Hsa_circ_0005729 enhances the accuracy in diagnosing parathyroid carcinoma

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Abstract

Background: Parathyroid carcinoma (PC), often misdiagnosed as parathyroid adenoma (PA), is prone to local relapse due to the initial surgery being restricted to parathyroid lesions instead of en bloc resection of parathyroid lesions with negative incision margins. However, it is very challenging to distinguish PC from PA preoperatively; hence, this study investigated an effective biomarker for increasing accuracy in PC diagnosis.

Method: First, differentially expressed the circular RNAs between three PC tissues and three PA tissues were screened by high-throughput circular RNA sequencing, and the expression of hsa_circ_0005729 was verified by qRT-PCR in 14 patients with PC and 40 patients with PA. Second, the receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to analyze the diagnostic efficiency of hsa_circ_0005729 in PC by combining with laboratory data. Third, RNF138 mRNA, the corresponding linear transcript of hsa_circ_0005729 was measured, and the relationship between hsa_circ_0005729 and RNF138 mRNA was analyzed in patients with PA and patients with PC.

Results: Hsa_circ_0005729 expression was significantly higher in patients with PC than in patients with PA. Serum calcium (p = 0.045), alkaline phosphatase (ALP) (p = 0.048), and creatinine levels (p = 0.036) were significantly higher in patients with PC than in patients with PA. The AUC increased to 0.86 when hsa_circ_0005729 combined with serum calcium, creatinine, and ALP. In addition, hsa_circ_0005729 was positively correlated with RNF138 mRNA in patients with PA but not in patients with PC.

Conclusion: The novel circular RNA hsa_circ_0005729 was found to have a higher expression in patients with PC, and indicating its usefulness for distinguishing PC from PA.

Introduction

Parathyroid carcinoma (PC) is an extremely rare disease, accounting for 0.005% of all
malignancies (1), and representing approximately 1%–5% of primary hyperparathyroidism cases (2-3). It is worth noting that the best approach for curing PC may be the en bloc resection of parathyroid lesions with negative incision margins (4-6), since PC is often insensitive to chemotherapy and radiotherapy (7-8). Most patients with PC suffer from lifetime distress due to repeated surgical resections, and have poor prognosis; most die of uncontrollable hypercalcemia due to excessive parathyroid hormone (PTH) secretion (5), with 5-year and 10-year survival rates between 77%–91% and 49%–77%, respectively (5-6, 9-15).

Nowadays, it is extremely difficult to distinguish PC from PA preoperatively (16), and patients with the PC and PA often have similar clinical manifestations and laboratory examinations (17). Most patients with PC have higher levels of serum calcium, PTH, and larger tumor size than the patients with PA. However, some PC cases had normal serum calcium or PTH levels. It is also difficult to identify benign or malignant parathyroid tumors by contrast-enhanced computed tomography (CT) scan, cervical ultrasonography, or 99mTc-Sestamibi scan (18), which may only help with localization of parathyroid lesions. Fine needle aspiration biopsy of the parathyroid mass is not recommended for parathyroid tumors, because of the risk of needle track implants (4, 19). Even intra-operative frozen section analysis may fail to identify a tumor as PC, because the pathological features of malignant lesions overlap with benign tumors. Unequivocal PC diagnosis must be confirmed via capsular invasion, vascular infiltration, nerve involvement, tumor infiltration of adjacent tissues, and postoperative local relapse, or metastasis (20-21).

*Cell division cycle 73 (CDC73)* germline mutations and loss of parafibromin staining occur in approximately 1/3 of the sporadic patients with PC (22-23), which may assist in diagnosing PC. However, the absence of *CDC73* mutations or loss of parafibromin staining cannot exclude PC (24). A recent study revealed that cancer-derived *immunoglobulin G* (cancer-IgG)
expression was higher in PC than in PA (25). However, immunohistochemistry was used to detect the cancer-IgG, which does not apply to the preoperative diagnosis PC.

Circular RNAs have recently been detectable noninvasively in peripheral blood specimens in the several cancers, such as breast cancer and colorectal carcinoma (26-28). Upregulated circular RNAs may help to identify an effective diagnostic biomarker for PC. To date, to our knowledge, only one study has described differentially expressed circular RNAs between patients with PC and patients with PA, including hsa_circRNA_0035563, hsa_circRNA_0017545, hsa_circRNA_0001687, and hsa_circRNA_0075005 (29). However, the global incidence of PC is extremely low, and more PC cases are required for identifying the effective PC biomarkers. This study identified a novel circular RNA hsa_circ_0005729 that may enhance diagnosing PC.

Materials and methods

1. Human tissue specimens

All tissue samples were collected immediately from the patients after a resection, snap-frozen in liquid nitrogen, and transferred to a −80°C refrigerator until RNA extraction, from the Beijing Chaoyang Hospital between October 2017 and September 2020. This study was approved by the Ethics Review Board of Beijing Chaoyang Hospital, Capital Medical University, and all the patients written informed consent was taken.

A total of 14 patients with PC and 40 patients with PA were enrolled, and the clinicopathological characteristics of the cohort are described in the Table 1. Two patients were diagnosed PC for the first time in our institution, one was pathologically confirmed with PC based on tumor capsular invasion, and the other one based on tumor infiltration of striated muscle and adipose tissues. We collected the two specimens of the primary parathyroid tumors. Other 12 patients with PC were admitted to our institution because of local relapse
after 1–6 times operations in other hospitals because of parathyroid tumors. All the 12 patients with PC underwent an extended en bloc resection in our institution (30), and we collected 12 specimens of the cervical tumor recurrence. The detailed information of the 14 patients with PC is shown in Table 2.

A total of 6 patients with PC had distant metastases, including the 4 cases with lung metastases, one with bone metastases, one with liver and lung metastases simultaneously. We collected 59 tissue samples from the 14 PA and 40 PC patients, including 5 matched distant metastases, with 4 lung metastases, and 1 liver metastases. All tissue samples were confirmed with PC pathologically according to the criteria of the WHO Classification of Tumors of Endocrine Organs, 4th Edition, Volume 10 (31).

