.011            | 8.61E-04±0.001           |
| Multifocal                  |                  |                           |                           |                           |                           |                           |                           |                           |                           |
| No                          | 31 (68.9)        | 1.19E-02±0.641            | 2.16E-03±0.148            | 4.24E-04±0.477            | 1.47E-04±0.961            | 2.32E-02±0.492            | 2.13E-02±0.941            | 8.52E-03±0.961            | 8.15E-04±0.902           |
| Yes                         | 14 (31.1)        | 1.29E-02±0.005            | 2.06E-03±0.001            | 4.47E-04±0.001            | 1.57E-04±0.000            | 2.37E-02±0.039            | 2.26E-02±0.019            | 8.61E-03±0.011            | 7.99E-04±0.001           |
| Ki67 <3                     | 26 (57.8)        | 1.21E-02±0.316            | 2.26E-03±0.725            | 4.34E-04±0.725            | 1.52E-04±0.116            | 2.39E-02±0.892            | 2.13E-02±0.705            | 8.81E-03±0.850            | 8.12E-04±0.110           |
| ≥3                          | 15 (33.3)        | 1.29E-02±0.005            | 2.06E-03±0.001            | 4.47E-04±0.001            | 1.57E-04±0.000            | 2.37E-02±0.039            | 2.26E-02±0.019            | 8.61E-03±0.011            | 7.99E-04±0.001           |
| VEGF(+)                     |                  |                           |                           |                           |                           |                           |                           |                           |                           |
| Yes                         | 41 (91.1)        | 1.27E-02±0.005            | 2.16E-03±0.001            | 4.47E-04±0.001            | 1.57E-04±0.000            | 2.37E-02±0.039            | 2.26E-02±0.019            | 8.61E-03±0.011            | 7.99E-04±0.001           |
| No                          | 0                |                           |                           |                           |                           |                           |                           |                           |                           |
| pT-stage                    |                  |                           |                           |                           |                           |                           |                           |                           |                           |
| x                           | 1 (2.2)          | 2.24E-03±0.375            | 2.16E-03±0.590            | 4.53E-03±0.098            | 1.57E-04±0.381            | 2.35E-02±0.319            | 2.25E-02±0.754            | 8.66E-03±0.237            | 7.81E-04±0.037           |
| 1                           | 29 (64.4)        | 1.25E-02±0.005            | 2.12E-03±0.001            | 4.45E-04±0.001            | 1.54E-04±0.000            | 2.32E-02±0.038            | 2.21E-02±0.018            | 8.51E-03±0.011            | 8.01E-04±0.001           |
| 2                           | 4 (8.9)          | 1.22E-02±0.006            | 2.21E-03±0.001            | 4.32E-04±0.001            | 1.50E-04±0.000            | 2.36E-02±0.039            | 2.16E-02±0.018            | 8.67E-03±0.011            | 7.94E-04±0.001           |
| 3                           | 11 (24.4)        | 1.23E-02±0.005            | 2.16E-03±0.001            | 4.27E-04±0.001            | 1.53E-04±0.000            | 2.41E-02±0.040            | 2.15E-02±0.018            | 8.76E-03±0.011            | 8.32E-04±0.001           |
| pN stage                    |                  |                           |                           |                           |                           |                           |                           |                           |                           |
| 0/x                         | 34 (75.6)        | 1.19E-02±0.291            | 2.16E-03±0.561            | 4.24E-04±0.428            | 1.47E-04±0.113            | 2.32E-02±0.398            | 2.13E-02±0.712            | 8.53E-03±0.653            | 8.15E-04±0.002           |
| 1                           | 11 (24.4)        | 1.27E-02±0.005            | 2.16E-03±0.001            | 4.53E-04±0.001            | 1.57E-04±0.000            | 2.35E-02±0.038            | 2.25E-02±0.018            | 8.66E-03±0.011            | 7.81E-04±0.001           |
Table III. Continued.

