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Research paper

Heparinase treatment of heparin-contaminated plasma from coronary artery bypass grafting patients enables reliable quantification of microRNAs

Kirill Kondratov[a], Dmitry Kurapeev[b], Maxim Popov[b], Marina Sidorova[c,d], Sarkis Minasian[b], Michael Galagudza[b], Anna Kostareva[a,e,f], Anton Fedorov[a,*]

[a] Institute of Molecular Biology and Genetics, Almazov Federal North-West Medical Research Centre, Saint-Petersburg, Russia
[b] Institute of Experimental Medicine, Almazov Federal North-West Medical Research Centre, Saint-Petersburg, Russia
[c] Department of Biotechnical Systems, Saint-Petersburg Electrotechnical University LETI, Saint-Petersburg, Russia
[d] Saint-Petersburg State University, Saint-Petersburg, Russia
[e] Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden
[f] Institute of Translational Medicine, ITMO University, Saint-Petersburg, Russia

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ABSTRACT

Background: microRNAs have recently been identified as powerful biomarkers of human disease. Reliable polymerase chain reaction (PCR)-based quantification of nucleic acids in clinical samples contaminated with polymerase inhibitor heparin requires deheparinization. However, the effects of deheparinization procedure on quantification of nucleic acids remain largely unknown. The aim of this study was to determine whether the deheparinization procedure completely eliminates the inhibition of amplification, while maintaining RNA integrity and technical variability of the measured microRNA levels.
Methods: Heparinized plasma from 9 patients undergoing coronary artery bypass grafting (CABG) and the heparin-free plasma from 58 rats were spiked with a synthetic RNA oligonucleotide and total RNA was extracted. The RNA solutions were then treated with heparinase I to remove contaminating heparin prior to reverse transcription. Levels of synthetic spike-in RNA oligonucleotide, as well as endogenous hsa-miR-1-3p and hsa-miR-208a-3p, were measured using quantitative reverse transcription PCR (RT-qPCR). The amplification efficiency and presence of inhibitors in individual samples were directly determined using calibration curves.
Results: In contrast to RNA samples from rat plasma, RNA samples derived from the CABG patient plasma contained inhibitors, which were completely eliminated by treatment with heparinase. The procedure caused a decrease in the amount of detected RNA; however, the technical variability of the measured targets did not change, allowing for the quantification of circulating endogenous hsa-miR-1-3p and hsa-miR-208a-3p in the plasma of CABG patients.
Conclusions: The heparinase treatment procedure enables utilization of RT-qPCR for reliable microRNA quantification in heparinized plasma.

1. Introduction

Heparin, a commonly used anticoagulant, seriously complicates polymerase chain reaction (PCR)-based analysis of nucleic acids in clinical samples, since it inhibits both reverse transcription (RT) and PCR amplification steps. The routine methods of RNA isolation, including column purification, do not remove heparin from RNA samples [1,2]. Currently, the only effective and universal approach to remove heparin is to treat RNA samples with heparinase [2].

The effects of treating RNA with heparinase for PCR-based studies have been estimated by several approaches that include

Abbreviations: PCR, polymerase chain reaction; CABG, coronary artery bypass grafting; EDTA, ethylenediaminetetraacetic acid; hsa-miR-1-3p, 3p strand of mature *Homo sapiens* microRNA-1; hsa-miR-208a-3p, 3p strand of mature *Homo sapiens* microRNA-208a; RT-qPCR, reverse transcription quantitative real-time PCR; RT, reverse transcription; Cq, quantification cycle; TP, time point; tRNA, transfer RNA; cel-miR-39-3p, 3p strand of mature *Caenorhabditis elegans* microRNA-39; RNase, ribonuclease; RIN, RNA integrity number; qPCR, quantitative real-time PCR.

* Corresponding author at: Institute of Molecular Biology and Genetics, Almazov Federal North-West Medical Research Centre, 197341, Akkuratova st., 2, Saint-Petersburg, Russia.
E-mail address: antonfedorow@gmail.com (A. Fedorov).

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evaluation of the electrophoretic band intensity of the PCR product [3], calculation of the proportion of target-positive samples [4], and monitoring changes in values of quantification cycle (Cq) [5–8]. These approaches are only sufficient to check the suitability of heparinase-treated samples for qualitative analysis of target nucleic acids because they measure reduced inhibition of the RT and PCR assays but do not prove complete elimination of heparin from the sample.

Reverse transcription quantitative PCR (RT-qPCR) is a conventional method for quantitative analysis of absolute and relative levels of target RNAs [9]. However, there are problems regarding the reliability of RT-qPCR analyses of RNA from heparinase-treated samples due to the lack of data on several critical parameters of deheparinized samples. These parameters are as follows: (i) efficiency of enzymatic reactions, which directly influences both accuracy and precision of RT-qPCR-based quantification, (ii) variability of RNA levels after additional pipetting steps, which influence precision, and (iii) changes in RNA integrity, which impede comparison of deheparinized and heparin-free untreated samples, as well as detection of low copy number transcripts. In the present study, we sought to characterize these parameters after heparinase treatment to determine whether the procedure completely removed the heparin, thus ensuring equal efficiencies in downstream enzymatic reactions, and whether the variability of measured RNA levels and integrity of the target RNA was preserved.