None of the recruited patients had any other type of cancer or had previously undergone chemotherapy, radiotherapy, or targeted therapy. Patients with multiple endocrine neoplasms, secondary hyperparathyroidism, tertiary hyperparathyroidism, or hyperparathyroidism-jaw tumor syndrome were also excluded according to familial medical histories, preoperative physical examination, laboratory studies, and imaging examinations, such as serum calcitonin, prolactin, insulin, cortisol, aldosterone, brain magnetic resonance imaging, abdominal ultrasound, or abdominal contrast-enhanced CT.

2. RNA extraction and RNA quality estimation

First, three tissue samples from three patients with PC and three tissue specimens from three patients with PA were used for high-throughput circular RNA sequencing. All three patients with PC underwent their first operation in other institutions and were pathologically diagnosed with PC before visiting our institution. They were diagnosed with PC pathologically in our institution due to cervical relapses and distant metastases. One patient had lung metastases, one had bone metastases, and another had lung and liver metastases simultaneously. All tumor tissue samples used for high-throughput circular RNA sequencing
were from the cervical relapse cases.

All the fresh-frozen tissues were ground using a tissue homogenizer. Ribosomal RNA (rRNA) was removed by using an Epicenter Ribo-zero™ rRNA Removal Kit (Epicenter, USA) and further treated with RNase R (Epicenter, USA). The total RNA from each tissue specimen was isolated by RNA extraction using TRIZol (Invitrogen, USA) according to the manufacturer’s recommendations. The concentration and purity of RNA were evaluated by a Nanodrop 2000 (Wilmington, DE, USA), and RNA integrity was assessed by native agarose gel electrophoresis by inspecting the 28S and 18S rRNA bands.

3. High-throughput circular RNA sequencing

After removal of rRNA and treatment with RNase R, six RNAs from three PC, and three PA tissue samples were prepared for a high-throughput circular RNA sequencing with Illumina sequencing technology from Novegene Inc. Briefly, mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads. First-strand cDNA was synthesized by using random hexamer primers and M-MuLV Reverse Transcriptase; second-strand cDNA synthesis was performed using DNA polymerase I and RNase H. PCR was performed with Phusion High-Fidelity DNA polymerase, universal PCR primers, and index (X) primer. The PCR products were purified, and the library quality was assessed on an Agilent Bioanalyzer 2100 system. The index-coded samples were clustered, and the library preparations were sequenced on an Illumina HiSeq platform, generating 125 bp/150 bp paired-end reads. The detailed methods are described in Supplementary Method 1.

4. Data analysis of the high-throughput circular RNA sequencing

Clean data (clean reads) were obtained by removing reads containing adapters and poly-N and those of poor quality from the raw data. At the same time, the Q20, Q30, and GC contents of the clean data were calculated. All downstream analyses were based on high-quality clean data. The expected number of fragments per kilobase of the transcript sequence per million
base pairs sequenced (FPKM) is currently the most used method for estimating gene expression levels. The number of FPKM of each gene was calculated based on the length of the gene and the read count mapped to this gene. Genes with an adjusted P-value less <0.05 were considered as differentially expressed. A corrected P-value of 0.005 and log 2 (fold change, FC) of one were set as the thresholds for a significantly differential expression. The detailed data analysis of the high-throughput circular RNA sequencing is described in the Supplementary Method 2.

5. Quantitative real-time PCR (qRT-PCR) validation

The primers for the circular RNAs were designed according to the Oligo 7 primer analysis software, and the human β-actin served as the endogenous control. The details of the primer sequences are shown in Supplementary Table 1. Total RNAs were isolated from fresh-frozen tissues, and the mRNAs were used to synthesis of cDNAs after removing rRNAs and treatment with RNase R. The PCR was implemented to determine the primer specificity of circular RNA novel_circ_0024633, hsa_circ_0027093, hsa_circ_0005729, novel_circ_0026113, novel_circ_0026101, which are the top five upregulated circular RNAs according to the results of high-throughput circular RNA sequencing.

The expression levels of hsa_circ_0027093, hsa_circ_0005729, and human β-actin were further measured in 14 PC tissues and 40 PA tissues using qRT-PCR. The qRT-PCR assays were performed with PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Code.DRR047) and SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Code.DRR820) in TaKaRa TP600 PCR Thermal Cycler Dice, and ABI StepOne Real-Time PCR System.

First, cDNAs were synthesized using the PrimeScript RT reagent Kit with a gDNA Eraser. Each reaction consisted of 1.5 µg of total RNA, 2 µL of 5×gDNA Eraser Buffer, 1 µL gDNA Eraser, and sufficient RNase Free dH2O to reach a total volume of 10 µL and was performed at 42°C for 2 mins. Second, RT products were synthesized by using SYBR Premix Ex Taq II
(Tli RNaseH Plus). Each reaction consisted of 10 µL of cDNA, 4 µL of 5×PrimerScript Buffer 2 (for Real-Time), 1 µL of PrimeScript RT Enzyme Mix I, 1 µL of RT Primer mix, and 4 µL of RNase Free dH₂O, and was performed at a temperature of 37°C for 15 s and then 85°C for 5 s. The PCR products were further synthesized with 2 µL of the RT products, 10 µL of SYBR Premix Ex Taq II (2×), 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), and 6 µL of RNase Free dH₂O at 95°C for 30 s, followed by the 40 cycles of 95°C for 5 s, and then 60°C for 30 s. All the reactions were performed in triplicate.

The expression levels of the circular RNAs were calculated using the ΔCT method as follows: Expression$_{\text{hsa_circ_0005729}} = -\Delta C T = -(\text{CT}_{\text{hsa_circ_0005729}} - \text{CT}_{\text{human \beta-actin}})$, Expression$_{\text{hsa_circ_0027093}} = -\Delta C T = -(\text{CT}_{\text{hsa_circ_0027093}} - \text{CT}_{\text{human \beta-actin}})$, and Expression$_{\text{RNF138 mRNA}} = -\Delta C T = -(\text{CT}_{\text{RNF138 mRNA}} - \text{CT}_{\text{human \beta-actin}})$.

