

Plasma concentration of piRNAs in breast cancer and its association with metastasis

Ping Yin^{1,†}, Zheng Wang^{1,†}, Yan Chen^{1,†}, Zhao-Quan Wen^{1,2}, Hong-Hai Hong^{1,*}, Yu-Ling Mao^{3,4,*}

¹Department of Clinical Laboratory, The Third Affiliated Hospital of Guangzhou Medical University, 510150 Guangzhou, China

²Department of Blood Transfusion, Dongguan Tung Wah Hospital, 523120 Dongguan, China

³Center for Reproductive Medicine, The Third Affiliated Hospital of Guangzhou Medical University, 510150 Guangzhou, China

⁴Key Laboratory for Reproductive Medicine of Guangdong; Key Laboratory of Reproduction and Genetics of Guangdong Higher Education Institutes; Key Laboratory for Major Obstetric Diseases of Guangdong Province, 510150 Guangzhou, China

*Correspondence: gaolaosao@126.com (Hong-Hai Hong); 2018683015@gzhmu.edu.cn (Yu-Ling Mao)

† These authors contributed equally.

DOI: [10.31083/j.ejgo.2021.02.2179](https://doi.org/10.31083/j.ejgo.2021.02.2179)

This is an open access article under the CC BY 4.0 license (<https://creativecommons.org/licenses/by/4.0/>).

Submitted: 27 June 2020 Revised: 18 November 2020 Accepted: 23 November 2020 Published: 15 April 2021

Objectives: Piwi-interacting RNAs (piRNAs), are a family of small, non-coding RNA transcripts, previously thought to silence gene expression in germ cells through the piRNA-PIWI complex. In recent studies, piRNAs were found to be prominently involved in the development and prognosis of malignant tumors. The study aims to identify differentially expressed circulating piRNAs in breast cancer patients. **Methods:** 8 piRNAs were tested using Real-time PCR assay in the plasma samples of 33 healthy subjects and 37 breast cancer patients. **Results:** In this study, we found that piR-651, piR-17458, and piR-20485 were significantly down-regulated in breast cancer patients when compared with healthy controls. Further, piR-20485 was found to be decreased in patients with metastatic disease relative to those with carcinoma *in situ*. **Conclusion:** These findings demonstrate that piRNAs are potential biomarkers in peripheral blood for breast cancer diagnosis.

Keywords

piRNA; Breast cancer; Plasma; Metastasis; Quick detection

1. Introduction

piRNAs are a novel class of small noncoding RNAs that are 24–31 nucleotides in length first found in germ cells of mammals. Generally, they form a complex with PIWI proteins and function to repress gene transcription and maintain germline genome integrity [1]. Recent findings reveal that piRNAs are differentially expressed in various types of tumors [2–5], a finding that prompted us to hypothesize that piRNAs may participate in the process of tumorigenesis.

Breast cancer is a major health threat to women in many countries [6, 7]. The role of noncoding RNAs in the epigenetic regulation of breast cancer has been emphasized in several published articles [8–12]. Weng *et al.* [13] proved that piR-1245 was overexpressed in colorectal cancer tissues and its expression level was significantly correlated with advanced and metastatic disease. Tan and coworkers [5] found that piRNA-36712 was significantly decreased in breast cancer tissues and likely functions in restraining can-

cer progression through interaction with SEPW1 pseudo-gene (SEPW1P) RNA. However, the expression pattern of circulating piRNAs in breast cancer patients has never been investigated before.

Herein, we aimed to measure the plasma concentration of 8 piRNAs, which were previously reported to be differentially expressed in breast cancer and normal breast tissues using qRT-PCR. Besides, we tried to investigate a possible relationship between piRNA and tumor metastasis.

2. Materials and methods

2.1 Clinical samples

Plasma samples were obtained from breast cancer patients at The Third Affiliated Hospital of Guangzhou Medical University. Age-matched healthy females served as controls. All plasma samples were collected and stored frozen at -20 °C immediately after clinical examination to assure sample integrity. Relevant clinical information was also collected after informed consent (Table 1). The study was approved by The Institutional Ethics Committee of The Third Affiliated Hospital of Guangzhou Medical University.

