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Original Article

Differential expression profiles of circRNAs in human prostate cancer based on chip and bioinformatic analysis

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Abstract: Background: Increasing evidence suggests that circRNAs are involved in the pathogenesis of multiple kinds of cancer. Nevertheless, the differential expression of circRNAs in prostate cancer (PCA) is rarely reported. Material/Method: In our present analyses, circRNAs expression profiles were identified in PCA, based on 5 pairs of PCA and matched non-PCA tissues using circRNA chips. Results: A number of 749 differential circRNAs were expressed between PCA tumor and paracancerous tissues (Fold Change, FC ≥ 2.0 and $P < 0.05$): 261 were up-regulated, whereas 487 were downregulated in PCA tissues. Gene ontology and KEGG pathway analyses indicated that many of the circRNAs are related to carcinogenesis. Circ_0033074 and circ_0016064 both showed changes of maximum magnitude among differentially expressed circRNAs. Conclusions: Our study detected a relative comprehensive differential map of circRNAs in PCA, which may become novel biomarkers for diagnosis, treatment and follow-up in the future.

Keywords: Prostate cancer, circRNA, microarray, expression

Introduction

The latest forecast data estimated that 174,650 new cases of prostate cancer (PCA) were diagnosed in the USA in 2019, causing 31,620 related deaths [1]. With dramatic economic growth and socio-cultural changes leading to an increased life expectancy and westernized lifestyle, the incidence and mortality frequency of PCA in China have shown a rapid growth trend [2]. Currently PCA has already ranked the sixth in incidence of the most frequent cancers, with about 72 thousand cases in 2015, and the tenth in cancer-related death, with about 31 thousand cases.

The androgen receptor (AR) pathway plays an essential role in the early stage of PCA. The androgen deprivation therapy (ADT) is effective for more than 80% patients, but after a median time of 14-30 months, lesions in almost all patients will gradually develop into an androgen independent state, namely castration resistant prostate cancer (CRPC) [3], which is a major cause of death in patients with advanced PCA with a median survival time less than 20 months. The mechanism of CRPC is very complicated, and recent studies revealed that the AR pathway still plays an important role [4]; in addition, the tumor related pathways, such as PI3K/AKT, RAS/MAPK, TGF-β were also reported in the process of CRPC [5-7]. Although docetaxel/prednisone and AR blocking drugs (such as abiraterone, enzalutamide) can delay the development of CRPC [8, 9], the clinical effect is limited, and the development of effective biomarkers for early detection and targeted treatment is receiving much attention.

So far, many novel biomarkers, including DNA, proteins, non-coding RNAs, and exosomes, have been reported [10-13]. Among them, the role of non-coding RNAs in cancer has been under wide consideration, with miRNAs, lncRNA, and circRNAs identified in the last five years [14, 15]. Accumulated evidence has suggested a close association between miRNAs/lncRNAs and the proliferation, invasion, and progression of cancer. Recent studies have focused on the circRNAs in cancer develop-

Table 1. Basic information of patients with PCA included in our study

NO	Gender	Age (Years)	Histologic type	Initial total PSA	Gleason score	TNM stage
13	Man	64	Adenocarcinoma	9.39	3+4	T2cN0M0
24	Man	50	Adenocarcinoma	15.84	4+3	T3bN0M0
26	Man	62	Adenocarcinoma	14	4+3	T3bN0M1
45	Man	54	Adenocarcinoma	9.13	4+3	T2cN0M0
67	Man	62	Adenocarcinoma	54.66	5+4	T3bN1M1

ment [16, 17]. However, studies about the association between circRNAs and PCA risk have not been large enough to reach a definitive conclusion.