6. Clinical, laboratory data collection, and analysis

Clinicopathological features and laboratory data of 14 PC and 40 patients with PA were collected and analyzed. Information were collected including age, sex, serum calcium, calcium on the first postoperative day, the decline in calcium levels (calcium on the first postoperative day relative to preoperative), serum intact PTH (iPTH), iPTH on the first postoperative day, the decline in the iPTH (iPTH on the first postoperative day relative to preoperative), phosphorus, alkaline phosphatase (ALP), creatinine, 25-hydroxy vitamin D, 24-hour urinary calcium, relapse times, the date of the initial operation, date of the operation in our institution, and sites of the distant metastases. We analyzed the differences of the above clinical parameters between 14 PC and 40 patients with PA. We also examined the above indexes between 8 patients with PC without the distant metastases and 6 PC cases with the metastases. The factors with the significant differences between patients with PC and patients with PA were further analyzed together with hsa_circ_0005729 and hsa_circ_0027093.
7. **ROC curves and AUC analysis of laboratory parameters were used to assess the diagnostic efficiency of PC**

Laboratory parameters that were significantly different between the patients with PC and patients with PA were further used to assess the diagnostic power of PC by these parameters alone and combined with *hsa_circ_0027093* and *hsa_circ_0005729* (32) according to the logistic regression, ROC curve analysis, and the AUC calculation. The cutoff values were identified according to the Euclidean index (33), along with the sensitivity, specificity, and 95% confidence intervals.

8. **Analyze the correlation between *hsa_circ_0005729* and *RNF138* mRNA in PC and patients with PA**

*RNF138* is the host gene of *hsa_circ_0005729*, and we measured the expression of *RNF138* mRNA by qRT-PCR in 14 patients with PC and 40 patients with PA. The relationship between the *hsa_circ_0005729* and *RNF138* mRNA in 54 patients was analyzed by a Pearson’s correlation analysis. In addition, the relationship between the *hsa_circ_0005729* and *RNF138* mRNA in 14 PC and 40 patients with PA were analyzed, respectively.

9. **Statistical analysis.**

Statistical analyses were performed with a SPSS version 23.0 (Chicago, IL, USA), GraphPad Prism 8.0 (La Jolla, CA, USA), and SigmaPlot 14.0 (Systat Software Inc, Beijing, China). All the measurements were repeated three times in the triplicate. Differences in *hsa_circ_0027093*, *hsa_circ_0005729*, *RNF138* mRNA, and laboratory parameters between patients with PC and patients with PA were determined using the Mann–Whitney U-test. The clinicopathological features of different groups were evaluated using Chi-square tests. Logistic regression, ROC curve, and AUC calculation were performed to assess the diagnostic value of serum calcium, ALP, creatinine, *hsa_circ_0027093*, and
hsa_circ_0005729 alone, or a combination of them in PC. P < 0.05 was considered as statistically significant.

Results

1. Clinical characteristic features of PC and patients with PA

This study included 14 patients with PC and 40 patients with PA. The PC group had an equal number of the males and females in our institution (7 vs. 7), and the mean age was 42.9±15.1 years (median 42 years, range 19–69 years) at the time of initial operation. The PA group had a female-to-male ratio of 2.3:1 (28 vs. 12), and the mean age was 49.4±11.2 years (median 52, range 28–66). There was no significant difference in either age or the sex distribution between the patients with PC and patients with PA, but the age at the initial operation among the patients with PC was approximately 7 years younger than that among the patients with PA. Shaha et al. (34) and Schulte et al. (35) established different TNM staging systems that differ in the T classification for identifying PC. The T classification is associated with the tumor size in the Shaha system, while the Schulte system utilizes the histopathological features. The Schulte system was used because the most patients with PC in this study had experienced recurrence, and the histopathological features were considered as the better indicators (Table 1).

2. Overview of the results of the high-throughput circular RNA sequencing

A total of three PC and three PA tissues were used for screening the differentially expressed circular RNAs by a high-throughput circular RNA sequencing on the Illumina HiSeq 2500 Platform. In total 550,623,586 raw reads were generated, including the 279,097,882 raw reads for PC tissues and 271,525,704 raw reads for the PA tissues. A total of 524,800,460 clean reads (266,092,644 for PC and 258,707,816 for PA) were filtered after removing the poor quality, poly-N-containing, and adapter-containing reads from the raw data. Finally,
16,038 circular RNAs were identified as differentially expressed between the PC and PA according to the find_circ software, and then used for further analysis.

3. Analysis of differentially expressed circular RNAs: PC vs. PA
A total of 274 significant differentially expressed circular RNAs were identified according to the threshold log 2(FC) greater than 1 or lesser than –1 and corrected P < 0.005, including 118 upregulated and 156 downregulated circular RNAs in PC relative to PA. Clustering heatmaps and volcano plots were constructed to show the differentially expressed circular RNAs (Figure 1A and 1B). The top 10 upregulated circular RNAs in PC are shown in the Supplementary Table 2.

4. Hsa_circ_0005729 and hsa_circ_0027093 were significantly higher in 14 PC than patients with PA
PCR was performed to assess the expression levels of the top five upregulated circular RNAs in the PC tissues, and only hsa_circ_0027093 and hsa_circ_0005729 were chosen to study further because of the good shape of the PCR amplification curve and the specific melt peak. Furthermore, PCR products were detected by a gel electrophoresis, and the sequences of hsa_circ_0027093 and hsa_circ_0005729 were also identified by a sequencing using gel extraction. qRT-PCR was performed to measure the expression of hsa_circ_0027093 and hsa_circ_0005729 in 14 patients with PC and 40 patients with PA. The results showed that both hsa_circ_0005729 and hsa_circ_0027093 were significantly upregulated in 14 patients with PC specimens compared to the 40 PA tissue specimens, with p = 0.0073 and p = 0.0359, respectively (Figure 2A and 2B).

5. Hsa_circ_0005729 expression was higher in local relapses than in matched distant metastases of the same PC patients
There was no significant difference of hsa_circ_0005729 expression between patients with PC with (n = 6) and without distant metastases (n = 8) (p = 0.2824), as well as
hsa_circ_0027093 \ (p = 0.8518) \) (Figure 2C and 2D). In addition, we compared the hsa_circ_0005729 expression between local relapse tissues \ (n = 4) \) and matched distant metastases specimens \ (n = 5) \), including the four lung metastases and one liver metastases. The results showed that hsa_circ_0005729 expression was higher in the local relapses than in the matched distant metastases of the same patient \ (p = 0.0226) \) (Figure 2E), but there was no significant difference of hsa_circ_0027093 expression between the two groups \ (p = 0.2764) \) (Figure 2F). There were two specimens from the first-operated patients with PC and 12 specimens from the multifold-operated patients with PC. Hsa_circ_0005729 and hsa_circ_0027093 between the first-operated and multifold-operated patients with PC were analyzed, and the results showed no significant differences in the hsa_circ_0005729 and hsa_circ_0027093 between the first-operated patients with PC and the multifold-operated PC patients, with \ p = 0.440 \) and \ p = 0.659 \), respectively.

