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Basic Study

Urine Exosome mRNA-based test for monitoring kidney allograft rejection: effects of sample transportation and storage, and interference substances

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Abstract

BACKGROUND

Exosomes are 30-150 nm nanovesicles with sophisticated nucleic acids cargo, actively secreted by all cells within human body, and found in abundance in all body fluids, including urine. These extracellular vesicles have tremendous potential for next generation diagnostics, theoretically enabling noninvasive assessment of organ and tissue function *via* liquid biopsy analysis.

AIM

Recently, feasibility of an exosomal molecular test was demonstrated for post-organ transplant monitoring: analysis of urine-derived exosomal mRNA cargo allowed early detection of kidney allograft rejection. Here, we further studied urine-derived exosomes and their mRNA content as a highly promising diagnostic modality. This included stability studies of urine samples and exosomal mRNA upon transportation from the point of collection to a centralized testing facility, short-term storage of urine at different conditions upon receipt till the point molecular assay is performed, and effects of various potentially interfering substances on the downstream qPCR assay.

METHODS

The urine specimens were stored at various conditions and pre-processed in different ways. Next, samples were passed through the columns to capture all extracellular vesicles, the vesicles were lysed to release their content and the exosomal RNA was purified on the mini-columns, reverse transcription was performed, next pre-amplification, followed by a qPCR analysis for a panel of mRNA markers.

RESULTS

To ensure exosomal RNA integrity, the harvested urine specimens should be shipped refrigerated, by overnight delivery. Urine can next be stored at the test site for up to 1 wk at 4C, and long term should be frozen at -80C. Urine specimens must be centrifuge at low G-force to deplete cells and debris, to ensure consistent top results in downstream molecular assays. All commonly used medications (tacrolimus, cyclosporin A, mycophenolic acid, everolimus, sirolimus, ascomycin, teriflunomide) were tested and confirmed that they do not cause assay interference.

CONCLUSION

mRNA from urine-derived exosomes was shown to be stable across a broad range of conditions and produced accurate results when analyzed *via* qPCR assay for kidney allograft rejection. We identified the most optimal conditions for every step of the process, ensuring pre-analytical sample integrity and robust qPCR results.

INTRODUCTION

There is an urgent need for advanced post-transplant monitoring molecular tests, allowing to rapidly determine allograft rejection at the earliest stage using non-invasively collected patients' samples. At the moment one of the most widely utilized tests is serum creatinine, which is suboptimal [1-2]. The next gen molecular assay, rapidly expanding in laboratories world-wide is a cell free DNA-based test [3-4]. This test analyses percentage of the donor-derived (dd) cfDNA in the blood of organ recipient,

and if the fraction increases over time and crosses a predetermined threshold (typically 1% for kidney transplants)- this indicates organ rejection. Although this test was already commercialized a few years ago, its specificity is suboptimal, as other-than-allograft rejection events can also trigger increase in dd cfDNA percentage. This test does not work well for detection of the early stage organ rejection (TCMR1a). Also, this approach is *minimally* invasive, rather than *non-invasive*- as it requires the collection of a patient's blood (which is an inconvenience for immunosuppressed individuals). Consequently, clinicians still need sensitive and specific biomarkers that overcome all the above listed limitations of cfDNA-based tests for post-transplant monitoring.

Exosomes are recently discovered nanovesicles secreted by all cells within human body and found in abundance in all body fluids. They contain very sophisticated cargo of nucleic acids and proteins, reflecting the content of parental cells [5-9]. Exosomes and other extracellular vesicles have tremendous potential as biomarkers for disease diagnosis, as they theoretically should allow access to the health state of all organs and tissues *via* liquid biopsy analysis, using not only blood, but also urine, saliva, and other non-invasively collected samples [10-13]. The value of urinary exosomes in post-transplant diagnostics was demonstrated in a recent study for early detection of kidney transplant rejection [14]. Notably, urine collections are better suited for active surveillance monitoring compared to frequent blood draws and urinary exosomes exhibit promising clinical utility for detecting early kidney allograft rejection and stratifying rejection etiology.

In this study, we further characterized the aforementioned assay to understand the preanalytical limitations for an eventual migration into the clinical diagnostic domain. An emphasis was placed on the viability of urine as a matrix to facilitate at home collections from post-kidney transplant patients and correlate expected outcomes for the advanced exosome assay. This included the stability of exosomal mRNA in urine upon transportation from the point of collection to a centralized testing facility, the storage of urine samples at different conditions upon receipt until the point molecular assay is performed, and the effect of various potentially interfering substances on the

downstream qPCR assay. mRNA from the urine-derived exosomes was shown to be stable in a broad range of conditions and produced accurate results when analyzed *via* qPCR assay for kidney allograft rejection. We identified the most optimal conditions for every step of the process, ensuring preanalytical sample integrity and robust qPCR results.