CircRNAs are a class of RNA molecules that lack 5'-3' ends and poly A tail and covalently form closed loops [18]. The advantage of circRNAs, rather than miRNAs and lncRNAs, is that they exist stably and are not easily degraded by exonuclease RNase R in the cells [19]. Growing evidence has shown that circRNAs are involved in the pathogenesis of variety of diseases, such as diabetes, Alzheimer's disease, and cancer through corresponding miRNAs [20-22]. Moreover, the stability and specificity of circRNAs in body fluids have made them new molecular biomarkers for cancer diagnosis and monitoring [23, 24]. Still, the expression and latent roles of circRNAs in PCA are still little understood. In the current analyses, we found a differential expression of circRNAs in PCA tissues, to identify several significant and potential biomarkers.

Materials and methods

Tissue samples

A total of 5 pairs of PCA and matched non-tumor normal tissues were collected from Huashan Hospital, Fudan University. Our study was approved by the ethics committee of Huashan Hospital, Fudan University, and written informed consent was acquired from all patients. All tissue was histologically identified, diagnosed as prostate adenocarcinoma, and the Gleason score, PSA value, TNM stage, and recurrence were according to the NCCN guidelines. The initial screening step (**Table 1**) was processed by microarray chip assay.

RNA extraction and purification

MirVana™ miRNA Isolation Kit without phenol (Ambion, Austin, TX, US) was applied to extracted and purified total RNA, following the manufacturer's instructions and Agilent Bioanalyzer 2100 was put to use to check for a RIN number to inspect RNA integration (Agilent Technologies, Santa Clara, US).

RNA labeling

Low Input Quick Amp WT Labeling Kit (Agilent Technologies, Santa Clara, US) was used to amplify and label the rRNA. Immediately afterwards, RNeasy mini kit was carried out to purify the labeled cRNA (QIAGEN, Germany).

Array hybridization

Gene Expression Hybridization Kit was used to hybridize each slide with 1.65 μg Cy3-labeled cRNA in Hybridization Oven (Agilent Technologies, Santa Clara, US). After hybridization, staining dishes (Thermo Shandon, Waltham, US) and Gene Expression Wash Buffer Kit (Agilent Technologies, Santa Clara, US) were employed to wash the slides. Differentially expressed circRNAs were analyzed with independent samples by t-test. Any fold change (FC) and *P*-value for circRNAs that were more than 2 or less than 0.05, respectively, were considered as significant differential expression.

Data acquisition

Agilent Microarray Scanner was applied to scan the slides (Agilent Technologies, Santa Clara, US) with default settings, Dye channel: Green, Scan resolution = 3 μm, PMT 100%, 20 bits. Feature Extraction software 10.7 was applied to extract data (Agilent technologies, Santa Clara, US). Raw data were normalized by Quantile algorithm (Limma packages in R).

Bioinformatics analysis

Differentially expressed circRNAs identified with profiling data were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Ge-

