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Droplet digital PCR as a novel detection method for quantifying microRNAs in acute myocardial infarction

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ABSTRACT

Background: micro-RNAs have shown promise as potential biomarkers for acute myocardial infarction and ischemia-reperfusion injury (I/R). Most recently droplet digital polymerase chain reaction (ddPCR) has been introduced as a more reliable and reproducible method for detecting micro-RNAs.
Aims: We aimed to demonstrate the improved technical performance and diagnostic potential of ddPCR by measuring micro-RNAs in ST-elevation myocardial infarction (STEMI).
Methods: A dilution series was performed in duplicate on synthetic *Caenorrhabditis elegans*-miR-39, comparing quantitative real-time PCR (qRT-PCR) and ddPCR. We used ddPCR and qRT-PCR to quantify the serum levels of miR-21, miR-208a and miR-499 between STEMI patients ($n = 24$) and stable coronary artery disease (CAD) patients ($n = 20$). In STEMI, I/R injury was assessed via measurement of ST-segment resolution.
Results: In the dilution series, ddPCR demonstrated superior coefficient of variation (12.1%vs.32.9%) and limit of detection (0.9325 vs.2.425copies/μl). In the patient cohort, ddPCR demonstrated greater differences in miR-21 levels (2190.5 vs. 484.7 copies/μl; $p = 0.0004$ for ddPCR and 136.4 vs. 122.8 copies/μl; $p = 0.2273$ for qRT-PCR) and in miR-208a (0 vs. 24.1 copies/μl, $p = 0.0013$ for ddPCR and 0 vs. 0 copies/μl, $p = 0.0032$ for qRT-PCR), with similar differences observed in miR-499 levels (9.4 vs. 81.5 copies/μl, $p < 0.0001$ for ddPCR and 0 vs. 19.41 copies/μl, $p < 0.0001$ for qRT-PCR). ddPCR also more accurately defined STEMI for all miRNAs (area under the curve (AUC) of 0.8021/0.7740/0.9063 for miR-21/208a/499 with ddPCR vs. AUC of 0.6083/0.6917/0.8417 with qRT-PCR). However, there was no association between miR-21/208a/499 levels and ischemia-reperfusion injury.
Conclusion: ddPCR demonstrates superiority in both technical performance and diagnostic potential compared to qRT-PCR. Ultimately, this supports its use as a diagnostic method for quantifying micro-RNAs, particularly in large multi-center trials.

1. Introduction

Ischemic heart disease is the leading cause of morbidity and mortality worldwide [1]. Early diagnosis and intervention is integral in reducing injury in acute myocardial infarction (AMI). The current gold standard for diagnosis uses high-sensitivity cardiac troponins (hs-cTn), however these markers are not without their limitations. hs-cTn have compromised specificity, with an increased detection of no-ischemic damage [2–4]. Moreover, raised troponins are not entirely AMI specific, and they can be chronically raised in patients with congestive cardiac failure or chronic kidney disease [2–5]. The establishment of novel biomarkers for AMI has therefore become an important focus of medical research. Furthermore, there is a relative paucity of established biomarkers for reperfusion injury. In recent years miRNAs have been identified as promising markers for a number of diseases, including