MATERIALS AND METHODS

Specimen transportation boxes

The following box was utilized in studies on transportation of urine specimens: Small box cat #56519 (Therapak); exterior dimensions: 11 x 9 x 11 in; inner dimensions: 8 x 6 x 8 in; wall thickness: 1.5 in. Gel packs (12 oz Gel Pack #PP12 (Sonoco)) were frozen overnight at -20C and placed in the boxes, to refrigerate urine specimens. Temperature monitor #40510 (DeltaTrak) was used to monitor temperature inside the box, up to 72 h.

Isolation of Exosomes from the human urine samples

Exosome isolation was performed according to the ExoLution protocol (Exosome Diagnostics, a Bio-Techne brand). In brief, 10 mL urine samples were combined with 2.5 mL of 5xBB buffer in 15 mL tubes. ISS-MHV positive extraction control was added to each sample and loaded onto ExoLution Columns in 50 mL tubes and centrifuged for 5 min at 5000 x g. Flow-through was discarded and columns were washed with 10 mL of buffer UWB and re-centrifuged at 5,000 x g. ExoLution Columns were then transferred to fresh 50 mL tubes and 550 µL of lysis mix was added directly onto the ExoLution Column membrane surface. Columns were then centrifuged for 5 min at 5,000 x g. Flowthrough containing exosomal RNA was collected and transferred to the RNA purification step.

RNA purification

RNA was further purified according to the ExoLution protocol using silica spin columns. Briefly, after a chloroform separation, the aqueous phase was transferred to 1.5 mL reaction tubes containing ethanol and loaded onto silica spin columns. Spin columns were centrifuged for 30 sec at 11,000 x g. The flow-through was discarded and the spin columns were re-assembled onto the next set of fresh 2 mL collection tubes. The previous steps were repeated until the entire sample was loaded onto each column.

Next, RNA Prep buffer was added to the silica spin columns, and columns were centrifuged for 30 sec at 11,000 x g. The flow-through was discarded, and the spin columns were re-assembled on the next set of fresh 2 mL collection tubes. RNA Wash buffer was next added to the spin columns, and columns were centrifuged for 30 sec at 11,000 x g. The flow-through was discarded, and the spin columns were re-assembled on the next set of fresh 2 mL collection tubes. The wash process was then repeated a second time.

The spin columns were centrifuged for 3 min at 16,000 x g to dry the membranes. The 2 mL collection tubes were discarded, and the spin columns were placed in fresh 1.5 mL reaction tubes. 15 μ L of TE buffer was added directly onto the silica membranes. The columns were incubated for 5 minutes. Purified exosomal RNA was finally eluted by column centrifugation for 1 min at 16,000 x g and immediately carried forward to reverse transcription.

Reverse Transcription

Reverse transcription (RT) reaction mix was prepared using SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, cat# 11754-250): 10x SuperScript Enzyme Mix 2 μ L, 5x VILO Reaction Mix 4 μ L, exosomal RNA 14 μ L. Samples were placed into thermocycler and were subject to the following cycling conditions: 25C: 10 min, 42C: 70 min, 85C: 5 min, hold: 4C.

Pre-Amplification

Pre-Amplification was carried out as follows: TaqMan™ PreAmp Master mix (Thermo Fisher Scientific, 4488593): 12.5 uL, Primer mix 0.5 uL, RT reaction samples 12 uL. Samples were placed into thermal cycler and the following cycling parameters were utilized: initial denaturation 95C for 10 min; 14 cycles: (95C for 15 sec, 60C for 4 min); hold: 4C.

qPCR

qPCR analysis was performed in triplicate, under the following conditions: TaqMan™ Fast Universal PCR Master Mix (Thermo Fisher Scientific, cat# 4367846): 10 uL, 20x assay mix: 1 uL ^[11], nuclease-free water: 7 uL, 5x diluted PreAmp sample: 2 uL. qPCR plates were analyzed on QuantStudio 5 (QS5) fast real-time PCR machine with 96-well 0.1 mL Block. Fast cycling conditions were utilized: initial denaturation 95C for 20 sec, 40 cycles: (95C for 1 sec, 60C for 20 sec).

Interference substances: medications commonly prescribed to transplant patients

All pure drug compounds were acquired from Fisher Scientific: Teriflunomide (cat# AC467112500), Cyclosporin A (cat# AAJ6319106), Sirolimus (cat# AAJ62473MF), Everolimus (cat# AAJ60139MB), Mycophenolic acid (cat# AAJ6190509), Tacrolimus (cat# AAJ63571MF), Ascomycin (cat# AAJ66751MC), dissolved in DMSO to produce 5000x stocks, and tested for potential interference with assay at concentrations 50x exceeding expected urinary excretion level transplant patients. Expected levels: Tacrolimus: 0.53 ug/mL; Cyclosporin A: 10.5 ug/mL; Mycophenolic acid: 16.67 ug/mL; Everolimus: 0.03 ug/mL; Sirolimus: 0.10 ug/mL; Ascomycin: 0.47 ug/mL; Teriflunomide: 18.33 ug/mL.