6. Several laboratory parameters were expressed higher in the 14 Patients with PC than that in 40 patients with PA

We analyzed several laboratory parameters between PC and patients with PA, and the results showed that the levels of serum calcium \ (p = 0.0447) \), ALP \ (p = 0.0481) \), and creatinine \ (p = 0.0358) \) were significantly higher in the 14 patients with PC than in 40 patients with PA (Figure 3A–3C). However, there was no significant difference in calcium on the first postoperative day \ (p = 0.1047) \), the decline in calcium levels \ (p = 0.4058) \), iPTH \ (p = 0.0562) \), iPTH on the first postoperative day \ (p = 0.0698) \), the decline in iPTH \ (p = 0.5913) \), phosphorus \ (p = 0.0745) \), 25-hydroxy vitamin D \ (p = 0.2510) \), 24-hour urinary calcium \ (p = 0.8052) \) between the PC and patients with PA (Supplementary Figure 1).

7. ROC curve analysis of laboratory parameters in PC patients

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Serum calcium, creatinine, and ALP had the AUC of 0.68, 0.69 and 0.68, respectively, while the AUC of $\text{hsa}_\text{circ}_0005729$ and $\text{hsa}_\text{circ}_0027093$ were 0.74 and 0.69, separately. The AUC was 0.86 when $\text{hsa}_\text{circ}_0005729$ was combined with serum calcium, creatinine, and ALP (Figure 3D–3G), while AUC was 0.79 when $\text{hsa}_\text{circ}_0027093$ was combined with the above three indexes. The median levels and the cutoff values according to the Euclidean index of all parameters are shown in Table 3.

8. The correlation between $\text{hsa}_\text{circ}_0005729$ and $\text{RNF138}$ mRNA in PA and PC patients

$\text{Hsa}_\text{circ}_0005729$ is translated from the $\text{RNF138}$ gene, and we measured $\text{RNF138}$ mRNA in 14 patients with PC and 40 patients with PA. The results showed no significant difference in $\text{RNF138}$ mRNA expression between patients with PC and patients with PA (Figure 4A). Additionally, we analyzed the relationship between $\text{hsa}_\text{circ}_0005729$ and $\text{RNF138}$ mRNA, and the results revealed that $\text{hsa}_\text{circ}_0005729$ expression was positively correlated with $\text{RNF138}$ mRNA (Pearson’s $r = 0.375$, $p = 0.005$) slightly in all 54 patients (Figure 4B). Subgroup analysis showed a positive correlation between $\text{hsa}_\text{circ}_0005729$ and $\text{RNF138}$ mRNA in 40 patients with PA (Pearson’s $r = 0.468$, $p = 0.002$) (Figure 4C). However, there was no correlation between $\text{hsa}_\text{circ}_0005729$ and $\text{RNF138}$ mRNA in 14 patients with PC (Pearson’s $r = 0.318$, $p = 0.268$) (Figure 4D).

9. The differences in the laboratory parameters between patients with PC with metastases and without metastases were analyzed

The laboratory parameters between patients with PC with and without metastases were analyzed. The results showed no significant difference in preoperative serum calcium ($p = 0.6870$), calcium on the first postoperative day ($p = 0.3277$), the decline in serum calcium ($p > 0.9999$), preoperative iPTH ($p = 0.4136$), the decline in iPTH ($p = 0.0813$), ALP ($p = 0.9497$), creatinine ($p = 0.5728$), phosphorus ($p = 0.6870$), 25-hydroxyvitamin D ($p = 0.2949$) between
the patients with PC with metastases and patients with PC without metastases. However, there was a significant difference in iPTH on the first postoperative day between the patients with PC with and without metastases \((p = 0.020)\), which showed the level of iPTH on the first postoperative day was higher in the patients with PC with metastases than that in patients with PC without the metastases (Figure 5A–5J). In addition, we analyzed the relationship between the \(hsa\_circ\_0005729\) and iPTH on the first postoperative day, and the results showed no correlation between \(hsa\_circ\_0005729\) and iPTH on the first postoperative day in 14 patients with PC (Pearson’s \(r = 0.149\), \(p = 0.612\)).

**Discussion**

PC is an intractable disease, and the only chance to cure it may depend on the initial en bloc resection of parathyroid lesions with ipsilateral hemithyroidectomy to achieve negative incision margins, avoid tumor rupture, and spillage (4-6). Studies showed that the PC is insensitive to the chemotherapy and radiotherapy in most patients with PC (5, 7, 15, 36), and chemoradiotherapy may be used when patients cannot tolerate surgery (8, 37). Sorafenib and Lenvatinib have been reported to be the effectively targeted drugs for patients with PC in two studies (38-39).

It is of paramount importance to diagnose the PC preoperatively, which may provide a basis for performing en bloc resection. However, there is no specific method for diagnosing the PC preoperatively so far. In this study, a novel circular RNA \(hsa\_circ\_0005729\) was found significantly higher in the PC than in patients with PA, and \(hsa\_circ\_0005729\) had the biggest AUC than any other biochemical indices such as serum calcium, ALP, and creatinine. Most studies have shown that PC mainly occurs in the fifth decade of life (40), and the mean age of the patients with PC in our cohort was 42.9 years, consistent with a study including the 70 patients with PC who had a mean age of 44.3 years (41). Other studies showed older ages
for the occurrence of the PC, such as 58.0 years in a survey including the 21 patients with PC (40) and 56.9 years in another study with >1022 patients with PC (15). The age at diagnosis of PC is approximately 10 years earlier than that of the PA (15, 40), this was confirmed in our studies, which showed that the mean age of patients with PC was 42.9 years at the time of the initial operation, approximately 7 years earlier than the mean age of patients with PA (49.4 years). In addition, most studies have shown that there is no sex predominance in the patients with PC, while patients with PA tend to be female of 3–4 times than the males (16, 19, 42-43). These results are consistent with our results, which showed no male-female difference among the 14 PC patients, but a female-to-male ratio of 2.3:1 among the 40 patients with PA. However, one study showed PC cases had male predominance with seven males and four females (44).