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AMI [6]. miRNAs are short (~22 nucleotide) non-coding single-stranded RNA molecules involved in the regulation of messenger RNA (mRNA) via inhibitory effects on translation and/or stability, ultimately influencing gene expression [7]. Under physiological or pathological conditions, miRNAs can be released from their cells of origin into the circulation and are thought to act upon cells and tissues at distant sites [8]. miRNAs are attractive candidates for disease biomarkers. They are highly stable in the circulation and measureable in a variety of bodily fluids [7,8]. Moreover, their levels can change significantly in pathological states, and some miRNAs show high tissue and disease specificity [7]. A number of miRNAs have been investigated as valid biomarkers in AMI. Of particular interest for this study are miRNA-21, miRNA-208a and miRNA-499. These miRNAs have been shown to be upregulated in AMI, and miRNA-499 has even shown potential as a biomarker for reperfusion injury [9–15]. Although results are encouraging, a variety of issues have arisen which have limited their use [16]. Foremost amongst these limitations is the current method used to quantify miRNAs, qRT-PCR [17]. ddPCR is a relatively novel method of PCR, which partitions a 20 μl sample into ~20,000 individual droplets [18]. The ddPCR system uses a Poisson statistical analysis of fluorescent signals from positive and negative droplets to allow for absolute quantification [18]. ddPCR has demonstrated a number of advantages over conventional qRT-PCR, which may aid in mitigating the current limitations on using circulating miRNAs as biomarkers. In experiments to date, ddPCR has demonstrated a high degree of linearity and quantitative correlation in measuring miRNAs within its dynamic range, and has shown greater reproducibility and less inter- and intra-assay variability compared to qRT-PCR [18,19]. Through these advantages ddPCR could offer greater day-to-day comparability and reliability and hence greater utility as both a diagnostic method as well as in validating miRNAs in large multi-center clinical trials. The aim of this study was to investigate ddPCR as a novel method of quantifying serum miRNAs and to determine whether its use leads to an improvement in the diagnostic potential of validated miRNAs for AMI and I/R injury.

2. Methods

2.1. Synthetic oligonucleotide dilution series of C. elegans-miR-39

A lyophilised 5′-phosphorylated synthetic oligonucleotide for Caenorhabditis elegans-miR-39 (C. elegans-miR-39) (Integrated DNA Technologies) (Table A.1), with a known starting mass of 7.6 nmol was centrifuged according to the company's protocol and diluted in molecular grade Tris-EDTA (TE) buffer (Thermo Fisher Scientific) to a final concentration of 10 pmol/μl.

A 12-step dilution series using nuclease-free water (Applichem Panreac) from 2500 copies/μl to 0 copies/μl was performed for C.elegans-miR-39 (Table A.2 and Fig. A.1). Each sample was briefly centrifuged for 10 s at 8000g before being diluted into the next sample in the dilution series. Each sample then underwent reverse transcription (RT) using 4.16 μl/well nuclease-free water, 1.50 μl/well RT buffer, 0.15 μl/well 100 nM dNTP, 0.19 μl/well RNAse inhibitor, 1.00 μl/well multi-scribe reverse transcriptase and 3 μl/well specific RT primer (Applied Biosystems, Inc. ID: 000200). For RT reaction 10 μl mastermix and 5 μl sample were combined and spun on a microplate centrifuge at 4 °C for 2 min at 2000g (Thermo Scientific). Samples underwent a 15 μl thermal cycling protocol using the C1000 Touch™ Thermal Cycler (BioRad) at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min and held at 4 °C. The RT product was then further used for qRT-PCR and ddPCR in equal volume.

For qRT-PCR, 7.67 μl/well nuclease-free water, 10 μl/well universal PCR master mix and 1 μl/well of a specific 20× hydrolysis primer/probe (Applied Biosystems, Inc. ID: 000200) were combined, briefly centrifuged and partitioned into 18.67 μl/well with 1.33 μl/well RT product. Each sample was prepared in duplicate. Samples were spun on a microplate centrifuge at 4 °C for 2 min at 2000g (Thermo Scientific). Samples underwent a 20 μl qRT-PCR protocol at 95 °C for 10 min, 95 °C for 15 s and 60 °C for 60 s, before being repeated for 39 more cycles and held at 4 °C. All qRT-PCR data was analyzed using CFX Manager™ (BioRad).

For ddPCR, 7.67 μl/well nuclease-free water, 10 μl/well ddPCR™ supermix for probes (no dUTP) (BioRad) and 1 μl/well 20× specific hydrolysis primer/probe were combined, briefly centrifuged and partitioned into 18.67 μl/well with 1.33 μl/well RT product. 20 μl of ***the*** sample was pipetted into each well in an 8-well cassette with 70 μl droplet generation oil for probes (BioRad) and placed in the QX200™ Droplet Generator (BioRad). 40 μl of the droplet-formed sample was pipetted into separate wells. Each sample was prepared in duplicate. Samples were transferred to the C1000™ Thermal Cycler (BioRad) and underwent thermal cycling set for 40 μl with 2.5 °C/s ramp rate at 95 °C for 10 min, 94 °C for 30 s, ***and*** 60 °C for 60 s, before being repeated for 39 more cycles and thereafter taken to 98 °C for 10 more minutes and held at 12 °C.

