Application of droplet digital PCR in quantitative detection of the cell-free circulating circRNAs

Dan-Feng Chen*, Lu-Jun Zhang*, Kezhe Tan and Qing Jing

Department of Cardiology, Changhai Hospital, Shanghai, PR China

ABSTRACT

Diagnostics based on circulating circular RNA (circRNA) is an emerging field for noninvasive molecular diagnosis, owing to the stable circular structure of circRNA. However, the uniquely circular structure still poses a challenge for circRNA quantification in the research community. Here, we verified the discrepancy in the circRNA quantification by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and reverse transcription-droplet digital polymerase chain reaction (RT-ddPCR), which is caused by the rolling reverse transcription (RT) product originating from the circular structure. In addition, we detected the stability of cell-free circRNA in serum/plasma and determined the pre-analysis sampling procedure that will compromise the quantification of circRNA. Our results showed that prolonged RT incubation time resulted in multiple PCR products from circular RNA, which will reduce the accuracy of circRNA quantification by RT-qPCR technology, whereas ddPCR can address this limitation and could be a good alternative to qPCR for circRNA quantification. CircRNA showed a high stability in promptly separated serum/plasma but not in delay-separated samples.

Introduction

Circular RNAs (circRNAs) are a newly discovered type of endogenous noncoding RNAs [1] characterized by a closed continuous loop formed by a covalent joint of the downstream 3' end and the upstream 5' end. The closed loop structure without 5'–3’ polarity or polyadenylated tail renders circRNA remarkably stable and insusceptible to degradation by RNA exonuclease or Ribonuclease R (RNase R) compared with mRNA [2]. Recent studies revealed that circRNAs are widely expressed in various tissues and have functional implications in development and diseases [3–7]. For example, brain circRNAs are positioned to respond to and regulate synaptic formation and function [1,8]. In addition, statistically based splicing detection revealed that tissue-specific RNAs in the heart and lung are markedly induced during human fetal development [3]. Recent studies in this field have revealed that circRNAs are involved in several diseases, such as Alzheimer’s disease [4], heart failure [9], Moyamoy disease [10], and cancers [5,6,11–14], whereby they function as miRNA sponges.

Furthermore, researchers have identified more than 400 circRNAs in human cell-free saliva (CFS) [15] and have detected thousands of circRNAs in human blood from healthy individuals [16], suggesting that circRNAs could exist in some body fluids. According to the findings yielded by a recent study [17], a typical circular RNA, Hsa_circ002059, was downregulated significantly in gastric cancer tissue. In addition, its levels in plasma were significantly different in postoperative and preoperative patients, while the lower expression levels significantly correlated with distal metastasis [17]. Given the promise of circRNA in serving as noninvasive diagnostic and prognostic biomarker, accurate quantification of circRNA should be the prerequisite for clinical applications and discovery of the role of circRNA in health and disease. Presently, northern blot and RT-qPCR (reverse transcription-quantitative polymerase chain reaction) are the primary techniques for circRNA detection. However, authors of an earlier study observed and sequenced the primary techniques for circRNA detection. However, authors of an earlier study observed and sequenced the rolling RT products of circRNA in cDNA [8]. This phenomenon, known as rolling-circle RT, can lead to a laddering effect on a gel after PCR but can also result in an overestimation of circRNA by qPCR [18].

Droplet digital PCR (ddPCR) is a newly developed technology for absolute quantification of nucleic acids with higher sensitivity and accuracy. More importantly, as the algorithm is based on Poisson distribution, it...
eliminates the effect of rolling RT products on circRNA quantification. Thus, we attempted to demonstrate the difference between RT-qPCR and RT-ddPCR technology in circRNA quantification. In addition, we tested the application of ddPCR in circRNA quantification to overcome this technical barrier. Finally, we applied ddPCR to determine the stability of circRNA, which is one of the important factors involved in biomarker research, in separated serum/plasma and delay-separated serum/plasma within 24 h.