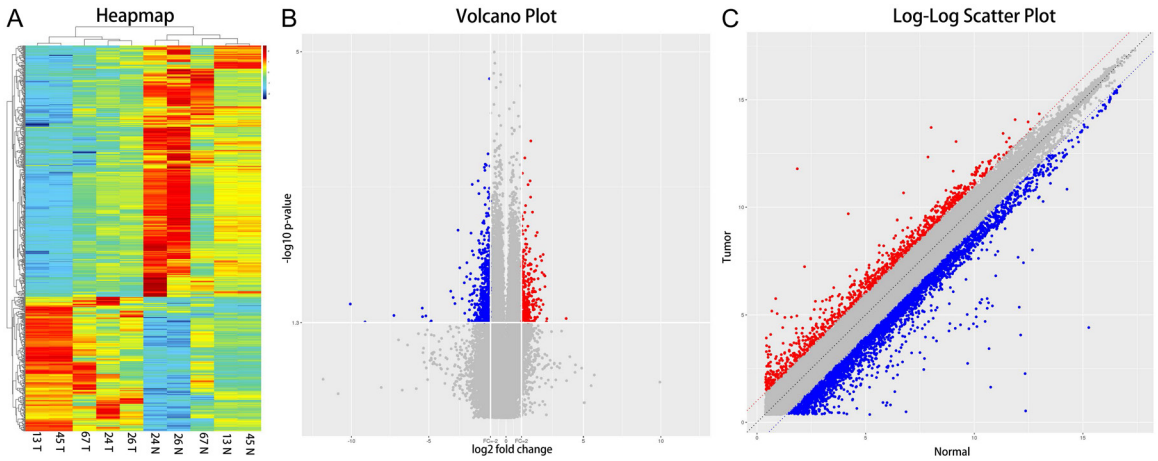

Figure 1. Hierarchical clustering, volcano plots, and scatter plots displayed the differentially expressed circRNAs in PCA tissues compared to paracancerous tissues. A. Hierarchical clustering: numbers were the samples used for the microarray assay. T: PCA tissues, N: paracancerous tissues. B. Differentially expressed circRNAs are shown as volcano plots. The blue and red parts indicate (FC more than 2 folds) downregulated and upregulated expression circRNAs in PCA tissues, respectively ($P < 0.05$). C. Differentially expressed circRNAs shown by scatter plots. The green and red parts indicate (FC more than 2 folds) downregulated and upregulated expression circRNAs in PCA tissues, respectively ($P < 0.05$).

nes and Genomes (KEGG) pathway analyses, and whose targeted miRNAs were predicted by miRanda software (http://miranda.org.uk/) coupled with statistical analysis substantially. The circRNAs expression profile microarray chip assay, plus data and bioinformatics analysis were produced, tested and analyzed by Shanghai Biotechnology Corporation (Shanghai, China).

Results

CircRNAs expression profiles in PCA

The microarray testing identified 88,750 kinds of circRNAs in PCA and or non-PCA tissues. As illustrated in **Figure 1**, 749 circRNAs were differentially expressed between PCA tumor and paracancerous tissues (FC ≥ 2.0 and $P < 0.05$) (Table S1): among which 261 were upregulated, and the other 487 were downregulated in PCA tissues. The circRNAs with the highest differential expression were the has_circ_0016064 among downregulated circRNAs (FC = 0.00656, $P = 0.0399$) and the has_circ_0033074 among upregulated circRNAs (FC = 14.85488, $P = 0.0439$), respectively. Hierarchical clustering (**Figure 1A**), volcano plot (**Figure 1B**), and scatter plots (**Figure 1C**) showed that the different expression profiles of circRNAs between PCA and non-PCA tissues were diverse. The top each twenty up- and downregulated circRNAs are listed in **Table 2**.

Bioinformatics analysis

All differentially expressed circRNAs could be located to all chromosomes, except for chromosomes 21 and Y (Table S1). The top each twenty up- and down-regulated circRNAs and corresponding three kinds of sponges of microRNAs are shown in **Table 2**. Moreover, each Top 30 enrichments according to GO and KEGG analyses suggested that these differentially expressed circRNAs were relevant to several vital physiologic processes, such as transmembrane receptor protein tyrosine kinase activity, spindle localization, glycolytic process, phosphatidylinositol signaling system, and gluconeogenesis. Many common pathways associated with invasion and metastasis of PCA, such as tight junction, focal adhesion, adherens junction, ECM-receptor interatom, and SNARE interactions in vesicular transport were also implicated (**Figure 2A**, **2B**).

Discussion

The most the popular biomarker for early detection of PCA is the PSA value. However, the specificity and stability of PSA are relatively low. Therefore, it is necessary to find new PCA diagnostic and monitoring markers for early screening of PCA [25, 26]. CircRNAs are natural endogenous RNAs, widely studied in recent five years. The mechanism of circRNAs is to function as miRNA sponges like competitive

Table 2. The top twenty kinds of decreased and increased differentially expressed circRNAs in PCA tissues compared to those in non-cancerous tissues and their highest frequency of MREs through sponge adsorption