To identify an effective biomarker distinguishing PC from PA, a novel circular RNA hsa_circ_0005729 expression was verified higher in PC patients, and the levels of preoperative serum calcium, ALP, and creatinine were also higher in PC than in the patients with PA. We calculated the AUC of each index and found the AUC of hsa_circ_0005729 was bigger than any index alone; the AUC increased to 0.86 when hsa_circ_0005729 was combined with serum calcium, ALP, and creatinine, with the cutoff value of 2.88mmol/L, 137 IU/L, and 61.25µmol/L, respectively. Our results were consistent with some findings of another study, which showed that the expression of serum calcium and ALP were significantly higher in PC than in patients with PA. However, they showed significant differences in iPTH between PC and patients with PA and revealed that ALP combined with tumor size could differentiate PC from PA with a cutoff value of 285 IU/L, and a tumor size >3.0 cm (44). Serum iPTH, a specific biomarker for hyperparathyroidism, being often higher in patients with PC than in patients with PA, was found no significant differences between patients with PC and patients with PA in this study. These differences may be due to
the small number of the PC cases enrolled in both the studies, with 14 PC cases in this study and 11 PC cases in another study. In addition, 86% of the patients with PC in this study were local relapses with the multiple cervical lesions, which could not be accounted for in terms of tumor size; even six patients with PC had distant metastases.

However, our results revealed that the level of iPTH on the first postoperative day was higher in the 6 patients with PC with metastases than in the 8 patients with PC without metastases. Up to now, there are no studies on molecules in the distant metastases of PC. This study collected four local cervical relapses, four matched lung metastases, and one matched liver metastases. We analyzed the hsa_circ_0005729 expression between the cervical relapses and distant metastases of the same patients. The results showed that the hsa_circ_0005729 expression level was higher in local relapses than in matched distant metastases. However, the underlying mechanisms were not studied.

RNF138 gene encodes hsa_circ_0005729. Recent studies revealed that RNF138 is an essential factor in maintaining chromosome integrity (45), and RNF138 mRNA is expressed at significantly higher levels in glioma tissues than in adjacent noncancerous brain tissues (46). We measured RNF138 mRNA expression in tissue samples of 14 PC and 40 patients with PA; however, we found no significant difference in RNF138 mRNA expression between patients with PC and patients with PA. In addition, correlation analysis revealed that hsa_circ_0005729 and RNF138 mRNA were not correlated in the 14 patients with PC, but positively associated in the 40 PA cases, indicating that the mechanisms underlying PC and PA are different. Costa Guda et al. reported that the deletion of chromosome 11q allele is the most common change in PA, but it has never been detected in PC, suggesting that the latter arises de novo rather than evolving from pre-existing benign parathyroid diseases (47).

This study has limitations. Firstly, only 14 patients with PC were enrolled in this study. Secondly, the results showed that hsa_circ_0005729 expression was higher in the PC than in...
PA, higher in local relapses than in the matched distant metastases, and \textit{hsa\textunderscore circ\textunderscore 0005729} was positively correlated with \textit{RNF138} mRNA in patients with PA, but not in the PC patients. However, the mechanisms have not been studied.

**Conclusions**

In summary, this was one of the few studies that revealed the circular RNAs in patients with PC. A novel circular RNA \textit{hsa\textunderscore circ\textunderscore 0005729} may be a promising biomarker for distinguishing the PC from PA. In addition, this is the first study to measure the circular RNAs in matched distant metastases of the patients with PC. More patients with PC need to be studied to unveil the underlying mechanisms of the PC gradually in future work.

**Declaration of competing interest**

Not applicable.

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**Availability of data and materials**

The datasets are available from the corresponding author upon reasonable request.

**Conflicts of interest**

The authors declare no conflicts of interest.

**Code availability**

Not applicable.

**Author contribution statement**

Bojun Wei and Qian Wang were responsible for the study concept and design, and Qian Wang performed all experiments and wrote the manuscript. Jiacheng Wang, Yunhui Xin, and
Ziyang He collected the data and analyzed. Xiang Zhou and Mulan Jin were responsible for the pathological diagnoses of PC. Xing Liu, Teng Zhao and Hong Shen assisted in tissue sample collection.

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**References**


7. Christakis, I, Silva, AM, Kwatampora, LJ, Warneke, CL, Clarke, CN, Williams, MD


15. Sadler, C, Gow, KW, Beierle, EA, Doski, JJ, Langer, M, Nuchtern, JG Parathyroid carcinoma in more than 1,000 patients: a population-level analysis. Surgery:Copyright © 2014 Elsevier Inc. All rights reserved.2014;156:1622-1629, 1629-1630.


30. Wei, B, Zhao, T, Shen, H, Jin, M, Zhou, Q, Liu, X Extended en bloc reoperation for


42. van der Zwan, JM, Mallone, S, van Dijk, B, Bielska-Lasota, M, Otter, R, Foschi, R Carcinoma of endocrine organs: results of the RARECARE project. Eur J
Cancer: Copyright © 2012 Elsevier Ltd. All rights reserved. 2012; 48: 1923-1931.


45. Yard, BD, Reilly, NM, Bedenbaugh, MK, Pittman, DL RNF138 interacts with RAD51D and is required for DNA interstrand crosslink repair and maintaining chromosome integrity. DNA Repair (Amst): Copyright © 2016 Elsevier B.V. All rights reserved. 2016; 42: 82-93.


Figure legends:

Figure 1. Heat map and volcano plot were constructed to show the differentially expressed circular RNAs between 3 parathyroid carcinoma (PC) and 3 parathyroid adenoma (PA) tissues.

(A) Heat map: Differentially expressed circular RNAs in 3 PC (PC1+PC2+PC3) and 3 PA (PA1+PA2+PA3) tissues were screened using high-throughput circular RNA sequencing. Each column represents one specimen, and each row shows one circular RNA. (B) Volcano plot: A total of 274 significant differentially expressed as a circular RNAs were identified,
including 118 upregulated and 156 downregulated circular RNAs in PC relative to PA, according to the threshold log 2 (FC) > 1 or ≤−1 and P < 0.005.