Subjects and methods

Blood processing

Peripheral blood was drawn from 30 healthy volunteers (recruited from ChangHai Hospital) in SST™ tubes and K₂EDTA tube for serum and plasma collection respectively (BD, NJ, USA). Blood sample collection was conducted in accordance with the Declaration of Helsinki. All volunteers had given informed consent according to the institutional review boards (IRBs).

To get promptly separated serum/plasma, the clotted blood and EDTA (ethylenediaminetetraacetic acid) blood were processed immediately by centrifugation at 1000 g for 10 min at 4°C using a swinging bucket rotor. Afterwards, the upper serum/plasma layer was transferred to a new tube without disturbing the lower hematocytes followed by centrifugation at 16,000 g for 10 min at 4°C in a fixed-angle rotor. The clarified serum/plasma was transferred and then equalized per tube, discarding the buffy coat. The equalized serum/plasma samples were left at room temperature (20–25°C) for pre-designed times (0, 6 and 24 h), followed by RNA extraction. For delay separated samples, the clotted blood and EDTA blood were left at room temperature for pre-designed times. After then, the samples were subjected to centrifugation and separation under the same conditions used in promptly separated serum/plasma, followed by RNA extraction (see Figure 1S in the Online Supplementary Appendix).

RNA extraction

The cell-free RNA was extracted and purified from serum/plasma using miRNeasy Serum/Plasma Kit (Cat # 217184, Qiagen, Helden, Germany) according to the manufacturer’s instructions. The total RNA extracted from 200 μL serum/plasma finally was eluted in 16 μL nuclease-free water for the dead volume of 2 μL for pipetting and passed over the column twice to finally obtain 14 μL RNA. The 14 μL RNA was processed into reverse transcription promptly.

Reverse transcription

All the 14 μL RNA from 200 μL serum/plasma was pooled firstly and mixed with reverse transcription reagents followed by equalization into 20 μL containing 4 μL 1 x RT Buffer, 1 μL RT Enzyme Mix, 14 μL RNA with 1 μL primer using The ReverTra Ace® qPCR RT Kit (Cat # FSQ-101, Toyobo, Osaka, JP) according to the manufacturer’s instructions, modified by different prolonged incubation time including 15, 30, 60 and 120 min at 37 °C under the needs of different experiments. The reaction was inactivated by heating at 98 °C for 5 min before PCR. To test the effect of RNase H (Ribonuclease H), 1 unit RNase H (Cat # EN0202, life, MA, USA) was added to the 20 μL reagent and co-incubated at 37 °C for 15 min.

PCR

PCR was done in a 10 μL reagent mixture containing 1 μL cDNA template, 2 μL PCR Hot-Start Master Mix II (5X) (Cat # RP02-HS-II, GeneMark, TWN), 0.2 μL Forward primer (10 μL), 0.2 μL Reverse primer (10 μmol/L) and 6.6 μL ddH₂O with a conventional thermal cycler (S100™, Bio-Rad, CA, USA). Thermal cycling conditions were as follows: 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, a final extension at 72 °C for 7 min. The specificity of the PCR products was verified by the predicted size using agarose electrophoresis and Sanger sequencing (Mapbioo, Shanghai, China).

Quantitative analysis of circRNA and mRNA

After reverse transcription, cDNA was followed by qPCR and ddPCR detection. The circRNA, circSMARCA5, was especially detected with divergent primers and Applied Biosystems™ TaqMan® MGB (minor groove binder) probe spanning the back-splice site designed by Primer Express® Software v3.0.1 (http://www.appliedbiosystems.com/absite/us/en/home/support/software-community/free-ab-software.html). The sequence of amplification primers and the dual-labeled MGB probe of circSMARCA5 were:

Forward primer: 5’-TCGAAGGAGAAGACTATAGACGAAAAACA-3’
Reverse primer:5’-GTTGCTCCATGTCTAATCATTTGAA-3’
MGB probe: 5’-(FAM) AAGGGAGGCTTGTGGAT(MGB)-3’

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and β-actin mRNA were detected by Applied Biosystems Taqman Gene expression assays (GAPDH,
conditions were as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was tested in triplicate for the last calculation and run with a non-template control (NTC) composed of sterile water instead of cDNA. The threshold was adjusted at 0.01 for all the performance for CT value.

ddPCR

ddPCR was performed with QX200 Droplet Digital PCR System (Bio-Rad, CA, USA), according to the manufacturer's protocol and the dMiQE guidelines [19]. First, each 20 μL ddPCR assay mixture containing 2 μL cDNA template, 10 μL TaqMan® Gene Expression Master Mix (Cat # 4369016, Applied Biosystems, Foster City, CA), 1.8 μL forward primers (10 μmol/L), 1.8 μL reverse primer (10 μmol/L), 0.3 μL of 10 pmol/L MGB probe and 4.1 μL ddH2O, with Corbett Roter-Gene™ 3000 system (Qiagen, Helden, Germany), according to the manufacturer’s protocol and the MiQE guidelines (http://www.rdml.org/miqe). Thermal cycling conditions were as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was tested in triplicate for the last calculation and run with a non-template control (NTC) composed of sterile water instead of cDNA. The threshold was adjusted at 0.01 for all the performance for CT value.

Results and discussion

Prolonged RT (reverse transcription) incubation time resulted in multiple PCR products of rolling cDNA from circRNA

The rolling cDNA that could not be detected from any linear RNA verified the existence of the circular structure of circRNA [8]. To validate this finding, we adjusted the RT incubation time from 15 to 120 min, as indicated. Agarose gel electrophoresis showed that the PCR products multiplied and resulted in three bands of approximately 100, 400 and 600 nt length, respectively (Figure 1 A). Unlike in circRNA, only one band was observed in linear RNA represented as β-actin mRNA (Figure 1(B)). Interestingly, the multiple PCR products of circRNA disappeared (Figure 1(C)) when the RT step was co-reacted with RNase H, an RNA enzyme that specifically degrades the RNA strand in DNA–RNA hybrids, and only one band of a length of 100 nt was shown. This phenomenon was not observed in β-actin mRNA (data not shown). To further confirm the presence of multiple PCR products of different lengths, we cloned and Sanger sequenced the circRNA-derived PCR products. The results indicated that the longest band of 643 nt contained the sequences of the shorter 374 and 105 nt bands. Moreover, the 374 nt band contained the sequence of the 105 nt band (Figure 1(D, E)). The sequences of 643 and 374 nt lengths contained the Exon2 and Exon3 of circSMARCA5. This result indicated that the multiple bands were the products of the RT reaction that produced rolling cDNA from circRNA exclusively.

ddPCR is highly accurate in circRNA quantification

To further determine whether the rolling RT product uniquely derived from circRNA might compromise the accuracy of quantitative analysis of the RT-qPCR technique, we mixed the RT reaction thoroughly and divided it into five equal portions, and then subjected it to different RT incubation times from 15 to 120 min. After that, qPCR was used to measure the cDNAs. We used the ΔΔCT method to calculate the qPCR results and found that the relative levels of circRNA increased proportionally to the RT incubation time (Figure 2(A)). A significant increase was observed in the 90-min group (p < 0.05) and 120-min group (p < 0.05).