RESULTS

Ideally, active surveillance testing for post-transplant monitoring should be facilitated through an at home non-invasive collection of the patients' specimens. Alternatively, the procedure could be performed with ease in a healthcare provider's office. Regardless of the point of collection, the specimen must be transported to a centralized laboratory, accessioned and temporarily stored prior to analysis.

The objective of this study was to explore stability of urinary exosomes and their diagnostic value with a previously described analytical method [14] for the detection and stratification of kidney allograft rejection. Briefly, in this assay the urine sample is first passed through the column to capture all extracellular vesicles, next the vesicles are lysed to release their content and the exosomal RNA is purified on the mini-column, reverse transcription is performed, next pre-amplification, followed by a qPCR analysis for a panel of mRNA markers. The output Ct values are analyzed by an algorithm that generates the scores that allow determination of rejection for kidney allograft, and type of rejection: TCMR *vs* ABMR. The entire assay takes less than 6h to complete, which enables the laboratory to produce rapid "same day" results.

Taking into account the novelty of urine as a sample of choice for this molecular assay, and also exosomes, particularly their RNA cargo, as markers of organ rejection, it is of high importance to characterize every step in more detail, to ensure subsequent smooth clinical implementation of this test. Here we explored: (1) stability of urine specimens and exosomal mRNA upon transportation from the point of collection to testing facilities, (2) storage of urine samples at different conditions upon receipt till the point molecular assay is performed, and (3) effect of various potentially interfering substances on the downstream qPCR assay.

Stability of exosomal mRNA upon urine transportation at different temperatures, and effects of various urine upfront processing techniques.

Stability of exosomal mRNA upon preanalytical conditions mimicking urine transportation at different temperatures, and effects of various urine upfront processing techniques were studied in detail. Results are shown in Figure 1. Urine specimens were

derived from 4 donors, and processed following the complete workflow, with qPCR assay performed for a panel of 9 mRNA targets. Preanalytical conditions mimicking specimen shipment and urine processing included: 2 days storage at +4C; 2 days at +20C; 2 days at +40C; whole urine frozen and thawed (mimicking transportation in frozen state); urine pre-processed to remove cells and debris by centrifugation for 20 minutes at 2,000 x g, and supernatant subject to single or double freeze/thaw cycles.

Storage of urine samples at elevated temperature, +20C and especially +40C caused gradual degradation of mRNA, as indicated by Ct (threshold cycles) shift that varied among targets and donors (as expected). While for some donors and certain mRNA targets Ct shift was minimal (< 0.5 Ct), for other donors and certain mRNA targets effects were substantial (>5 Ct). Importantly, variable increases in Ct values introduce unpredictable changes to the outputs of algorithms analyzing levels of multiple mRNA targets and as a consequence will decrease accuracy of calculated outcomes. Normalization to PGK1 or other RNA largely addresses this issue. However, overall it is recommended that urine specimens be transported refrigerated, to ensure temperatures are consistently maintained below +20C.

Freezing urine specimens on dry ice and -80C, followed by defrosting, caused mild increase in Ct values compared to unfrozen specimens, presumably due to partial mRNA degradation upon sample thawing; 2 freeze/thaw cycles did not have significant further effects on mRNA integrity.

Specimens which were frozen without initial centrifugation at low G-force to deplete cells and debris generated somewhat variable Ct values for some samples and mRNA targets. This indicates the need for preanalytical centrifugation of urine specimens to ensure removal of interfering cellular RNA fraction (including blood-derived: see discussion below), consistent qPCR results, and assay algorithmic output scores.

In summary, for exosome-based molecular assays, the preanalytical process plays an important role in downstream assay integrity. Urine specimens must be transported under refrigeration, and centrifugation of the samples ensures depletion of cells and debris, and consistent, reproducible results. Effects of urine freezing and prolonged

storage on the accuracy and reproducibility of the assay will be further discussed below.

Next, temperature of the urine specimens was monitored during conditions mimicking transportation, to identify the optimal gel pack volume that would ensure sample refrigeration (Figure 2). One, two, three or four 12 oz gel packs were frozen overnight at -20C and placed in the 1.5-inch thick styrofoam box, that was stored at ambient temperature. Results are shown in Figure 2.