ProbeName	*P*-values	Fold change	Regulation	Circ_ chrom	Hostgene	CircRNA		
						MRE1$ (combine bit points)	MRE2$ (combine bit points)	MRE3$ (combine bit points)
hsa_circ_0033074	0.043966188	14.85487618	Up	chr14	SERPINA3	hsa-miR-6889-3p$2	hsa-miR-1273e$2	hsa-miR-296-5p$1
hsa_circ_0057549	0.044251247	6.259110255	Up	chr2	TMEFF2	hsa-miR-6838-5p$4	hsa-miR-195-5p$4	hsa-miR-16-5p$4
hsa_circ_0076303	0.011520736	5.749538882	Up	chr6	PGC	hsa-miR-198$2	hsa-miR-4779$2	hsa-miR-520a-5p$2
hsa_circ_0076304	0.016135493	5.747004796	Up	chr6	PGC	hsa-miR-6799-3p$2	hsa-miR-6789-3p$2	hsa-miR-3173-5p$2
hsa_circ_0076305	0.011034354	5.461548042	Up	chr6	PGC	hsa-miR-6865-5p$3	hsa-miR-6815-5p$3	hsa-miR-936$3
hsa_circ_0040583	0.00586104	5.295847499	Up	chr16	CENPN	hsa-miR-619-5p$4	hsa-miR-6506-5p$4	hsa-miR-661$4
hsa_circ_0024925	0.028275165	5.28313187	Up	chr11	ACAD8	hsa-miR-7112-5p$1	hsa-miR-6828-3p$1	hsa-miR-3615$1
hsa_circ_0008053	0.01034582	5.025655906	Up	chr6	PAK1IP1	hsa-miR-4252$2	hsa-miR-3144-5p$1	hsa-miR-6810-5p$1
hsa_circ_0007405	0.007097955	4.940850802	Up	chr16	CENPN	hsa-miR-7110-3p$2	hsa-miR-554$1	hsa-miR-1185-5p$1
hsa_circ_0062627	0.024770482	4.810985476	Up	chr22	GGT1	hsa-miR-3605-5p$2	hsa-miR-3065-3p$2	hsa-miR-3679-5p$2
hsa_circ_0004390	0.03341108	4.801446853	Up	chr1	LPAR3	hsa-miR-198$3	hsa-miR-711$2	hsa-miR-6512-3p$2
hsa_circ_0040578	0.006241681	4.676950834	Up	chr16	CENPN	hsa-miR-619-5p$5	hsa-miR-6506-5p$5	hsa-miR-146a-3p$4
hsa_circ_0070475	0.017644399	4.574571538	Up	chr4	PDLIM5	hsa-miR-4480$2	hsa-miR-5695$2	hsa-miR-8485$2
hsa_circ_0005917	0.030493374	4.458396676	Up	chr6	PAK1IP1	hsa-miR-572$1	hsa-miR-324-5p$1	hsa-miR-6501-5p$1
hsa_circ_0070466	0.023657495	4.306640303	Up	chr4	PDLIM5	hsa-miR-544b$4	hsa-miR-378g$3	hsa-miR-4324$3
hsa_circ_0013059	0.042001336	4.089111212	Up	chr1	LPAR3	hsa-miR-198$4	hsa-miR-6720-5p$3	hsa-miR-6512-3p$3
hsa_circ_0062625	0.002930265	4.055779738	Up	chr22	GGT1	hsa-miR-4778-3p$3	hsa-miR-4469$2	hsa-miR-324-5p$2
hsa_circ_0075601	0.024302633	3.988707364	Up	chr6	PAK1IP1	hsa-miR-572$1	hsa-miR-324-5p$1	hsa-miR-6501-5p$1
hsa_circ_0004646	0.009677779	3.774169933	Up	chr1	UAP1	hsa-miR-6772-3p$3	hsa-miR-6801-3p$2	hsa-miR-6810-3p$2
hsa_circ_0072904	0.044529246	3.764492045	Up	chr5	MCCC2	hsa-miR-1306-5p$4	hsa-miR-6514-3p$2	hsa-miR-3160-5p$2
hsa_circ_0020064	0.048931868	0.236283669	Down	chr10	ABLIM1	hsa-miR-4731-5p$5	hsa-miR-3972$5	hsa-miR-1202$5
hsa_circ_0090179	0.005928275	0.232902566	Down	chrX	DMD	hsa-miR-4635$3	hsa-miR-378g$2	hsa-miR-3664-3p$2
hsa_circ_0090180	0.005242068	0.220318677	Down	chrX	DMD	hsa-miR-4687-3p$1	hsa-miR-7113-5p$1	hsa-miR-3664-3p$1
hsa_circ_0057896	0.047006842	0.214726487	Down	chr2	NRP2	hsa-miR-23b-5p$1	hsa-miR-23a-5p$1	hsa-miR-454-5p$1
hsa_circ_0029996	0.028345901	0.214352917	Down	chr13	DCLK1	hsa-miR-3189-5p$1	hsa-miR-4434$1	hsa-miR-5703$1
hsa_circ_0032813	0.012751171	0.198553084	Down	chr14	NRXN3	hsa-miR-181a-5p$2	hsa-miR-181b-5p$2	hsa-miR-181d-5p$2
hsa_circ_0020060	0.025564507	0.192676458	Down	chr10	ABLIM1	hsa-miR-3972$4	hsa-miR-1202$4	hsa-miR-3194-5p$4
hsa_circ_0074026	0.047967039	0.188441006	Down	chr5	PITX1	hsa-miR-6784-5p$4	hsa-miR-1292-3p$4	hsa-miR-4747-3p$4
hsa_circ_0032812	0.007589741	0.172980926	Down	chr14	NRXN3	hsa-miR-181a-5p$2	hsa-miR-181b-5p$2	hsa-miR-181d-5p$2
hsa_circ_0087142	0.011816017	0.17097621	Down	chr9	PIP5K1B	hsa-miR-7110-3p$4	hsa-miR-6873-3p$4	hsa-miR-6817-3p$4
hsa_circ_0087140	0.008909913	0.125440984	Down	chr19	HSPB6	hsa-miR-6861-5p$2	hsa-miR-1229-5p$2	hsa-miR-5589-5p$2
hsa_circ_0087144	0.002724689	0.116309931	Down	chr9	PIP5K1B	hsa-miR-6735-3p$2	hsa-miR-100-3p$2	hsa-miR-3141$1