Since ddPCR determines the absolute concentration via the ratio of positive to negative droplets only, we used ddPCR to quantify circRNA-derived cDNAs that had

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6.01 (GraphPad Software, CA, USA). Analysis of variance (ANOVA) was used for multiple comparison. P < 0.05 was considered statistically significant.
undergone different prolonged RT incubation times. We first tested the ddPCR by gradual dilution of RNA and cDNA, noting that ddPCR exhibited high sensitivity and precision, with the R value of 0.99552 and 0.9996, respectively (data not shown). We subsequently quantified the cDNA samples used in the qPCR experiment. The results revealed no significant differences among the five sample groups ($p > 0.05$) (Figure 2(A)). This finding indicates that the quantitative analysis by ddPCR is not affected by the rolling RT products of circRNA produced by prolonged RT incubation time.

A similar experiment was performed in β-actin mRNA. As expected, no statistical differences were observed in the five groups in either RT-qPCR ($p > 0.05$), or RT-ddPCR ($p > 0.05$) (Figure 2(B)).

Theoretically, the level of RNA is invariant under different RT conditions as adjusted by inter-controls, but inevitable variation occurs in RT-qPCR when applied for circRNA quantification due to the rolling cDNAs of different lengths, different circRNAs containing a different amount of bases and the lack of matched inter-controls. To the best of our knowledge, this is the first time that the influence of this rolling RT product on quantitative analysis by RT-qPCR was investigated. We posit that this deviation is due to the multiple fluorescence signals produced by a single cDNA from one circRNA (Figure 3(A)). ddPCR, nevertheless, produced more consistent results due to its endpoint detection method (Figure 3(B)) and is considered more precise in circRNA quantification compared with qPCR.
In addition, other studies indicate that ddPCR has several favourable advantages: (1) it performs absolute quantification based on the principles of sample partitioning and Poisson statistics, overcoming normalization and calibration, and thus can simplify the experimental procedures. (2) It has shown increased sensitivity while detecting low target copies without the need for any pre-amplification steps. (3) It is relatively insensitive to potential PCR inhibitors [20–23].

Detection and quantification of circRNA is a prerequisite for further exploration of the function and related mechanisms of circRNA, and for utilizing it as diagnostic and predictive disease biomarker. Thus, ddPCR has the potential as a circRNA quantification method.

**Cell-free circulating circRNA is stable within 24 h in promptly centrifuged serum but not in delay-centrifuged serum**

To determine whether circulating circRNA was stable in separated serum, we measured the circRNA serially in promptly-centrifuged sera that had been incubated at room temperature for pre-designed periods up to 24 h. Multiple comparisons revealed no significant changes in the level of circRNA over the time period measured ($p > 0.05$). To further study the effect of time delay in centrifugation on the concentration of circRNA, the clotted whole blood was conserved at room temperature for a pre-designed time before centrifugation and RNA extraction. A significant decrease was found when the...
uncentrifuged clotted blood was left at room temperature for 24 h (p < 0.05) (Figure 4(A)). Apart from circRNA quantification, the concentration of GAPDH mRNA was measured simultaneously. As shown in Figure 4(B), no significant differences were observed up to 24 h in promptly separated serum (p > 0.05), while in delay-centrifuged serum, the GAPDH mRNA concentration showed a significant increase at 6 h (p < 0.05) (Figure 4(B)).

Cell-free circulating circRNA is stable within 24 h in promptly centrifuged plasma but not in delay-centrifuged plasma

Similar studies were carried out for plasma and no significant difference was observed in either circRNA or GAPDH mRNA in promptly-centrifuged plasma. In contrast, when the EDTA blood was left for 0, 6 and 24 h at room temperature, circRNA showed a significant decrease at 24 h (p < 0.05), whereas GAPDH mRNA showed a significant increase at 6 h (p < 0.05) (Figure 4(C, D)).

Recently, fetal RNA was found in maternal plasma [24], and tumour-associated circulating mRNA was detected in serum/plasma [25], both of which indicate that circulating cell-free RNA (cfRNA) in blood can be utilized in non-invasive diagnosis and prognosis. Nevertheless, several factors appear to limit the effective use of cfRNA in clinical testing: (a) the degradation of cfRNA due to serum/plasma nucleases; (b) the variation of sample handling; and (c) the interference of the hematocytes RNA release.