| Clinico-pathologic parameters | No. of cases (%) | chr5:161330882-161336769+ | chr12:129699809-129700698+ | chr9:22046750-22097364+ | chr20:17456347-17465553+ | chr7:116699070-116700284+ | chr8:18765448-18804898+ | chr7:22308338-22318037+ | chr5:38523418-38530666+ |
|------------------------------|------------------|-----------------------------|----------------------------|-----------------------------|----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                              | Mean ± SD        | P-value                     | Mean ± SD                  | P-value                     | Mean ± SD                  | P-value                     | Mean ± SD                  | P-value                     | Mean ± SD                  | P-value                     |
| **BRAF mutation**            |                  |                             |                            |                             |                            |                             |                            |                             |                             |                             |
| Yes                          | 36 (80.0)        | 1.27E-02± 0.015±           | 2.16E-03± 0.865            | 4.53E-04± 0.041±           | 1.57E-04± 0.955           | 2.35E-02± 0.496            | 2.25E-02± 0.036±           | 8.66E-03± 0.670            | 7.81E-04± 0.444            |
| No                           | 9 (20.0)         | 9.71E-03± 0.012            | 1.95E-03± 0.006            | 3.96E-04± 0.001            | 1.38E-04± 0.000           | 2.14E-02± 0.037            | 1.75E-02± 0.016            | 8.59E-03± 0.011            | 7.24E-04± 0.001            |
| Capular invasion             |                  |                             |                            |                             |                            |                             |                            |                             |                             |                             |
| Yes                          | 34 (75.6)        | 1.25E-02± 0.025±           | 2.12E-03± 0.005            | 4.45E-04± 0.059            | 1.54E-04± 0.937           | 2.32E-02± 0.771            | 2.21E-02± 0.561            | 8.51E-03± 0.979            | 8.01E-04± 0.544            |
| No                           | 11 (24.4)        | 1.19E-02± 0.006            | 2.25E-03± 0.001            | 4.33E-04± 0.000            | 1.50E-04± 0.000           | 2.18E-02± 0.037            | 2.13E-02± 0.018            | 8.38E-03± 0.011            | 8.68E-04± 0.001            |
| With thyroiditis             |                  |                             |                            |                             |                            |                             |                            |                             |                             |                             |
| Yes                          | 9 (20.0)         | 1.27E-02± 0.610            | 2.16E-03± 0.061            | 4.53E-04± 0.222            | 1.57E-04± 0.364           | 2.35E-02± 0.570            | 2.25E-02± 0.212            | 8.66E-03± 0.395            | 7.81E-04± 0.281            |
| No                           | 36 (80.0)        | 1.06E-02± 0.013            | 1.89E-03± 0.006            | 4.07E-04± 0.001            | 1.59E-04± 0.000           | 2.18E-02± 0.039            | 2.03E-02± 0.019            | 8.39E-03± 0.011            | 7.89E-04± 0.001            |

*Associations between the mean ± standard deviation of circRNA expression in the tumor and the clinical characteristics of the 45 PTC patients were analyzed with nonparametric statistics Mann-Whitney U test and Kruskal-Wallis test followed with post-hoc Dunn's multiple comparison test respectively for comparison between two or multiple groups, and P<0.05 was considered to indicate statistically significant differences. circRNA, circular RNA; PTC, papillary thyroid cancer; BRAF, B-type Raf kinase; SD, standard deviation.
Table IV. Area under the curve (AUC) of the circRNA mRNA expression with the clinical characteristics of the 45 cases of PTC.

| circRNA | Clinical characteristics | AUC   | 95% CI              | Std. Error | P-value |
|---------|--------------------------|-------|---------------------|------------|---------|
| chr5:161330882-161336769- | BRAF mutation             | 0.7654| 0.6142 to 0.9167    | 0.07714    | 0.01471 |
| chr9:22046750-22097364+    | BRAF mutation             | 0.7222| 0.5562 to 0.8882    | 0.08468    | 0.0411  |
| chr8:18765448-18804898-    | BRAF mutation             | 0.7284| 0.5757 to 0.8811    | 0.0779     | 0.0358  |
| chr12:129699809-129700698-  | Capsular invasion         | 0.7273| 0.5480 to 0.9066    | 0.09146    | 0.02482 |
| chr5:38523418-38530666-    | pN Stage                  | 0.8209| 0.6905 to 0.9512    | 0.0665     | 0.001538|

PCT, papillary thyroid cancer; circRNA, circular RNA; CI, confidence interval; BRAF, B-type Raf kinase.

Figure 5. (A) Gene Ontology (GO) terms. N, normal thyroid tissues; T, tumour thyroid tissues. (B) Kyoto Encyclopedia of Genes and Genomes pathways of the differentially expressed circRNA genes in 20 pathways. BP, biological process; CC, cellular component; MF, molecular function.
The BRAF V600E mutation is the most common genetic aberration in PTC. The associations between the circRNA expression profile and clinical characteristics in PTC were then explored, and it was observed that chr5:161330882-161336769-(P=0.015), and clinical characteristics in PTC were then explored, and in PTCs with microarray profiling (51), which was consistent (hsa_circ_0002111) was found to be significantly upregulated and apoptosis inhibited PTC cell proliferation and promoted cell cycle arrest and apoptosis in vitro (52), which may constitute the evidential basis for further research.