Table 1. Clinical characteristics of all participants in this study.

Variables	Healthy controls (N = 33)	Breast Cancer (N = 37)
Age, mean (SEM)	48.9 (5.3)	52.1 (4.2)
TNM stage, N (%)		
Stage I	/	5 (13.5)
Stage II	/	25 (67.6)
Stage III	/	7 (18.9)
Adjuvant chemotherapy, N (%)		
Yes	/	30 (81.1)
No	/	7 (18.9)

All participants in our study are female.

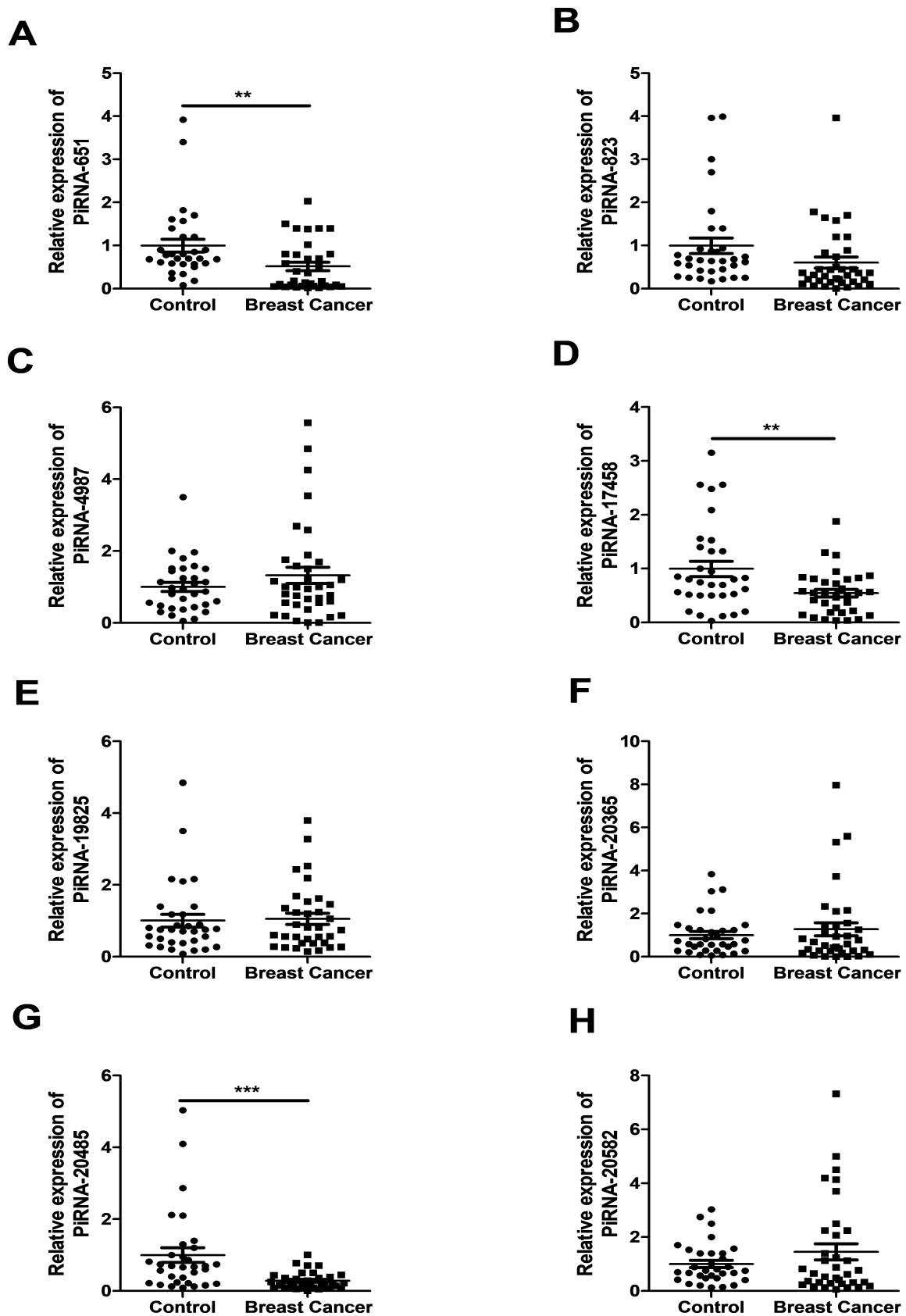


Fig. 1. The relative expression of piRNAs in Controls and Breast Cancer patients. (A-H) The relative expression of piRNA-651, 823, 4987, 17458, 19825, 20365, 20485, 20582, small RNA U6 was used as reference. **, $P < 0.01$, ***, $P < 0.001$.