Except for small RNAs, circulating RNA has been thought to be labile and easily prone to degradation due to the presence of blood RNases; a free RNA liability experiment shows that >99% of the free added RNA is degraded after 15 s incubation time [26]. In this study, we have demonstrated that circulating circRNA is sufficiently stable to allow detection within 24 h at room temperature in the promptly centrifuged serum and
plasma. These findings suggest that the cell-free circulating circRNA may be protected by some mechanisms and it will be a promising tool for diagnosis.

It is worth noting that circRNA, as well as GAPDH mRNA, varied significantly in both uncentrifuged serum and uncentrifuged plasma. The inconsistent results between centrifuged and uncentrifuged serum/plasma suggest that lower hematocytess may be a contributing factor. Furthermore, we observed a different variation between circRNA and GAPDH mRNA in uncentrifuged serum/plasma, whereby an increase occurred in GAPDH mRNA but not in circRNA. This discrepancy may be explained by the far greater concentrations of the housekeeping gene GAPDH in the lower hematocytes, which can be eliminated by promptly centrifuging and separating the serum/plasma from lower hematocytes. We thus infer that the final concentration of circRNA depends on the comprehensive effect of the RNA release and RNase degradation from lower hematocytes.

Moreover, the circulating specific miRNA abundance is dramatically influenced by the duration of the EDTA treatment and cellular miRNAs expression profiles [27,28]. From this aspect, serum may be preferred for biomarker research because of its lower interference with the specific cell-free circulating RNA from hematocyte contamination and EDTA when compared to plasma.

Conclusions

This study has demonstrated that RT-qPCR technology has some notable deficiencies in circRNA quantification, which should be treated with caution in future research. ddPCR will probably be an appropriate method for circRNA quantitative detection. CircRNA remains stable in the promptly separated serum/plasma within 24 h and is a promising candidate for biomarker research. In addition, because the concentration of circRNA will be affected if the clotted blood and EDTA blood are not centrifuged promptly, samples should be handled in a timely manner to reduce the hematocyte-derived variation. However, as this study is based on plasma and serum obtained from healthy individuals, ascertaining whether the stability of circulating circRNA can be generalized to all cases requires further investigation.

Funding

The work was supported in part by the National Key Research and Development Program of China (2017YFA0103700) and the National Natural Science Foundation of China (91339205).

Acknowledgments

The authors gratefully acknowledge the technical assistance provided by Bio-Rad Laboratory (Shanghai, China) for this study. We thank Yulai Liang and Jian Chen (Shanghai Institutes for Biological Sciences, CAS) for help during this study and thank Chen Wang (Shanghai Institutes for Biological Sciences, CAS) for his valuable advice and critical reading of this manuscript.

Disclosure statement

The authors declare no competing interests.