hsa_circ_0007499	0.02749466	0.101227605	Down	chr3	VEPH1	hsa-miR-216b-5p$3	hsa-miR-6511a-3p$2	hsa-miR-6511b-3p$2
hsa_circ_0067802	0.022712773	0.093496121	Down	chr3	VEPH1	hsa-miR-211-3p$3	hsa-miR-6754-5p$3	hsa-miR-4270$3
hsa_circ_0067804	0.018611184	0.083830657	Down	chr3	VEPH1	hsa-miR-8070$2	hsa-miR-653-3p$2	hsa-miR-4666b$2
hsa_circ_0057223	0.047480592	0.034978971	Down	chr2	TTN	hsa-miR-4659a-3p$17	hsa-miR-4659b-3p$17	hsa-miR-4778-3p$14
hsa_circ_0057213	0.040500989	0.027514635	Down	chr2	TTN	hsa-miR-6715b-5p$32	hsa-miR-4269$32	hsa-miR-4266$20
hsa_circ_0057224	0.041513118	0.023651164	Down	chr2	TTN	hsa-miR-4660$10	hsa-miR-5581-5p$8	hsa-miR-4474-3p$8
hsa_circ_0057222	0.031686665	0.023389371	Down	chr2	TTN	hsa-miR-4659a-3p$14	hsa-miR-4659b-3p$14	hsa-miR-4778-3p$11
hsa_circ_0016064	0.03996311	0.006560389	Down	chr1	MYBPH	hsa-miR-1911-3p$2	hsa-miR-6753-5p$2	hsa-miR-516b-5p$2

FC: Fold changes. MRE: microRNA response element. $: the number of combination sites.