The technical performance of ddPCR versus qRT-PCR was compared statistically by a number of methods.

Coefficient of variation was calculated by the equation:

$$CV\% = (\text{Standard deviation}/\text{Mean}) \times 100\%$$

Before calculating the CV% for each diluent in qRT-PCR, the Ct values were converted to absolute copies using Pfaffl analysis [20]:

$$R = E_{target}^{\Delta CPtarget\ (control-sample)}$$

where E_{target} represents the efficiency of each individual qRT-PCR run according to the slope of the line of best fit:

$$E_{target} = -1 + 10^{(-1/slope)}$$

The control for the Pfaffl equation was calculated by calculating the mean of the highest concentration (i.e. 2500 copies/μl), and comparing that to each sample. Each sample could then be converted to absolute copies by the following equation:

$$\text{Absolute copies} = R \times 2500$$

The qRT-PCR data could then be analyzed for CV% and compared with ddPCR. Reduction in CV% was calculated by the equation:

$$\text{Difference in } CV\% = (CV\%_{qRT-PCR} - CV\%_{ddPCR})/(CV\%_{qRTPCR})$$

The lower limit of linear range (LLLR) was determined by runs-testing [21], removing successive dilution points until the p-value was >0.05 (Prism Version 5.0c software). The limit of detection (LOD) was calculated by the following equation [22]:

$$LOD = \langle x \rangle_{blank} + 1.645\sigma_{blank} + 1.645\sigma_{low}$$

where

$\langle x \rangle_{blank}$ = mean of negative controls
σ_{blank} = standard deviation of negative controls
σ_{low} = standard deviation of lowest concentration measurement

The limit of quantification (LOQ) was defined as the lowest concentration tested which still remained above or equal to both the LLLR and the LOD.

Linearity of ddPCR and qRT-PCR for each dilution series was assessed using the r-value for each line of best fit (GraphPad Prism v5.0).

Values of quantification are presented as log transformed for both qRT-PCR and ddPCR measurements in order to display every dilution point.

2.2. Patient samples

2.2.1. Patient population

In this single-center prospective study, peripheral venous blood was obtained from 24 STEMI patients and 20 patients with stable CAD between October 2013 and August 2015. Patients with STEMI as evidenced by ST-elevation >0.1 mV in at least two contiguous leads were included. Major exclusion criteria included LVEF <30% and cardiogenic shock. The final study population consisted of 44 patients. The protocol of this study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional ethical committee of the University Medical Center of Freiburg (CF16/1546-2016000808). Written informed consent was obtained from all patients.

2.2.2. Percutaneous coronary intervention

All STEMI patients received aspirin (minimum of 250 mg) and an ADP receptor blocker (prasugrel 60 mg, ticagrelor 180 mg or clopidogrel 600 mg). Unfractionated heparin (5000 U) was administered prior to angiography. Eptifibatide was given at the discretion of the cardiologist. Peripheral blood was obtained 5 ± SD 2.7 h after PCI. Blood was drawn post-PCI in order to also assess for an association between miRNA levels and ST-resolution post-PCI, indicating clinically relevant I/R-injury.

2.2.3. miRNA extraction

Serum samples underwent miRNA extraction according to the manufacturer's instructions (Qiagen miRNeasy Serum/Plasma Kit, ID: 217184). After the initial Qiazol denaturation step, all samples were spiked with 5 μl of 9.635×10^5 copies/μl *C. elegans*-miR-39 to act as an exogenous control.

2.2.4. Reverse transcription, qRT-PCR and ddPCR

Samples underwent reverse transcription, qRT-PCR and ddPCR according to the above-mentioned protocols, using specific hydrolysis primer/probes for each miRNA analyzed (ThermoFisher, ID: 000397, 000511, 001352). Every sample also underwent RT and qRT-PCR/ddPCR for *C. elegans*-miR-39, performed in duplicate in separate wells to act as an exogenous control. Final ddPCR concentrations for each miRNA were equal to the results