ORCID

Qing Jing http://orcid.org/0000-0001-9834-1214

References

[1] Rybak-Wolf A, Stottmeister C, Glazar P, et al. Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. Mol Cell. 2015;58(5):870–885.
[2] Petkovic S, Muller S. RNA circularization strategies in vivo and in vitro. Nucleic Acids Res. 2015;43:2454–2465.
[3] Szabo L, Morey R, Palpant NJ, et al. Statistically based splicing detection reveals neural enrichment and tissue-specific induction of circular RNA during human fetal development. Genome Biol. 2015;16:126. DOI: 10.1186/s13059-015-0690-5
[4] Lukiw WJ. Circular RNA (circRNA) in Alzheimer’s disease (AD). Front Genet. 2013;4:307. DOI: 10.3389/fgene.2013.00307
[5] Hansen TB, Kjems J, Damgaard CK. Circular RNA and miR-7 in cancer. Cancer Res. 2013;73(18):5609–5612.
[6] Li Y, Zhang J, Huo C, et al. Dynamic organization of IncRNA and circular RNA regulators collectively controlled cardiac differentiation in humans. EBioMedicine. 2017;24:137–146.
[7] Xu T, Wu J, Han P, et al. Circular RNA expression profiles and features in human tissues: a study using RNA-seq data. BMC Genomics. 2017;18(Suppl 6):680. DOI: 10.1186/s12864-017-4029-3
[8] You X, Vlatkovic I, Babic A, et al. Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. Nat Neurosci. 2015;18(4):603–610.
[9] Wang K, Long B, Liu F, et al. A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. Eur Heart J. 2016;37(33):2602–2611.
[10] Zhao M, Gao F, Zhang D, et al. Altered expression of circular RNAs in Moyamoya disease. J Neurol Sci. 2017;381:25–31.
[11] Wang X, Zhang Y, Huang L, et al. Decreased expression of hsa_circ_001988 in colorectal cancer and its clinical significances. Int J Clin Exp Pathol. 2015;8(12):16020–16025.
[12] Li F, Zhang L, Li W, et al. Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/beta-catenin pathway. Oncotarget. 2015;6(8):6001–6013.
[13] Wang F, Nazarali AJ, Ji S. Circular RNAs as potential biomarkers for cancer diagnosis and therapy. Am J Cancer Res. 2016;6(6):1167–1176.
Kristensen LS, Hansen TB, Veno MT, et al. Circular RNAs in cancer: opportunities and challenges in the field. Onco- gene. Forthcoming 2017; DOI: 10.1038/onc.2017.361

Bahn JH, Zhang Q, Li F, et al. The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva. Clin Chem. 2015;61(1):221–230.

Memczak S, Papavasileiou P, Peters O, et al. Identification and characterization of circular RNAs As a new class of putative biomarkers in human blood. PloS One. 2015;10(10):e0141214. DOI: 10.1371/journal.pone.0141214

Li P, Chen S, Chen H, et al. Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clin Chim Acta. 444:132–136.

Barrett SP, Salzman J. Circular RNAs: analysis, expression and potential functions. Development (Cambridge, England). 2016;143(11):1838–1847. DOI: 10.1242/dev.128074

Huggett JF, Foy CA, Benes V, et al. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin Chem. 2013;59(6):892–902.

Hindson CM, Chevillet JR, Briggs HA, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods. 2013;10(10):1003–1005.

Ma J, Li N, Guarnera M, et al. Quantification of plasma miRNAs by digital PCR for cancer diagnosis. Biomark Insights. 2013;8:127–136.

Hudecova I. Digital PCR analysis of circulating nucleic acids. Clin Biochem. 2015;48(15):948–956.

Campomenosi P, Gini E, Noonan DM, et al. A comparison between quantitative PCR and droplet digital PCR technologies for circulating microRNA quantification in human lung cancer. BMC Biotechnol. 2016 [cited 2017 Oct 21];16(1):60. DOI: 10.1186/s12896-016-0292-7.

Poon LL, Leung TN, Lau TK, et al. Presence of fetal RNA in maternal plasma. Clin Chem. 2000 Nov;46(11):1832–1834.

Hasselmann DO, Rapp G, Rossler M, et al. Detection of tumor-associated circulating mRNA in serum, plasma and blood cells from patients with disseminated malignant melanoma. Oncol Rep. 2001;8(1):115–118.

Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. Clin Chem. 2002;48(10):1647–1653.

Leidinger P, Backes C, Rheimheimer S, et al. Towards clinical applications of blood-borne miRNA signatures: the influence of the anticoagulant EDTA on miRNA abundance. PloS One. 2015 [cited 2017 May 22];10(11):e0143321. DOI: 10.1371/journal.pone.0143321

Duttagupta R, Jiang R, Gollub J, et al. Impact of cellular miRNAs on circulating miRNA biomarker signatures. PloS one. 2011;6(6):e20769. DOI: 10.1371/journal.pone.0020769