In the present study, RNA samples from rat plasma were used as a control to assess variability of the measured Cq in the absence of both inhibition and heparinase treatment. The inhibitory action of heparin in RNA samples from the plasma of coronary artery bypass grafting (CABG) patients collected at different timepoints during surgery was analyzed and the effect of heparinase treatment on the efficiency of enzymatic reactions was determined. RT-qPCR analysis of either heparinase-treated or untreated aliquots of target RNA aqueous solutions allowed us to determine the effect of heparinase treatment on RNA integrity and Cq variability. Finally, we tested whether treatment with heparinase allows for the detection of endogenous high- and low-abundance microRNAs in plasma samples of CABG patients. Hsa-miR-1-3p was selected as one of the most abundance microRNAs in both skeletal and cardiac muscles and hsa-miR-208a-3p was selected as relatively low-abundance cardiospecific microRNA [10]. Both microRNAs are known to be upregulated in the plasma of patients after acute myocardial infarction [11].

2. Materials and methods

2.1. Study cohort and blood sampling

Written informed consent was obtained from all patients undergoing CABG. The study has been approved by the local Ethics Committee and conforms to the principles outlined in the Declaration of Helsinki. Patients included in this study (n = 9) had cardiac troponin I levels less than 0.011 ng/mL before surgery and greater than 1.249 ng/mL within 120 min after aortic declamping. Patients received bolus dose of unfractionated heparin (B. Braun Medical Inc., Melsungen, Germany) approximately 30 min after thoracotomy. Some patients received maintenance dose of heparin during cardiopulmonary bypass. Heparin doses were adjusted to maintain activated clotting time during cardiopulmonary bypass between 300 and 450 s. The average total heparin dose was 312 IU/kg (range 261–450 IU/kg). Whole blood samples were collected in EDTA tubes at the following time points: 40 min before heparin bolus dose administration (time point 1 (TP1)) as well as 20 min (TP2), 140 min (TP3), and 260 min (TP4) after heparin bolus dose administration (Supplementary Fig. 1). Samples at TP1, TP4 and TP2, TP3 were drawn from the peripheral vein and from the coronary sinus, respectively. Clinical data and information on exact heparin doses are provided in the Supplementary Table 1.

2.2. Animals and blood sampling

Fifty eight male Wistar rats (weight 300–350 g) were enrolled in the study. Whole blood was collected in EDTA tubes from the aorta immediately after thoracotomy (control group, n = 10) or 120 min after thoracotomy (ischemia-reperfusion group, n = 48). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996), European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The study protocol was approved by the local Ethics Committee. The details on animal care and surgery procedures used to induce myocardial ischemia-reperfusion are provided in the Supplementary methods.

2.3. Plasma preparation

Human and rat whole blood samples were stored at 4 °C for a maximum of 30 min after collection. Plasma was isolated using double centrifugation at 1600*g* for 10 min at 4 °C. Samples were aliquoted, snap frozed in liquid nitrogen and stored at −80 °C.

2.4. Isolation of total RNA

Total RNA, including small RNA species, was isolated from 200 μL of plasma using 600 μL of TRIzol LS reagent (Life Technologies Co., Carlsbad, USA) that was pre-mixed with 1 μg of *Escherichia coli* tRNA (Sigma-Aldrich Co., Missouri, USA) and 10^8 molecules of the synthetic RNA oligonucleotide synth-cel-miR-39 (Syntol, Moscow, Russia), which is identical to mature *Caenorhabditis elegans* microRNA cel-miR-39-3p (miRBase accession: MIMAT0000010). RNA was isolated according to the manufacturer's recommendations. The RNA pellet was air dried, and dissolved in 10 μL of RNase-free water and stored at −80 °C. The details on RNA integrity analysis are provided in the Supplementary methods and Supplementary Fig. 2.

2.5. Heparin elimination procedure

Heparin was eliminated from RNA isolated from CABG patient plasma samples using the protocol described by Izraeli et al. [2] with some modifications as described in the Supplementary methods. To test the effect of heparinase treatment on Cq variability and RNA integrity, we compared the amplification of synth-cel-miR-39 from heparinase-treated with that of a control synth-cel-miR-39 water solution. The details of these methodologies are provided in the Supplementary methods.

2.6. RT-qPCR quantification

Target RNAs were detected using hydrolysis probe-based TaqMan MicroRNA Assays. Reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies), amplification was performed using TaqMan Universal PCR Master Mix (Life Technologies) according to the manufacturer's recommendations. The details of these assays are provided in the Supplementary.

2.7. Data analysis

The Shapiro-Wilk test was used to check the normality of data. The Student's *t*-test (for normally distributed data and