**Figure 2. Validation of hsa_circ_0005729 and hsa_circ_0027093 expression in 14 PC and 40 PA tissues by qRT-PCR.**

(A–B) Boxplots: *Hsa_circ_0005729* and *hsa_circ_0027093* were verified significantly higher in PC than in the patients with PA by qRT-PCR using the −ΔCT on the y-axis. (C–D) Boxplots: There was no significant difference in *hsa_circ_0005729* expression between patients with PC with distant metastases (PC-M1) (n=6) and without distant metastases (PC-M0) (n=8), as well as *hsa_circ_0027093*. (E) Scatter plot: *Hsa_circ_0005729* expression was higher in the local relapses than in the matched distant metastases of the same patient. (F) Scatter plot: There was no significant difference in *hsa_circ_0027093* expression between the local relapses and the matched distant metastases.

**Figure 3. Hsa_circ_0005729 enhanced the accuracy in diagnosing PC by combination with serum calcium, alkaline phosphatase (ALP), and creatinine.**

(A) Boxplot: Serum calcium was significantly higher in 14 patients with PC than in the 40 patients with PA using the −ΔCT on the y-axis. (B) ROC curve: AUC was 0.81 when *hsa_circ_0005729* was combined with serum calcium (AUC 0.68). (C) Boxplot: ALP was significantly higher in the 14 patients with PC than in 40 patients with PA. (D) ROC curve: AUC was 0.81 when *hsa_circ_0005729* was combined with ALP (AUC 0.68). (E) Boxplot: Creatinine was significantly higher in 14 patients with PC than in 40 patients with PA. (F) ROC curve: AUC was 0.83 when *hsa_circ_0005729* was combined with creatinine (AUC 0.69). (G) ROC curve: The maximum AUC was 0.86 when *hsa_circ_0005729* was combined with ALP, serum calcium, and creatinine.

**Figure 4. Hsa_circ_0005729 expression was positively correlated with RNF138 mRNA in patients with PA but not in PC patients.**
(A) Boxplot: RNF138 mRNA, the corresponding linear transcript of hsa_circ_0005729, was measured by qRT-PCR, and there was no significant difference in RNF138 mRNA expression between 14 patients with PC and 40 patients with PA using the −ΔCT on the y-axis. (B) Scatter plot with trend lines: The relationship between hsa_circ_0005729 and RNF138 mRNA was analyzed by Pearson’s correlation analysis, and hsa_circ_0005729 expression was positively correlated with RNF138 mRNA (Pearson’s r = 0.375, P = 0.005) slightly in all 54 patients. (C) Scatter plot with trend lines: Hsa_circ_0005729 expression was positively correlated with RNF138 mRNA in 40 patients with PA (Pearson’s r = 0.468, P = 0.002). (D) Scatter plot with trend lines: There was no significant correlation between hsa_circ_0005729 and RNF138 mRNA in 14 patients with PC (Pearson’s r = 0.318, P = 0.268).

Figure 5. The differences in laboratory parameters between patients with PC with metastases and without metastases were analyzed.

(A) Scatter plots: The results showed a significant difference in the iPTH on the first postoperative day between 6 patients with PC with the metastases and 8 patients with PC without metastases (p = 0.0200), which showed the level of iPTH on the first postoperative day was higher in patients with PC with the metastases than in patients with PC without metastases. (B-J) Scatter plots: There was no significant difference in preoperative serum calcium (p = 0.6870), calcium on the first postoperative day (p = 0.3277), the decline in serum calcium (p > 0.9999), preoperative iPTH (p = 0.4136), the decline in iPTH (p = 0.0813), phosphorus (p = 0.6870), ALP (p = 0.9497), creatinine (p = 0.5728), 25-hydroxyvitamin D (p = 0.2949) between 6 patients with PC with metastases and 8 patients with PC without the metastases.
### Table 1. Clinicopathological characteristics in patients with pathologically confirmed parathyroid carcinoma (PC) and parathyroid adenoma (PA).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number (N = 54)</th>
<th>PC (N = 14)</th>
<th>PA (N = 40)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(N = 54)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>35 (65%)</td>
<td>7 (50%)</td>
<td>28 (70%)</td>
<td>0.153</td>
</tr>
<tr>
<td>Male</td>
<td>19 (35%)</td>
<td>7 (50%)</td>
<td>12 (30%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>29 (87%)</td>
<td>11 (79%)</td>
<td>18 (45%)</td>
<td>0.060</td>
</tr>
<tr>
<td>≥50</td>
<td>25 (13%)</td>
<td>3 (21%)</td>
<td>22 (55%)</td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>2 (14%)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
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<td>N/A</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>1 (7%)</td>
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<td></td>
</tr>
<tr>
<td>T2</td>
<td>9 (64%)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>2 (14%)</td>
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<td>N/A</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>2 (14%)</td>
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<td>N/A</td>
<td></td>
</tr>
<tr>
<td>N classification</td>
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<td></td>
</tr>
<tr>
<td>N0</td>
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<td>N/A</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>3 (21%)</td>
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<td>N/A</td>
<td></td>
</tr>
<tr>
<td>M classification</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>8 (57%)</td>
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<td>N/A</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>6 (43%)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>TNM stage (Schulte)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5 (36%)</td>
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<td>N/A</td>
<td></td>
</tr>
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<td>III</td>
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</tr>
<tr>
<td>IV</td>
<td>6 (43%)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

N/A: Not Applicable. TNM staging systems for PC by Schulte: T1 Evidence of capsular invasion, T2 Invasion of surrounding soft tissues excluding the vital organs trachea, larynx, and esophagus, T3 Evidence of vascular invasion, T4 Invasion of vital organs.
i.e., hypopharynx, trachea, esophagus, larynx, recurrent laryngeal nerve, carotid artery.

No regional lymph node metastases, N1 Regional lymph node metastases, M0 No evidence of distant metastases, M1 Evidence of distant metastases. I: T1 or T2N0M0, II: T3N0M0, III: Any T, N1M0, or T4, IV: Any N, M1.
Table 2. Information of 14 patients with pathologically confirmed parathyroid carcinoma (PC).