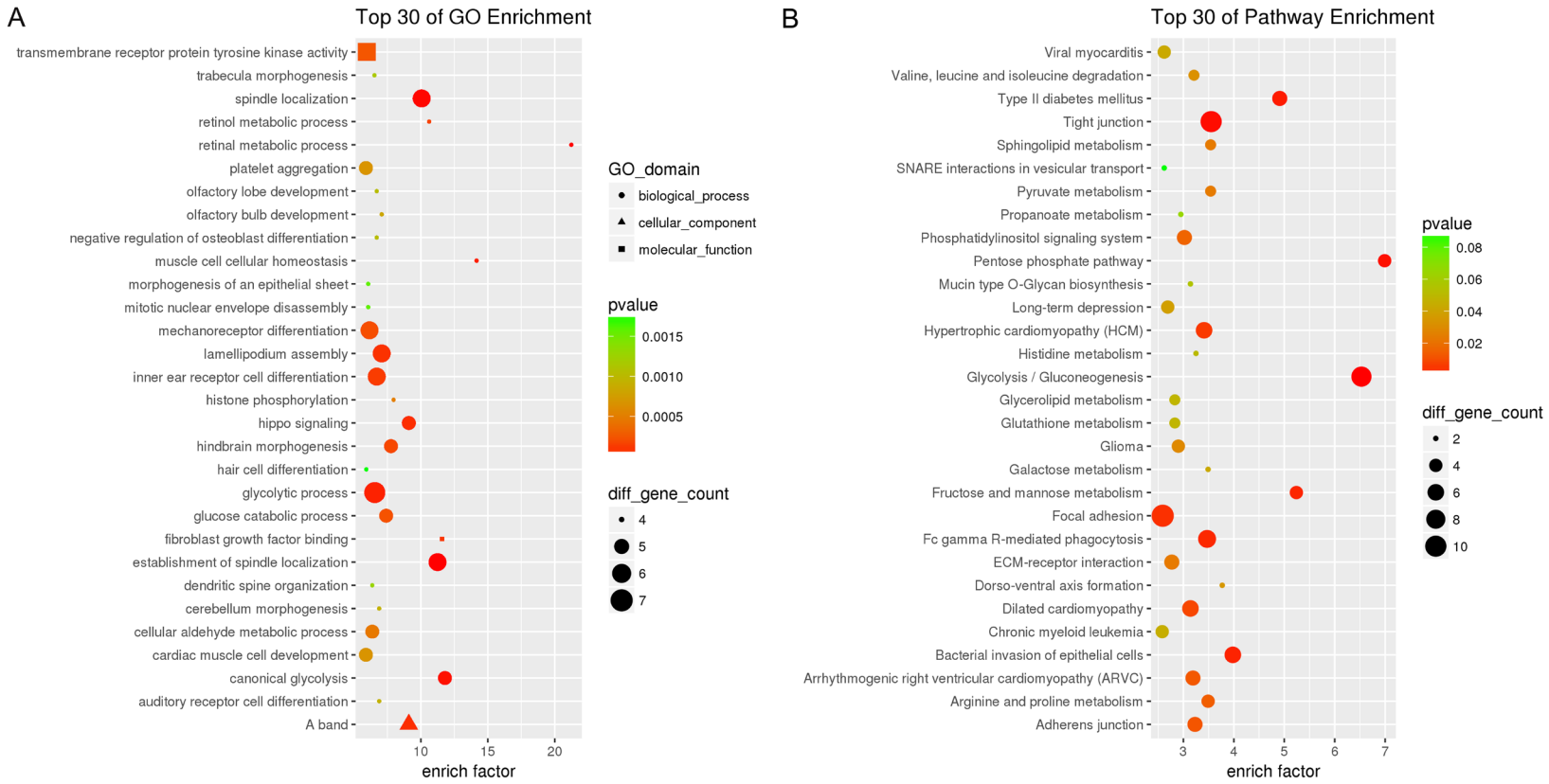

Figure 2. GO and KEGG pathway analysis of differentially expressed circRNAs. A. GO enrichment terms of top 30 classes. B. KEGG pathway enrichment terms of top 30 classes.

endogenous RNA molecules [27]. miRNAs are important regulators in gene expression and play a crucial role in cancer progression. Based on its stable properties, it may become a biomarker in many samples: such as tissues, blood, urine, saliva, secretions, and feces.