Additionally, chr8:18799294-18804898- (hsa_circ_0004458) was reported to be upregulated in PTC tumours (51) and cells (52), which was consistent with our sequencing results. Another study verified that the silencing of hsa_circ_0004458 cells (52), which was consistent with our sequencing results. Furthermore, the host genes of chr5:161330882-161336769-, had the highest area under the curve (AUC) (0.878) and may be considered a putative biomarker for the progression of PTC. Furlan et al results suggested that the presence of capsular invasion does not adversely affect the biological behaviour of PTC or patient survival (58), but another study indicated that it is associated with a higher recurrence rate (59) and reduced survival time and quality of life (60). Capsular invasion, advanced pT stage and pathological positive lymph node metastasis were independent predictors of multifocal PTC (61). Additionally, transmembrane protein 132D (TMEM132D), the host gene reported that hsa_circRNA_007148 is likely to be associated with the BRAFV600E mutation. The BRAFV600E mutation is the most common genetic alteration in PTC and has been found in up to 68% of PTCs in adults with aggressive disease characteristics (53).

Table V. Predicted miRNAs for the top upregulated or downregulated circRNAs in PTC.

| circRNA                  | circBase ID | Predicted miRNAs       |
|--------------------------|-------------|-------------------------|
| chr5:161330882-161336769-| /           | miR-1273g-3p miR-498 miR-1268b miR-363-5p miR-566 |
| chr12:129699809-129700698-| /           | miR-4726-3p miR-4793-3p miR-766-3p miR-1343 miR-1226-5p |
| chr9:22046750-22097364+  | hsa_circ_0008796 | miR-490-5p miR-224 miR-1228 miR-576-5p miR-611 |
| chr20:17456347-17465553+| /           | miR-939 miR-576-3p miR-3925-5p miR-5189 miR-4732-5p |
| chr7:116699070-116700284+| hsa_circ_0082002 | miR-512-5p miR-515-3p miR-519e miR-609 miR-653 |
| chr8:18765448-18804898- | hsa_circ_0002111 | miR-647 miR-532-3p miR-526b miR-1263 miR-432 |
| chr7:22308338-22318037-  | /           | miR-5095 miR-5096 miR-3159 miR-4524a-3p miR-5699 |
| chr5:38523418-38530666-  | hsa_circ_0072309 | miR-1277 miR-331-5p miR-409-3p miR-515-5p miR-1276 |

PCC, papillary thyroid carcinoma; circRNAs, circulating RNAs; miRNAs, microRNAs.

In addition, the results of the present study also demonstrated that chr12:129699809-129700698- was associated with capsular invasion (P=0.025), which is considered a putative biomarker for the progression of PTC. Furlan et al results suggested that the presence of capsular invasion does not adversely affect the biological behaviour of PTC or patient survival (58), but another study indicated that it is associated with a higher recurrence rate (59) and reduced survival time and quality of life (60). Capsular invasion, advanced pT stage and pathological positive lymph node metastasis were independent predictors of multifocal PTC (61). Additionally, transmembrane protein 132D (TMEM132D), the host gene reported that hsa_circRNA_007148 is likely to be associated with the BRAFV600E mutation. The BRAFV600E mutation is the most common genetic alteration in PTC and has been found in up to 68% of PTCs in adults with aggressive disease characteristics (53).

Although it was reported that the major driver mutation of follicular variant PTC and classical PTC was RAS-related mutation and BRAFV600E mutation, respectively (2), other studies indicated that the follicular variant PTC also harbours the BRAFV600E mutation (54,55). In addition to our study, Ren et al reported that hsa_circRNA_047771 is likely to be associated with the BRAFV600E mutation (35). The results indicated that BRAFV600E may be associated with multiple circRNAs, and whether those circRNAs are involved in tumourigenesis should be the focus of further investigation.

Furthermore, the host genes of chr5:161330882-161336769-, chr9:22046750-22097364+ (hsa_circ_0008796) and chr8:18765448-18804898- (hsa_circ_0002111) were expressed in multiple circRNAs, and whether those circRNAs are involved in tumourigenesis should be the focus of further investigation.

Furthermore, the host genes of chr5:161330882-161336769-, chr9:22046750-22097364+ (hsa_circ_0008796) and chr8:18765448-18804898- (hsa_circ_0002111) were expressed in multiple circRNAs, and whether those circRNAs are involved in tumourigenesis should be the focus of further investigation.