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age at the first OP(Y)</th>
<th>Gender</th>
<th>Relapse times</th>
<th>Date of the initial OP</th>
<th>Date of OP in our institution</th>
<th>iPTH (pg/mL)</th>
<th>Calcium (mmol/L)</th>
<th>ALP (IU/L)</th>
<th>Creatinine (µmol/L)</th>
<th>Distant Metastasis</th>
<th>Specimen source</th>
</tr>
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<tbody>
<tr>
<td>PC1</td>
<td>46</td>
<td>M</td>
<td>3</td>
<td>2012.02</td>
<td>2019.12</td>
<td>78.4</td>
<td>2.89</td>
<td>68</td>
<td>78.4</td>
<td></td>
<td>Cervical</td>
</tr>
<tr>
<td>PC2</td>
<td>39</td>
<td>F</td>
<td>2</td>
<td>2016.11</td>
<td>2019.08</td>
<td>112.5</td>
<td>2.55</td>
<td>60</td>
<td>112.5</td>
<td></td>
<td>Cervical</td>
</tr>
<tr>
<td>PC3</td>
<td>38</td>
<td>F</td>
<td>6</td>
<td>2008.05</td>
<td>2019.04</td>
<td>81.6</td>
<td>2.64</td>
<td>72</td>
<td>81.6</td>
<td>Lung + Lung</td>
<td>Cervical</td>
</tr>
<tr>
<td>PC4</td>
<td>44</td>
<td>F</td>
<td>3</td>
<td>2011.06</td>
<td>2020.07</td>
<td>458.8</td>
<td>3.88</td>
<td>155</td>
<td>458.8</td>
<td>Lung</td>
<td>Cervical</td>
</tr>
<tr>
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<td>2019.04</td>
<td>988.2</td>
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<td>562</td>
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<td>M</td>
<td>3</td>
<td>2013</td>
<td>2018.06</td>
<td>1204.5</td>
<td>3.68</td>
<td>198</td>
<td>723.3</td>
<td>Bone</td>
<td>Cervical</td>
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<td>2018.07</td>
<td>2019.10</td>
<td>199.2</td>
<td>2.18</td>
<td>161</td>
<td>824.2</td>
<td>Lung</td>
<td>Cervical</td>
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<tr>
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<td>42</td>
<td>M</td>
<td>1</td>
<td>2015.12</td>
<td>2017.11</td>
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<tr>
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<td>2018.05</td>
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<td>199.2</td>
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</tr>
<tr>
<td>PC11</td>
<td>65</td>
<td>F</td>
<td>3</td>
<td>2015.01</td>
<td>2018.02</td>
<td>880.8</td>
<td>2.96</td>
<td>80</td>
<td>889.1</td>
<td>Lung</td>
<td>Cervical</td>
</tr>
<tr>
<td>PC12</td>
<td>63</td>
<td>M</td>
<td>4</td>
<td>2015</td>
<td>2018.11</td>
<td>723.3</td>
<td>3.88</td>
<td>423</td>
<td>2000</td>
<td>Lung</td>
<td>Cervical + Lung</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
<td>---</td>
<td>----</td>
<td>------</td>
<td>---------</td>
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<td>------</td>
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<td>------</td>
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<td>-----------------</td>
</tr>
<tr>
<td>PC13</td>
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<td>M</td>
<td>0</td>
<td>2018.01</td>
<td>81.0</td>
<td>2.75</td>
<td>63</td>
<td>81</td>
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</tr>
<tr>
<td>PC14</td>
<td>19</td>
<td>F</td>
<td>3</td>
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<td>2019.05</td>
<td>344.2</td>
<td>3.23</td>
<td>175</td>
<td>344.2</td>
<td>Cervical</td>
<td></td>
</tr>
</tbody>
</table>

Y, year; M, male; F, female; ALP, alkaline phosphatase; iPTH, intact parathyroid hormone; OP, operation;
Normal range: iPTH (18.5-88.0pg/mL); Calcium (2.11-2.52mmol/L); ALP (35-100IU/L); Creatinine (41.0-73.0µmol/L)
Table 3. ROC curve and AUC analysis of laboratory parameters in parathyroid carcinoma (PC).

<table>
<thead>
<tr>
<th>Parameters (Reference range)</th>
<th>PC (Median value)</th>
<th>PA (Median value)</th>
<th>Cutoff value</th>
<th>Sensitivity (95% CIs)</th>
<th>Specificity (95% CIs)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsa_circ_0005729 (−ΔCT)</td>
<td>-9.04</td>
<td>-11.24</td>
<td>-10.26</td>
<td>71.43% (0.4190 to 0.9161)</td>
<td>72.5% (0.5611 to 0.8540)</td>
<td>0.74</td>
</tr>
<tr>
<td>Has_circ_0027093 (−ΔCT)</td>
<td>-6.20</td>
<td>-8.07</td>
<td>-6.36</td>
<td>57.14% (0.2886 to 0.8234)</td>
<td>82.5% (0.6722 to 0.9266)</td>
<td>0.69</td>
</tr>
<tr>
<td>Serum Calcium (2.11-2.52 mmol/L)</td>
<td>3.10</td>
<td>2.69</td>
<td>2.88</td>
<td>64.29% (0.3514 to 0.8724)</td>
<td>77.5% (0.6155 to 0.8916)</td>
<td>0.68</td>
</tr>
<tr>
<td>Creatinine (41.0-81.0 µmol/L)</td>
<td>92.90</td>
<td>55.35</td>
<td>61.25</td>
<td>64.29% (0.3514 to 0.8724)</td>
<td>77.5% (0.6155 to 0.8916)</td>
<td>0.69</td>
</tr>
<tr>
<td>ALP (45-125 U/L)</td>
<td>158</td>
<td>95</td>
<td>137</td>
<td>64.29% (0.3514 to 0.8724)</td>
<td>82.50% (0.6722 to 0.9266)</td>
<td>0.68</td>
</tr>
<tr>
<td>Has_circ_0027093 combined with serum calcium, creatinine and ALP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>Hsa_circ_0005729 combined with serum calcium, creatinine and ALP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.86</td>
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</table>