2.2 RNA extraction and reverse transcription

A miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) was used for purification of total RNA, including miRNA, from patient and control plasma according to the manufacturer's instructions. A Nanodrop 2000 spectrophotometer (Thermo, TX, USA) was used to measure the concentration and purity of RNA samples. cDNA was synthesized using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany).

2.3 Real-time PCR assay for detection of piRNAs

Real-time polymerase chain reaction (PCR) was performed using the miScript SYBR Green PCR Kit (Qiagen) on a Roche Lightcycler 480 (Roche, Basel, Switzerland). 20 μ L PCR mixture included QuantiTect[®] SYBRGreen PCR Master Mix (10 μ L), miScript Universal Primer (2 μ L), RNAase-free water (4 μ L), template cDNA (2 μ L), and transcript-specific piRNA primer (2 μ L). The primers are listed in Table 2. The thermocycling program used was 95 °C for 15 min, followed by 40 amplification cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. Small RNA U6 was used as reference in the analysis of piRNA expression.

Table 2. Primers of piRNAs.

piRNA	Primer sequences
piR-651 Forward	AGCGTTGGTGGTATAGTGGT
piR-823 Forward	AGCGTTGGTGGTATAGTGGT
piR-4987 Forward	TCGCCGTGATCGTATAGTGGTTAGTACTCTG
piR-17458 Forward	TTCACGTGATGAGAGCATTGTTCTGAGC
piR-19825 Forward	GCATTGGTGGTTCAGTGGTAGAATTCTCAC
piR-20365 Forward	GGCCGTGATCGTATAGTGGTTAGTACTCTG
piR-20485 Forward	GGGGATGTAGCTCAGTGGTAGAGCGCATGCT
piR-20582 Forward	GGTTCATGGTGTAAATGGTTAGCACTCTG
U6 Forward	TGCGGGTGCTCGCTTCGGCAGC
Common Reverse	CTTATGGAGCCTGGGACTCTGACC

2.4 Statistical analysis

Statistical analysis was performed using SPSS software 18.0 (SPSS Inc., Chicago, USA). Statistical differences between two variables were analyzed using student *t* test. $P < 0.05$ was considered significant.

3. Results

3.1 Expression level of piRNAs in breast cancer patients compared to healthy controls

33 healthy subjects and 37 breast cancer patients were included in this study. Relative levels of plasma piRNAs expression were measured using the U6 transcript as an internal reference.

Results showed that the relative expression of piR-651 ($P = 0.0089$), piR-17458 ($P = 0.0058$), and piR-20485 ($P = 0.0005$) were each significantly down regulated in breast cancer patients vs healthy controls. piRNA-20485 expression was even lower in patients with metastatic disease, but the difference failed to reach statistical significance as the num-

ber of cases is insufficient to establish significance (Fig 1).

4. Discussion

piRNAs have received attention in the last decades because of their potential role in pathogenesis, such as tumorigenesis and spine morphogenesis [13–16]. Recently, studies focused on differential expression patterns of piRNAs in malignant tumor tissue and healthy controls have emerged [17, 18]. Evidence suggest that piRNA may work through epigenetic mechanisms to maintain genome integrity [2]. Despite these advancements, circulating piRNA expression is less known. In this study, we evaluate the expression of eight piRNAs that have been previously reported to be dysregulated in breast tumor. Results showed significant differences in the expression of piRNAs-651, 17458, 20485 in plasma between breast tumor and healthy subjects confirming that piRNA are secreted into the bloodstream and rapid detection of these small transcripts is feasible.

