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LETTER TO THE EDITOR

Detection of piRNA-54265 in human serum: evidence and significance

Dear Editor,

An increasing number of studies have shown that PIWI-interacting RNAs (piRNAs) are aberrantly expressed in many types of human cancer and involved in regulating the malignant progression, piRNAs would be expected to serve as molecular biomarkers for cancer screening and early diagnosis.

In a previous study, we have identified the presence of piRNA-54265 in human serum [1]. Further research has demonstrated that it is a promising and specific blood molecular marker for colorectal cancer (CRC) [2]. In these studies, we have developed a stem-loop primer reverse transcription-quantitative PCR (RT-qPCR) approach to determine the piRNA-54265 levels, which included the reverse transcription of piRNA-54265 to cDNA using a stem-loop primer and the quantitative detection of cDNA by PCR with the primers and probe specific for piR-54265 [2].

However, a recent report suggested that piR-54265 we detected in serum could be a full-length (72 nt) small nucleolar RNA-SNORD57 [3]. To clarify this important issue, we performed further experiments to validate our method of piRNA detection and verify the identity of piR-54265 in human serum samples. The new results further confirmed our previous findings that the RNA detected in human serum was indeed mature piR-54265, but not SNORD57, based on the following evidences.

The stem-loop primer RT-PCR method we developed for detecting piR-54265 is shown in Figure 1A. As the name implies, the stem-loop reverse transcription primer includes two segments completely complementary with each other to form a stem loop, as indicated by the black boxes. Besides, our primer contains 9 bases complementary with piR-54265 at 3' end (highlighted in yellow) and the rest bases of piR-54265 from its 5' end to form the sequence of forward primer (highlighted in purple). For stem-loop primer RT-PCR of small RNA, the reverse primer is usually derived from the sequence of the corresponding reverse transcription primer as highlighted in green or double underline for piR-54265 (Figure 1A, upper panel). We could easily determine the sequence of cDNA (Figure 1A, middle panel) and the resultant PCR product (Figure 1A, lower panel). One of the cDNA chains comprises whole piR-54265 sequence and the inverse complementary sequence of the reverse transcription primer (between the black boxes). Thus, using this cDNA template, the PCR will produce a 77-base pair resultant product of piR-54265 (Figure 1A, lower panel).

We further examined in silico the feasibility of our stem-loop primer RT-PCR approach to detect SNORD57. As shown in Figure 1B, although the sequence of the first 29 bases at 5' end is the same for both piR-54265 and SNORD57, the last 10 bases at 3' end of SNORD57 is different from that of piR-54265. We designed a 9-base primer complementing the 3' end sequence of piR-54265 for reverse transcription (Figure 1B, upper panel). If SNORD57 could also be amplified by this method, the PCR product should be 120 base pairs (Figure 1B, lower panel). To address the potential concern that there were far more products of SNORD57 rather than piR-54265 via this stem-loop RT PCR method [3], we have performed additional experiments as described below.

In our previous studies establishing the stem-loop primer RT-PCR method for the detection of piR-54265 in serum samples, we have already confirmed that the PCR product is piR-54265 by TA-cloning sequencing [2]. As shown in Figure 1C, only the sequence that was identical to the expected PCR products of piR-54265 presents in the serum samples from both healthy controls and CRC patients, demonstrating that our stem-loop primer RT-PCR method was specific for the detection of piR-54265 but not SNORD57. To validate again our stem-loop primer RT-PCR method, we created another stem-loop primer RT-PCR method with the new primer set (Supplementary Table S1) to detect piR-54265 in serum samples. Sequencing

List of abbreviations: piRNA, PIWI-interacting RNA; RT-qPCR, reverse transcription-quantitative PCR; RT, reverse transcription; CRC, colorectal cancer; nt, nucleotide.

