Annals of Medical and Clinical Oncology
Wang F, et al. Ann med clin Oncol 5: 143.
www.doi.org/10.29011/2833-3497.000143
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Short Commenty

Commentary Referring to Hsa_circ_0003258 Promotes Prostate Cancer Metastasis by Complexing with IGF2BP3 and Sponging miR-653-5p

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Citation: Wang F, Sun Y, Chen J, Chen C (2022) Commentary Referring to Hsa_circ_0003258 Promotes Prostate Cancer Metastasis by Complexing with IGF2BP3 and Sponging miR-653-5p. Ann med clin Oncol 5: 143. DOI: 10.29011/2833-3497.000143

Received Date: 11 December, 2022; **Accepted Date:** 27 December, 2022; **Published Date:** 30 December, 2022

Commentary

Prostate cancer (PCa) ranks as the 2nd most common cause of cancer death in men [1]. Radical prostatectomy or radiation therapy was considered the traditional therapy for localized prostate cancer but not with metastatic stage. Although current mainstay therapy for metastatic PCa included surveillance of PSA doubling time, androgen deprivation, targeting PI3K/AKT signaling axis, DNA damage repair, immune checkpoint, etc, currently identified biomarkers were not robustly validated for prognostication or direct therapy, therefore, exploring novel biomarkers for precision medicine in PCa is imperative [2]. This elegant article we commented focuses on several potential drug targets, such as non-coding RNAs circular RNAs (circRNAs), microRNAs (miRNAs), and coding proteins ARHGAP5, and IGF2BP3. Each target had promising clinical value.

Abnormal expression of circRNA has been associated with a wide range of human cancers. CircRNA has specific biological functions in tumor cells including cell proliferation, invasion, and migration [3]. CircRNAs are single-stranded, covalently closed RNA molecules, formed by back-splicing of pre-mRNAs, they are essential regulators of gene expression generally through sponging microRNA (miRNA), interacting with RNA binding protein (RBP), transcriptional modification, and splicing, or affecting protein stability and certain signaling pathways, etc [4].

The authors in this published article started with researching human plasma samples to sequence and screen differentially expressed (DEGs) circular RNA by the heatmap and a volcano plot, among which highly expressed circ003258 were proportional to TNM stage and malignancy in clinicopathological features of table1 (prostate cancer patients' tissues). Circ_0003258 was verified among paired PCa, normal tissue, and PCa cells by qRT-PCR, circ_0003258 was highly expressed in both PCa cells and tissue. The genomic length of the circ_0003258 is 731bp and the spliced length is 261bp according to the UCSC genome database (http://genome.ucsc.edu/) [5]. Circ_0003258 consisted of exons 4 and 5 amplified by the convergent primer and confirmed by Sanger sequencing. Circ0003258 was derived from ZNF652, located on chromosome 17q21 in CircBase (http://www.circbase.org/) website [6]. Circ_0003258 could be amplified by both convergent primers in cDNA and gDNA, but not by divergent primers in gRNA. In addition, RNase R treatment digested linear gene ZNF652, circ_0003258 can be reserved. Collectively, circ_0003258 was screened and identified as a stable circular RNA.

The authors next determined to illustrate whether circ_0003258 promoted PCa cell metastasis. Wound healing and Transwell migration assays were set to evaluate this feature. On the one hand, small interfering RNA (siRNA) silencing circ_0003258 significantly reduced migration in two PCa cell types. On the other hand, overexpression of circ_0003258 substantially increased migratory cell numbers compared to the negative control vector

group. Genetic manipulation including loss-and gain-of-function study displayed that circ_0003258 enhanced PCa cell migration. Epithelial to Mesenchymal Transition (EMT) is a normal physiological process often accompanied by the downregulation of E-cadherin and the upregulation of Vimentin, weakening cell adhesion, cytoskeleton remodeling, wound healing, and enhancing cell migration and invasion. Pathologically, cancer cells hijacked the EMT process to help them dissociate from the primary tumor, intravasation, circulating tumor cells penetrated into the blood vessel, and extravasation, metastasized to the distant organ to form new tumor colonization by reversing EMT to Mesenchymal to Epithelial Transition (MET) [7].