Results showed that piR-651, piR-17458, and piR-20485 were decreased in the plasma of breast cancer patients when compared with normal control subjects (Fig 1). Although piR-651 expression was reported to be stimulated by estrogen in breast cancer cell lines MCF-7 and MDA-MB-231 [19] and piR-823 was found to be upregulated in gastric cancer tissues [20], we measured that that the plasma levels of piR-651 in breast cancer patients is decreased and piR-823 levels showed no difference between breast cancer and healthy patients. Huang *et al.* found that piR-20485 was increased in breast tumor tissue compared with non-tumor tissue [21], yet in this study circulating piR-20485 was decreased in tumor patients. In addition, the relative concentration of piR-4987, 20365, and 20582, which were also upregulated in tumor tissue [21], we found no significant difference in patient plasma. Of note, we found expression of piR-20485 is possibly associated with metastasis. Overall, the expression pattern of piRNAs in plasma commonly did not match previously measured levels in tumor tissue, and the underlying regulatory mechanism responsible for this should be explored in the future.

The detection of circulating tumor markers has been facilitated due to ease in specimen acquisition [22, 23]. Furthermore, results were found to be readily reproducible. Circulating piRNAs in breast tumor patients has never been previously reported, and may provide new potential biomarkers for breast cancer. However, it is worth noting that the expression of piR-651, piR-17458, and piR-20485 in the control group were quite divergent, which may limit their clinical application for distinguishing breast cancer from healthy patients.

In summary, our research confirms that there exists detectable levels of piRNAs in peripheral blood plasma, and that some of which piRNAs are differentially expressed in breast cancer patients versus normal controls. The results may suggest that circulating piRNAs are a potential breast cancer biomarker.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by PY, ZW, YC and ZQW. HHH and YLM conceived the idea for the project and wrote the paper and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of The Third Affiliated Hospital of Guangzhou Medical University (approval number: [2020]174).

Acknowledgment

We would like to express our gratitude to all those who helped me during the writing of this manuscript. Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This study was supported by National Natural Science Foundation of China (81701417, 81901557, and 81902693); The Doctor start-up program of The Third Affiliated Hospital of Guangzhou Medical University (2017B10); Natural Science Foundation of Guangdong Province (2018A030310298) and Educational Commission of Guangdong Province (2017KTSCX155), and Guangzhou Municipal Health Bureau (20171A011313). The funding agencies had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