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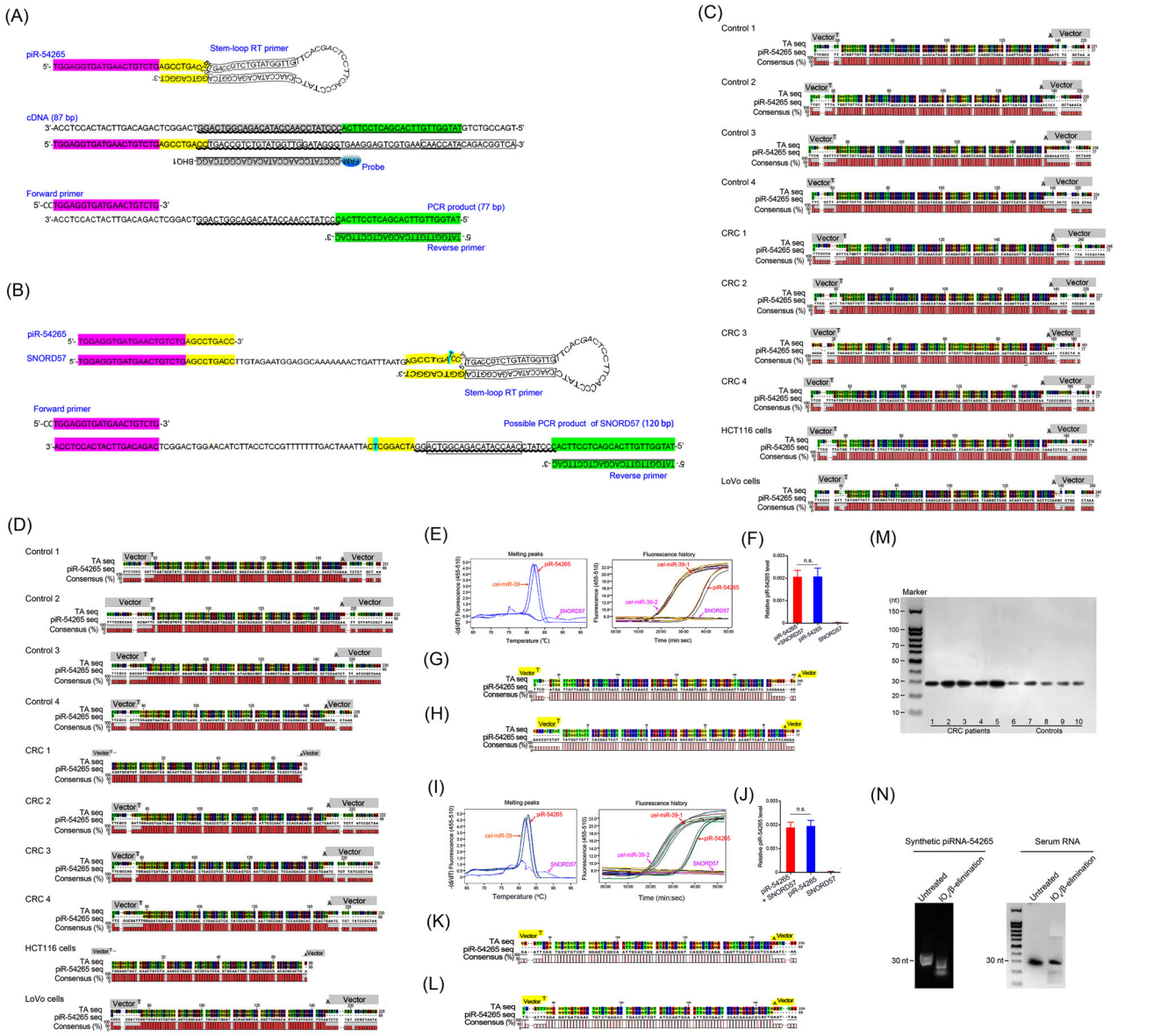

FIGURE 1 **The validation of the stem-loop primer RT-PCR method for detecting piR-54265 and the presence of piR-54265 in human serum. (A-B)** The piR-54265 sequence and schematic of stem-loop primer RT-PCR method for detecting piR-54265 (A) and in silico analysis of the stem-loop primer RT-PCR method for detecting SNORD57 (B). Shown are the sequences of piR-54265 or SNORD57 and stem-loop reverse transcription primers (upper), cDNA (middle), and PCR product produced by the stem-loop primer RT-PCR (lower). **(C-D)** TA clone sequences of the stem-loop primer RT-PCR products from piR-54265 (C) and the products produced by another stem-loop primer RT-PCR method for piR-54265 (D). Controls 1–4 were the serum samples from healthy individuals and CRC 1–4 were the serum samples from colorectal cancer (CRC) patients. **(E-H)** Detection of synthesized piR-54265 or SNORD57 with the stem-loop primer RT-PCR method and TA-cloning analysis of the PCR products. (E) Shown are melting (left panel) and amplification (right panel) curves of piR-54265, SNORD57, mixture of piR-54265 and SNORD57, and *cel*-miR-39. *Cel*-miR-39-1 and *cel*-miR-39-2 were the internal reference for piR-54265 and SNORD57 template sample, respectively. Quantitative statistics of piR-54265 levels in the sample of the mixture of piR-54265 and SNORD57, piR-54265 only, or SNORD57 only (F). The sequences of the PCR product from the mixture of piR-54265 and SNORD57 (G). The sequences of the PCR product from the sample of piR-54265 only (H). TA seq, TA-cloning sequence of the PCR product from each sample; piR-54265 seq, the expected sequence of piR-54265 PCR product. Data are mean ± SEM from 3 independent detections. n.s., not significant of Student *t*-test. **(I-L)** Detection of synthesized piR-54265 or SNORD57 with another stem-loop primer RT-PCR method and TA-cloning analysis of the PCR products. **(M)** Northern blot analysis with the specific probe indicates the presence of piR-54265 in the serum samples from health controls and CRC patients. **(N)** Analysis of 2'-O-methylation at the 3' end of piR-54265 by periodate and *β*-elimination treatment. Synthesized piR-54265 samples with treatment were separated and stained with ethidium bromide. Treated serum samples were detected by Northern blot with the probe specific for piR-54265. Bands indicate synthesized piR-54265 or serum piR-54265 and its degradation products. Abbreviations: bp, base pair; TA seq, TA-cloning sequencing; nt, nucleotide.