The mechanism of circ_0003258 mediated PCa cell migration, especially the EMT process, had been explored. The cellular process (GO analysis) and KEGG signaling pathways had been screened under lentiviral-mediated stable transfection of shRNA-circ_0003258 in PCa cells versus the negative control vector using RNA sequencing. ERK is a critical biomarker in the MAPK pathway. E-cadherin was selected as the major epithelial marker, while Vimentin was considered the mesenchymal marker. ZEB-1 was an EMT-related transcription factor. Therefore, knockdown or overexpression of circ_0003258 successfully decreased or increased p-ERK and EMT. It suggested circ_0003258 positively regulated the active form of ERK, overexpression of circ_0003258 with ERK inhibitor SCH772984 significantly impaired p-ERK protein and rescued the expression of E-cadherin, restoring the EMT function in both PCa (DU145 and C4–2) cells. In conclusion, circ_0003258 promoted cell migration via ERK in the MAPK pathway, facilitating the EMT process in PCa cells.

CircRNA is usually bound to miRNA via complementary base pair matching. The authors found miR-653-5p sponging with circ_0003258 promoted PCa metastasis. 3 miRNAs (miR-1278, miR-502-5p, miR-653-5p) were predicted to have the highest potential to bind circ_0003258 via the Circinteractome database. Circ_0003258 enriched mRNA expression of miR-653-5p and miR-502-5p validated by the pull-down assay. MiR-653-5p was chosen due to the innovation. The authors inserted wild-type (wt) and mutant sequences of 3'UTR regions of circ_0003258 into the plasmid containing luciferase and renilla double enzyme system, the miR-653-5p mimic significantly reduced the luciferase activity of the wt-circ_0003258. However, loss-and gain-of-function studies on either circ_0003258 or miR-653-5p had no effect on each other, which suggested that both RNAs may not be the linear regulatory relationship or they perform biological function independently or circ_0003258 and miR-653-5p forming complex associated with the additional molecules and synergistically accelerating PCa cell metastasis. Subsequently, 8 overlapped DEGs from 4 websites (miRDB, DIANA-microT, Targetscan, and Starbase) predicted the miRNA-targets relationship. Two proteins (SCHIP1 and ARHGAP5) were selected because of the downregulation of both mRNA expression of these 2 molecules after the knockdown of circ_0003258. Silence or overexpression of circ_0003258 significantly reduced ARHGAP5 mRNA and protein expression. In a word, circ_0003258 sponging with miR-653-5p complex to target ARHGAP5 proteins.

Next, the authors aim to investigate how circ_0003258/miR-653-5p-ARHGAP5 mediated PCa cell migratory function. MiR-653-5p mimics or inhibitors critically impaired or augmented the mRNA and protein expression of circ_0003258/miR-653-5p targets (ARHGAP5, SCHIP1). Furthermore, miR-653-5p mimics or inhibitors significantly decreased or increased PCa cell migration, respectively. Overexpression of circ_0003258 plus miR-653-5p mimics successfully restored the decreased migration and ARHGAP5 protein in PCa cells. They concluded that circ_0003258 boosted the migration capacity of PCa by protecting ARHGAP5 protein from inhibition of miR-653-5p. In addition, siRNA- ARHGAP5 decreased cell migration as well as the rise of E-cadherin and the decline of Vimentin protein, which verified that ARHGAP5 affected the migration ability by affecting the EMT process of cancer cells. In a summary, circ_0003258 sponging with miR-653-5p to target ARHGAP5 promoted PCa cell migration. Besides this signaling axis, circ_0003258 was also bound to RNA-binding protein to affect the PCa cell invasion and metastasis.

First, the authors aim to identify interactive molecules with hsa_circ_0003258 using RBPmap databases (http://rbpmap.technion.ac.il/) [8] and circinteractome (https://circinteractome.nia.nih.gov/) databases [9] pinpointed IGF2BP3 protein. The region of 0-80 nucleotides in the circ_0003258 sequence had been predicated with the highest potential via the catRAPID website (http://s.tartaglialab.com/page/catrapid_group). Furthermore, direct binding experiments (RNA pull down and RIP) were performed to validate the binding of circ_0003258 and IGF2BP3. The immunofluorescence image also confirmed that circ_0003258 colocalized with IGF2BP3 enriched in the cytosols. The author previously proved circ_0003258 was mainly located in the cytosol, both immunofluorescence and qPCR analysis added the piece of evidence to this puzzle. When authors overexpressed circ_0003258 but failed to detect any change in IGF2BP3 protein levels, it indicated 2 molecules may not function as up or downstream regulatory relationships, they independently exist, or they may bind through the intermediate molecules to perform the required biological function. Due to the features of IGF2BP3's m6A reader, the m6A modification site of circ_0003258 had been predicted via the SRAMP website (http://www.cuilab.cn/sramp) [10]. 2 sites in circ_0003258 region with the highest and moderate confidence showed relative higher modification probability, methylated RNA immunoprecipitation (MeRIP) assay successfully proved the authors' hypothesis that m6A precipitated fraction enriched circ_0003258 expression, it not only provided the strong