Xia et al. screened differentially expressed circRNAs using SBC-ceRNA array in 4 pairs of prostate tumor and paracancerous tissues [28]. 1021 differentially expressed circRNAs were identified. They demonstrated that combination of PSA level and two differentially expressed circ_0057558 and circ_0062019 showed significantly increased AUC, sensitivity, and specificity compared to PSA alone. However, the clinical information about PSA value, Gleason score, and TNM stage were missing, so heterogeneity may be enlarged. Zhang et al. analyzed differential circRNAs among three kinds of PCA cells (RWPE-1, 22RV1 and PC-3) by high-throughput circRNAs sequencing [29]. 9545 circRNAs were detected and hundreds of differentially expressed circRNAs were recognized. Our study is a timely and updated study combining gene chip and bioinformatic analyses. In this study, we identified 749 circRNAs differentially expressed between PCA and non-cancerous tissues by circRNAs chips. Has_circ_0033074 had the highest magnitude of upregulation, whereas has_circ_0016064 had the lowest expression in PCA tissue compared to the corresponding normal tissue.

SERPINA3 (serpin family A member 3), locates on 14q32.13 is the host gene for circ_0033074, which is a plasma protease inhibitor and member of the serine protease inhibitor class, which acts as an oncogene based on previous studies. Kulesza et al. reported SERPINA3 was a novel STAT3 target gene, involved in regulation of melanoma migration and invasion [30]. Cao et al. showed that SERPINA3 silencing may inhibit the migration, invasion, and liver metastasis of colon cancer cells [30]. Yang et al. provided insight that SERPINA3 promoted endometrial cancer cell growth by regulating cell cycle checkpoint and inhibiting apoptosis [31]. Based on the lowest expression of circ_0016064, MYBPH (myosin binding protein H) is its host gene, located at the 1q32.1 position. Hosono et al. validated that MYBPH, as a transcriptional target of TTF-1, could inhibit ROCK1 and reduce cell motility and metastasis in lung adenocarcinoma [32]. In addition, Zhu et al. found MYBPH could inhibit vascular smooth muscle cell migration and attenuate neointimal hyperplasia in a rat carotid balloon-injury model [33]. In summary, MYBPH acts as a anti-cancer molecule. Bioinformatics analyses of the trends for both circ_0033074 and circ_0016064 are according to the function of their host genes.

CircRNAs in PCA act as a double-edged sword. On one hand, circRNAs are proven to be related to the behavior of cancer cells' tumorigenesis and malignancy, such as proliferation, migration, and invasion. For example, Chen et al. illustrated that circHIPK3 was overexpressed in PCA tissue, and its higher expression was associated with tumor stage. Additionally, circHIPK3/miR-193a-3p-MCL1 signaling promoted PCA development and progression [34]. On the other hand, circRNAs have been identified to play vital roles in suppressing cell proliferation and arresting tumor progression. For instance, Huang et al. considered that circ-ITCH was downregulated in PCA tissue, and its low expression correlated with clinical characteristics, such as advanced pathologic T stage, high lymph mode metastasis risk, and poor overall survival [34]. Moreover, Song et al. revealed circ_0001206 played a suppressive role in the pathogenesis of PCA [35].

The current study used the combination of circRNAs chips with bioinformatic analyses of PCA tissues. There remain several limitations to the study. First, the top 20 differentially expressed circRNAs should be verified by real-time PCR. Second, the clinical PCA patients undergoing radical prostatectomy should be increased to compare the significant circRNAs to corresponding non-tumor tissues. Other evaluation-indicators such as overall survival, disease-free survival, Gleason score, surgical margin status, and lymph node metastasis, should be included in the analysis. Third, some PCA cells should be added to evaluate to the function and deep mechanism of significant circRNAs, so that they may be become novel biomarkers for diagnosis and treatment.

Conclusion

Our study provided a new landscape of circRNA differential expression in PCA vs. benign tissue. Further studies are required to show their potential functions as biomarkers for PCA.

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Disclosure of conflict of interest

None.

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