References

- [1] Iwasaki YW, Siomi MC, Siomi H. PIWI-interacting RNA: its biogenesis and functions. *Annual Review of Biochemistry*. 2015; 84: 405–433.
- [2] Moyano M, Stefani G. piRNA involvement in genome stability and human cancer. *Journal of Hematology & Oncology*. 2015; 8: 38.
- [3] Muller S, Raulefs S, Bruns P, Afonso-Grunz F, Plotner A, Thermann R, *et al*. Next-generation sequencing reveals novel differentially regulated mRNAs, lncRNAs, miRNAs, sdRNAs and a piRNA in pancreatic cancer. *Molecular Cancer*. 2015; 14: 94.
- [4] Ai L, Mu S, Sun C, Fan F, Yan H, Qin Y, *et al*. Myeloid-derived suppressor cells endow stem-like qualities to multiple myeloma cells by inducing piRNA-823 expression and DNMT3B activation. *Molecular Cancer*. 2019; 18: 88.
- [5] Tan L, Mai D, Zhang B, Jiang X, Zhang J, Bai R, *et al*. PIWI-interacting RNA-36712 restrains breast cancer progression and chemoresistance by interaction with SEPW1 pseudogene SEPW1P RNA. *Molecular Cancer*. 2019; 18: 9.
- [6] Fan L, Strasser-Weippl K, Li J, St Louis J, Finkelstein DM, Yu K, *et al*. Breast cancer in China. *The Lancet Oncology*. 2014; 15: e279–e289.
- [7] Harbeck N, Gnant M. Breast cancer. *The Lancet*. 2017; 389: 1134–1150.
- [8] Hamam R, Hamam D, Alsaleh KA, Kassem M, Zaher W, Alfayez M, *et al*. Circulating microRNAs in breast cancer: novel diagnostic and prognostic biomarkers. *Cell Death & Disease*. 2017; 8: e3045.
- [9] Nassar FJ, Nasr R, Talhouk R. MicroRNAs as biomarkers for early breast cancer diagnosis, prognosis and therapy prediction. *Pharmacology & Therapeutics*. 2017; 172: 34–49.
- [10] Li Z, Hou P, Fan D, Dong M, Ma M, Li H, *et al*. The degradation of EZH2 mediated by lncRNA ANCR attenuated the invasion and metastasis of breast cancer. *Cell Death & Differentiation*. 2017; 24: 59–71.
- [11] Niknafs YS, Han S, Ma T, Speers C, Zhang C, Wilder-Romans K, *et al*. The lncRNA landscape of breast cancer reveals a role for DSCAM-as1 in breast cancer progression. *Nature Communications*. 2016; 7: 12791.
- [12] Zhang H, Ren Y, Xu H, Pang D, Duan C, Liu C. The expression of stem cell protein Piwil2 and piR-932 in breast cancer. *Surgical Oncology*. 2013; 22: 217–223.
- [13] Weng W, Liu N, Toiyama Y, Kusunoki M, Nagasaka T, Fujiwara T, *et al*. Novel evidence for a PIWI-interacting RNA (piRNA) as an oncogenic mediator of disease progression, and a potential prognostic biomarker in colorectal cancer. *Molecular Cancer*. 2018; 17: 16.
- [14] Ng KW, Anderson C, Marshall EA, Minatel BC, Enfield KSS, Saprunoff HL, *et al*. Piwi-interacting RNAs in cancer: emerging functions and clinical utility. *Molecular Cancer*. 2016; 15: 5.
- [15] Mai D, Ding P, Tan L, Zhang J, Pan Z, Bai R, *et al*. PIWI-interacting RNA-54265 is oncogenic and a potential therapeutic target in colorectal adenocarcinoma. *Theranostics*. 2018; 8: 5213–5230.
- [16] Shen S, Yu H, Liu X, Liu Y, Zheng J, Wang P, *et al*. PIWIL1/piRNA-DQ593109 regulates the permeability of the blood-tumor barrier via the MEG3/miR-330-5p/RUNX3 axis. *Molecular Therapy*. 2018; 10: 412–425.
- [17] Han Y, Li Y, Xia S, Zhang Y, Zheng J, Li W. PIWI proteins and PIWI-interacting RNA: emerging roles in cancer. *Cellular Physiology and Biochemistry*. 2017; 44: 1–20.
- [18] Martens-Uzunova ES, Böttcher R, Croce CM, Jenster G, Visakorpi T, Calin GA. Long noncoding RNA in prostate, bladder, and kidney cancer. *European Urology*. 2014; 65: 1140–1151.
- [19] Oner C, Turgut Cosan D, Colak E. Estrogen and androgen hormone levels modulate the expression of PIWI interacting RNA in prostate and breast cancer. *PLoS ONE*. 2016; 11: e0159044.
- [20] Cheng J, Deng H, Xiao B, Zhou H, Zhou F, Shen Z, *et al*. PiR-823, a novel non-coding small RNA, demonstrates in vitro and in vivo tumor suppressive activity in human gastric cancer cells. *Cancer Letters*. 2012; 315: 12–17.
- [21] Huang G, Hu H, Xue X, Shen S, Gao E, Guo G, *et al*. Altered expression of piRNAs and their relation with clinicopathologic features of breast cancer. *Clinical and Translational Oncology*. 2013; 15: 563–568.
- [22] Blackman S, Stafford-Allen B, Hanson EK, Panasiuk M, Brooker AL, Rendell P, *et al*. Developmental validation of the ParaDNA® Body Fluid ID System-A rapid multiplex mRNA-profiling system for the forensic identification of body fluids. *Forensic Science International: Genetics*. 2018; 37: 151–161.
- [23] Chang W, Wang C, Lin C, Wu J, Lee MS, Lee G. Rapid detection and typing of live bacteria from human joint fluid samples by utilizing an integrated microfluidic system. *Biosensors and Bioelectronics*. 2015; 66: 148–154.