support for direct binding of circ_0003258 and IGF2BP3, but it also suggested that circ_0003258 binding to IGF2BP3 via m6A modification to affect their downstream effectors 'pathological process.

Subsequently, circ_0003258/IGF2BP3 complex may functionally change the mRNA stability of the certain molecule to influence PCa cell metastasis, therefore they initiated RNA-sequencing between the silence of circ_0003258 and the negative control group in PCa cells. 532 genes were significantly decreased when knockdown circ_0003258 with the cut-off criteria of log FC>2. Next, 16 mRNAs bound to IGF2BP3 had been narrowed down from the previously identified 532 genes using IGF2BP3 binding to 3'UTR regions (http://starbase.sysu.edu.cn/starbase2/) [11] and published IGF2BP3 enhanced-RBP CLIP-SEQ datasets. Literature novelty overlapping with genes associated with metastasis ruled out another 6 molecules. In the end, HDAC4 was successfully selected as the target of circ_0003258 after qPCR and WB validation as well as RIP assay in 2 PCa cells. Of note, SRAMP website tools and MeRIP assay identified HDAC4 bound to IGF2BP3, it was highly enriched in the m6A precipitation. Then, the authors designed the rescue experiments to illustrate the functional role of IGF2BP3/HDAC4 complex mediating metastasis in PCa cells. Firstly, overexpression of circ_0003258 and transient knockdown siRNA against IGF2BP3 substantially elevated and diminished HDAC4 protein level, which suggested IGF2BP3 had the potential to degrade HDAC4 mRNA stability. Secondly, si-IGF2BP3 successfully decreased HDAC4 expression at both transcriptional and translational levels, especially, in a time-dependent manner. Thirdly, circ_0003258 and IGF2BP3 complex can stabilize HDAC4 mRNA. This complex increased cell migration. Therefore, the authors further evaluated whether HDAC4 can enhance the metastatic ability of PCa cells. Transient knockdown of HDAC4 significantly attenuated PCa cell migration. In addition, western blot analysis also confirmed that the loss of HDAC4 strongly decreased Vimentin and p-ERK proteins, key molecules in the EMT process and in the MAPK signaling pathway. It suggested that circ_0003258 promoted EMT via activation of HDAC4 and MAPK signaling pathways in PCa cells. To evaluate the role of circ_0003258 in tumor metastasis in vivo, shRNA-circ_0003258 in DU145 cells was injected intravenously into the caudal vein of BALB/c nude mice. The author then examined the metastatic tumor nodules in the lungs. shRNA-circ_0003258 cells had fewer metastatic nodules in the lungs than those mice injected with the control cells. This was consistent with the results of Transwell and wound healing assays in the cells when silencing circ_0003258, and also proved that circ_0003258 was positively correlated with the migration of PCa cells. In addition, down-regulation of circ_0003258 expression could prolong the survival time of the mice. shRNA-circ_0003258 tumor tissues significantly repressed HDAC4 and ARHGAP5 histopathological staining by immunohistochemistry. In a summary, circ_0003258/IGF2BP3 complex enhanced the stability of HDAC4 mRNA by forming an RNA-protein ternary complex, thereby stimulating p-ERK to induce PCa metastasis.