In brief, the AUCs of the 8 dysregulated circRNAs indicated that they were specific to PTC, and among them, the 5 circRNAs associated with BRAFV600E, capsular invasion, advanced pT
stage and lymph node metastasis, may be putative biomarkers for the diagnosis and evaluation of progression of PTC.

For circRNAs that were found to be associated with the cellular RNA network and negatively regulating the inhibitory effects of miRNAs on their target mRNAs, it was necessary to analyse the downstream miRNAs and mRNA of circRNAs to gain insight into the molecular mechanisms of action and the role of circRNAs in tumourigenesis and cancer progression. Although the literature on the circRNA-miRNA network in regard to other identified circRNAs is limited, there was a considerable amount of literature on those miRNAs. Therefore, in our results, miR-498 was predicted to be a target mRNA of chr1:156330882-161336769+, and the expression of miR-498 was also markedly downregulated in ovarian cancer cells and tumours, acting as a new tumour suppressor by targeting the FOXO3 gene to inhibit cell proliferation in ovarian cancer (46). The present research demonstrated that chr5:161330882-161336769- was significantly upregulated in PTC, although further study is required to determine whether miR-498 could be consequently downregulated in PTC as a result of regulatory disinhibition, and the effects of dysregulation on cancer-related target genes should be the focus of further research.

In addition, miR-766-3p and miR-661, the predicted miRNAs of chr12:129699809-129700698- and chr9:22046750-22097364+ (has_circ_0008796), may also be consequently dysregulated in PTC as a result of regulatory disinhibition. For example, it was previously demonstrated that miR-766-3p inhibits tumour progression by targeting Wnt3a in HCC (47). MiR-661 was shown to inhibit metastatic tumour antigen 1 in breast cancer cells (50), and to inhibit cell proliferation, migration and invasion by targeting hTERT in glioma cells (49). Furthermore, as the predicted miRNA of chr5:38523418-38530666- (hsa_circ_0072309), miR-409-3p may promote epithelial-to-mesenchymal transition and tumourigenesis via the RSU1 or STAG2 pathway in prostate cancer (66,67). However, the specific function and mechanism of action of circRNAs and miRNAs in PTC have not been fully elucidated, and further studies should focus on the circRNA/miRNA/mRNA axis in the regulation of PTC tumourigenesis, in order to conclusively determine cancer-related target genes and facilitate further biomarker identification for PTC.

In general, the function of the parental genes may have some effect on the circRNA, but the effects on tumours are not only associated with the parental genes, as circRNAs may also play several important roles in biological processes, such as regulating gene expression by acting as competitors of pre-mRNA splicing, as decoys for microRNAs, as sponges for RNA-binding proteins, possibly also as substrates for translation (68), and may regulate the transcription of their parental genes (69). Ours is a preliminary study of the expression profile of circRNAs in thyroid cancer. In future studies, we would like to focus more on the mechanisms of action of the dysregulated circRNAs and their sponging miRNAs and potential mRNA targets, which may provide clues to the molecular mechanisms of action of circRNAs in PTC.

Besides, there were certain limitations to the present study, such as the small sample size, different histological subtypes of PTC, and the fact that signaling pathways were not studied. Thus, the results require verification using larger sample sizes and more detailed investigation of the differential expression of circRNAs between the two PTC subtypes and their role in diagnosis, progression and evaluation of prognosis.

In conclusion, the present study demonstrated that several circRNAs are differentially expressed in PTC and were found to be associated with the BRAFV600E mutation, capsular invasion, advanced pT stage and lymph node metastasis, thereby indicating that circRNAs may participate in tumourigenesis and cancer progression. The detailed role of circRNAs as putative biomarkers for the diagnostic and evaluation of progression of PTC should be further explored and validated. Furthermore, those differentially expressed circRNAs may participate in the cellular RNA network with miRNAs and mRNAs, and further research should be performed to gain a better insight into the molecular mechanisms of circRNAs in PTC.

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Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

DG carried out the study design and manuscript preparation. FL conducted the manuscript preparation and data analysis/interpretation. XZ carried out the experimental studies and data analysis. BL carried out the manuscript editing and revision. SZ conducted the experimental studies. AW performed the statistical analysis. DC conducted the experimental studies. JS was responsible for the specimen acquisition and diagnosis. BL was responsible for the study conception and clinical studies.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Peking Union Medical College Hospital (Beijing, China). Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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