analysis of the TA clones showed that the RT-PCR products were exclusively the piR-54265 but by no means SNORD57 (Figure 1D). Furthermore, we performed additional experiments to examine if our stem-loop primer RT-PCR methods can concurrently detect SNORD57 using synthesized piRNA-54265 or snoRNA-SNORD57 (Supplementary Table S1) or their mixture as the template. TA-cloning sequencing of the RT-PCR products did not show any SNORD57 amplification (Figure 1E-L). These results further demonstrate that the stem-loop primer RT-PCR methods we established are specific only for the piR-54265 detection.

Northern blotting assays in our previous study already demonstrated the presence of piR-54265 in serum samples [1]. The specific probe we used for the Northern blotting of serum samples contains a fragment that inversely complements with the piR-54265 sequence, which may also theoretically recognize snoRNA-SNORD57. However, no SNORD57 was detected by this probe in serum samples (Figure 1M), indicating that no SNORD57 was present in the serum and that the probe was specifically towards piR-54265 only. To further demonstrate serum piR-54265 as a bona fide mature piRNA, we analyzed whether it has a 3-terminal 2'-O-methylation, the characteristic feature of piRNAs [4]. We treated synthesized piRNA-54265 that did not have any modification and total RNA that was isolated from human serum samples with periodate (IO_4) followed by β-elimination. Synthesized piR-54265 without 3-terminal 2'-O-methylation was sensitive to IO_4 + β-elimination treatment as indicated by the significant reduction in the piR-54265 level (Figure 1N, left panel); however, no significant reduction in the piR-54265 level was observed for that isolated from human serum samples (Figure 1N, right panel). Hence, piR-54265 we detected in serum samples is a bona fide mature piRNA with 3-terminal 2'-O-methylation, but not a fragment of other non-coding RNAs.

The small RNA-sequencing dataset that Tosar *et al.* [3] used in their paper was from the plasma exosomes [5], which contains only exosome-derived small RNAs rather than whole-plasma small RNAs. Therefore, using such an exosome piRNA dataset might be problematic to see piR-54265 in serum. Furthermore, cautions should be taken in using piRNA datasets, since it has been shown that thermostable group II intron reverse transcriptase (TGIRT) sequencing might not be appropriate for piRNA detection because of the 3'-end sequencing bias [6]. TGIRT sequencing exploits TGIRT and TGIRT can catalyze template-switching reverse transcription reactions without adaptor ligation during cDNA synthesis [7]. However, the 3' ends of mature piRNAs are 2'-O-methylated by S-adenosylmethionine-dependent methyltransferases [8], which might inhibit TGIRT template-switching [9, 10]. In addition, Qin *et al.* [6] reported that they did not measure mature piRNAs, not only piR-54265 but also longer transcripts in the piRNA cluster, for establishing their dataset. Such a sequencing bias further indicates the lethal disadvantage of TGIRT-sequencing for the mature piRNA detection.

Based on the new data from both experimental and *in silico* analyses, we have concluded that piR-54265 is present in human serum samples. The results reported in the present study together with our previous findings clearly demonstrate that the stem-loop primer RT-PCR methods we established can specifically detect this piRNA, but not SNORD57.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Written informed consent was obtained from each subject and this study was approved by the Internal Review Boards of Sun Yat-sen University Cancer Center (YB2016-39).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The key raw data in this study have been uploaded onto the Research Data Deposit of Sun Yat-sen University Cancer Center (Approval number RDD2022001033, http://www.researchdata.org.cn)

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

Dongxin Lin conceptualized and supervised the research. Dongmei Mai and Lisha Zhuang designed and performed most experiments. Ying Ye was responsible for the bioinformatics analyses. Dongmei Mai, Ying Ye, Jian Zheng, and Dongxin Lin prepared the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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