Conclusion

All in all, two direct regulatory mechanisms of circ_0003258-mediated cell metastasis had been established in prostate cancer:

1. Circ_0003258 sponging with miR-653-5p targeting ARHGAP5 to promote PCa cell metastasis. Circ_0003258 was identified as candidate DEG when screening human samples. High expression of circ_0003258 related to the increased TNM stages and enhanced PCa cell metastasis. The authors then verified the function of circ_0003258 which positively correlated with cancer migration. Circ_0003258 promoted the PCa cell migration via ERK in the MAPK pathway, enhancing the EMT process in PCa cells. miR-653-5p was identified by the Circinteractome database. Dual luciferase assay proved the binding relationship between circ_0003258 and miR-653-5p, but there was no quantitative regulatory relationship between circ_0003258 and miR-653-5p. ARHGAP5 was selected from the overlaps of miRDB, Dia-MicroT, Targetscan, and Starbase, as well as the significantly decreased protein after circ_0003258 knocking out. Three biomarkers circ0003258, miR-653-5p, and ARHGAP5 related to prostate cancer were found. It is of great significance for the development of potential biomarkers and drugs for prostate cancer metastasis.

2. Circ_0003258/IGF2BP3 complex enhanced the stability of HDAC4 protein to activate p-ERK enhancing PCa cell metastasis.

The author overexpressed or knocked down circ_0003258 to find the molecule associated with it on the expression level. If a molecule is significantly associated with changes in circ_0003258 expression at both the RNA and protein levels, there is a high probability that these two molecules may interact. RNA pull-down and RIP verified whether IGF2BP3 and circ_0003258 can interact to bind. Both RNA pull-down and RIP are classical methods to verify the interaction between RNA and protein. The author not only used the bioinformatics websites to predict the distribution of m6A sites on circ_0003258 but also used MeRIP assay to investigate methylation sites. MeRIP is a technique based on the principle of a specific antibody binding to the methylated base, RNA immunoprecipitation-enriched methylated fragments, followed by high-throughput sequencing, to study the methylated RNA region within the range of the whole transcriptome and obtain results.

Pros and Cons

A. CircRNA originated from the linear gene ZNF652 and served as a biomarker in cancer, proved as a circular RNA. Divergent and convergent primers amplifying cDNA and gDNA, RNase R

digestion is a standard evaluation of circRNA since lncRNAs or microRNAs were easily digested by RNase R. In situ hybridization and immunofluorescence techniques provide a way to speculate the molecular function because recognizing the structure and location of the interested molecule leads to a better hypothesis of follow-up experiments, which can greatly enhance the credibility and persuasiveness of the results.

B. Traditionally, circRNA affected microRNA by complementary base pairing or up/down-regulation of each other to affect certain biological processes. This paper gave us an excellent example that circ_0003258 had no quantitative regulatory relationship with miR-653-5p, but circ_0003258 binding to miR-653-5 and released ARHGAP5 to promote cell migration and invasion via EMT.

C. This paper displayed a few direction interactions of RNA-RNA, RNA-protein, etc. RNA-protein interaction can be used in many research areas, RNA pull-down and RIP assays were good experiments, the former pull-down RNA with known protein molecules, then enrich and identify the RNA. RIP is to pull down protein with known RNA molecules, then identify the protein. Luciferase activity can be used to detect whether microRNAs regulate circRNA, and 3'UTR of circRNA can be inserted into an empty vector, after transfection to the cancer cells, miR mimics or inhibitors were added to the cells if luciferase activity changed, it suggests both RNAs may have binding sites to interact.

D. *In vivo* studies usually contained subcutaneous, xenograft, tail vein injection, and transgenic mouse models. Subcutaneous is easy and good to evaluate tumor growth but required a large number of tumor cells injection, the whole tumor mass will not reflect the *In vivo* environment. Xenografts provided a clear way to mimic the *In vivo* environment, we can observe tumor growth and metastasis. Tail vein injection often explores the problems of lung metastasis. Transgenic mice are trying to answer the importance of certain molecules, either completely knockout or conditional knockout such as the cre-loxp system, etc. The slight problem of this paper is the beginning when the author used plasma to screen circRNAs but validated this potential circRNA- circ_0003258 in the prostate cancer cells or tissues instead of plasma. In summary, the mechanism of the circRNA-miR-target gene, and circRNA-TF-target gene in this paper is excellent and mutually exclusive. The author finished two storylines and presented us with two full stories.

Acknowledgment

We appreciate the funding sources to support us to download the paper either commented on or cited with sponsorship for this short commentary to publish. (Phase II to Jinghua Chen) Top-notch Academic Programs Project of Jiangsu Higher Education Institutions. 2020M681492 (to Chen Chen) Chinese Post-Doc Science Fundss.

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