Receiver operating characteristic (ROC), Parathyroid adenoma (PA), 95% confidence intervals (CIs), the area under the curve (AUC), alkaline phosphatase (ALP)
Figure 1. Heat map and volcano plot were constructed to show the differentially expressed circular RNAs between 3 parathyroid carcinoma (PC) and 3 parathyroid adenoma (PA) tissues. (A) Heat map: Differentially expressed circular RNAs in 3 PC (PC1+PC2+PC3) and 3 PA (PA1+PA2+PA3) tissues were screened using high-throughput circular RNA sequencing. Each column represents one specimen, and each row shows one circular RNA. (B) Volcano plot: A total of 274 significant differentially expressed circular RNAs were identified, including 118 upregulated and 156 downregulated circular RNAs in PC relative to PA, according to the threshold log 2 (FC) > 1 or <-1 and P < 0.005.
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110x90mm (600 x 600 DPI)
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Figure 5. The differences in laboratory parameters between patients with PC with metastases and without metastases were analyzed. (A) Scatter plots: The results showed a significant difference in the iPTH on the first postoperative day between 6 patients with PC with the metastases and 8 patients with PC without metastases (p = 0.0200), which showed the level of iPTH on the first postoperative day was higher in patients with PC with the metastases than in patients with PC without metastases. (B-J) Scatter plots: There was no significant difference in preoperative serum calcium (p = 0.6870), calcium on the first postoperative day (p = 0.3277), the decline in serum calcium (p > 0.9999), preoperative iPTH (p = 0.6870), ALP (p = 0.9497), creatinine (p = 0.5728), 25-hydroxyvitamin D (p = 0.2949) between 6 patients with PC with metastases and 8 patients with PC without the metastases.
Supplemental Method 1. The detailed methods of circular RNA sequencing.

NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) were used according to manufacturer’s constructions. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer(5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated. Clean data (clean reads) were obtained by removing reads containing adapter, reads containing poly-N and low-quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Data analysis contained quality control, mapping to reference genome, and circRNA identification.

Supplemental Method 2. Data analysis of circular RNA sequencing.

Raw data (raw reads) of FASTQ format were firstly processed through in-house perl
scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from genome website directly. Index of the reference genome was built using Bowtie v2.2.3 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. We selected Hisat2 as the mapping tool for that Hisat2 can generate a database of splice sets based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools. HTSeq was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most common used method for estimating gene expression levels (Trapnell, Cole, et al., 2010). Differential expression analysis was performed using the edgeR package. The edgeR is one of the most popular Bioconductor packages for assessing differential expression in RNA-seq data. It is based on the negative binomial (NB) distribution and it models the variation between biological replicates through the NB dispersion parameter. This method is immediately able to handle complex experimental designs. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed (Robinson MD, et al., 2010). (For DESeq2 with biological replicates) Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package. DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed. (For DEGSeq without biological replicates) Differential expression analysis of two conditions was performed using the DEGSeq R package. The P values were adjusted using the Benjamini &
Hochberg method. Corrected P-value of 0.005 and log2(Fold change) of 1 were set as the threshold for significantly differential expression. Function enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway analysis. The GO project provides the most comprehensive resource currently available for computable knowledge regarding the functions of genes and gene products, mainly covering three aspects of biology: cell components, molecular functions and biological processes. GO terms with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/). Reactome is a collection database of articles on human reactions and biological pathways by experts and peer reviewed.
**Supplementary Table 1.** The primer sequences of all transcripts.

<table>
<thead>
<tr>
<th>Circular RNA ID</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>hsa_circ_0005729-F</td>
<td>TCTCTCAAGATTCAGTAGGGAACAGT</td>
</tr>
<tr>
<td>hsa_circ_0005729-R</td>
<td>CCGTTCAGGACATGCTCTCTC</td>
</tr>
<tr>
<td>hsa_circ_0027093-F</td>
<td>AGACTGGGAAGATGATTCAGATGA</td>
</tr>
<tr>
<td>hsa_circ_0027093-R</td>
<td>TAACATCCTTACTGTCTTCAACACAA</td>
</tr>
<tr>
<td>RNF138 mRNA-F</td>
<td>GTCCCCTGTGTCAAGAATCAAAT</td>
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<tr>
<td>RNF138 mRNA-R</td>
<td>GAAATTTCTGGTAATCTGGCTAGGAT</td>
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<tr>
<td>Human Actin-F</td>
<td>TGGCACACACACCTTCTACAATGA</td>
</tr>
<tr>
<td>Human Actin-R</td>
<td>GATAGCACACAGCCTGGATAGCAAC</td>
</tr>
</tbody>
</table>

F: Forward. R: Reverse
Supplementary Table 2. The top 10 up-regulated circular RNAs were screened by
the high throughput circular RNA sequencing.

<table>
<thead>
<tr>
<th>Circular RNA ID</th>
<th>Log₂ (fold change)</th>
<th>Regulation</th>
<th>P value</th>
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<tbody>
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<td>up</td>
<td>0.00000000062</td>
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<tr>
<td>hsa_circ_0027093</td>
<td>6.3252</td>
<td>up</td>
<td>0.00015324</td>
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<tr>
<td>hsa_circ_0005729</td>
<td>6.1217</td>
<td>up</td>
<td>0.00027597</td>
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<td>novel_circ_0026113</td>
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<td>0.00088732</td>
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<td>novel_circ_0026101</td>
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<td>0.0043225</td>
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<td>0.0039639</td>
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<td>novel_circ_0030363</td>
<td>4.7327</td>
<td>up</td>
<td>0.0092749</td>
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</table>
Supplementary Figure 1. The differences in laboratory parameters between patients with PC and with PA

Several laboratory parameters between 14 PC and 40 patients with PA were analyzed, and the results showed there was no significant difference in calcium on the first postoperative day (p = 0.1047), the decline in calcium (p = 0.4058), iPTH (p=0.0562), iPTH on the first postoperative day (p = 0.0698), the decline in iPTH (p = 0.5913), phosphorus (p = 0.0745), 25-hydroxy vitamin D (p = 0.2510), 24-hour urinary calcium (p = 0.8052) between patients with PC and patients with PA.
A. Calcium on the first postoperative day (mmol/L): P=0.1047
B. The decline in calcium: P=0.4058
C. Serum PTH: P=0.0562
D. PTH on the first postoperative day (pg/mL): P=0.0698
E. The decline in PTH: P=0.5913
F. Phosphorus (mmol/L): P=0.0745
G. 25-hydroxy vitamin D (ng/mL): P=0.2510
H. 24-hour urinary calcium (mmol/L): P=0.8052