At 24h, which is typical for overnight sample shipment, temperature inside the box with single gel pack (12 oz) was ~14C; two, three or four gel packs successfully maintained temperature below 4C. By 48h, temperature inside the boxes with 1-3 gel packs (12 oz) was >15C; only four gel packs secured temperature of ~8C. By 72h, temperature inside all boxes with 1-4 gel packs (12 oz) was >18C.

Overall, for molecular tests, harvested urine specimens should be shipped by next day overnight delivery, in Styrofoam boxes ≥ 1.5 inch thick with gel packs totaling 24-48 oz, to ensure optimal refrigeration and specimen temperature for 24- 48h.

Stability of exosomal mRNA upon prolonged urine storage at different temperatures.

Stability of exosomal mRNA was next studied upon prolonged urine storage at different temperatures. Results are shown in Figure 3. Urine samples were derived from 4 donors, and processed following the complete workflow, with qPCR assay performed for a panel of 9 mRNA targets. Conditions included: 2 days at +4C; 7 days at +4C; 14 days at +4C; 4 days at -80C; 30 days at -80C.

Storage of urine samples at +4C for up to one week did not affect mRNA integrity and assay outcome; at 2 wk of storage gradual degradation of mRNA is occurring, as indicated by Ct shift that varied for different targets and donor-to-donor (as expected). Thus, unfrozen urine specimens should ideally be tested within 7 days of specimen receipt and refrigerated storage.

Once samples were frozen at -80C, and then defrosted and tested in assay, Ct values typically shift up compared to fresh samples, and for some donors and certain mRNA

targets the change is significant (> 2 Ct). Prolonged sample storage at -80°C does not cause any further Ct increase. Thus, for the purpose of consistency and reproducibility, all urine samples should be either processed unfrozen (within a week, as stated above) or all subject to centrifugation at low G-force, frozen, then defrosted and tested at any time point. As mentioned above, normalization to PGK1 or other RNA, will ensure consistent assay performance.

Effect of interference substances on qPCR assay

First, medications commonly prescribed to transplant patients, were studied for potential interference with exosome molecular assay. Urine samples derived from 4 donors were utilized, and various parent drugs and metabolites were spiked in before exosome purification, at concentrations exceeding 50-fold the expected urinary excretion levels for transplant patients. Expected urine levels for immunosuppressants and other common medications were: Tacrolimus: 0.53 ug/mL; Cyclosporin A: 10.5 ug/mL; Mycophenolic acid: 16.67 ug/mL; Everolimus: 0.03 ug/mL; Sirolimus: 0.10 ug/mL; Ascomycin: 0.47 ug/mL; Teriflunomide: 18.33 ug/mL.

Samples were processed following the complete workflow, and qPCR assay was performed for a panel of 9 mRNA targets. Results are displayed in Figure 4. None of the drugs caused major interference with assay at the dose significantly exceeding the typical circulating levels in bodily fluids, as indicated by Ct values within ± 1 compared to controls. This demonstrates that molecular assay is robust, and all common medications are depleted from the mRNA analytes during the exosome and RNA purification workflow, and the trace amounts remaining do not have negative impact on reverse transcription or real-time PCR.

Next, effect of trace amount of blood in urine was studied. Hematuria is a fairly common condition, and blood was expected to impact the performance of exosome molecular assay. Urine samples derived from 4 donors were utilized, and blood (0-1%) was spiked into urine before exosome purification. Results are shown in Figure 5.

Blood in urine did not inhibit mRNA target detection but rather added its own “signal” (originating from blood-derived cellular mRNA) to that of the urinary exosomes. For all mRNA analytes, Ct values significantly decrease indicating several fold increase in mRNA levels due to blood-derived mRNA co-purifying with exosomal RNA. However, once the urine specimens are centrifuged (which is an obligatory part of the exosome assay, as was described above), the bulk of blood cells are successfully depleted and Ct values generated are very similar to normal urine specimens. Spiking in serum instead of blood confirmed the results - assay interference is coming from blood-derived components that can be successfully removed by centrifugation.

In case blood cells are lysed, the contents obviously cannot be easily spotted or removed from urine; however such RNA is rapidly degrading in urine that contains high levels of RNases, and thus interference with assays should be minimal – if any.

Overall, centrifugation of the urine samples ensures depletion of blood-derived cells and large debris, and consistent, reproducible results. As a precaution, urine samples with visible hematuria should be excluded for this molecular test.

DISCUSSION

see above - Results and Discussion, combined

CONCLUSION

To conclude, we characterized molecular assay utilizing urine-derived exosomes. This included stability of urine samples upon transportation from the point of collection to a centralized testing facility, storage of urine at different conditions upon receipt till the point molecular assay is performed, upfront processing, and effect of various interference substances on the downstream qPCR assay.

mRNA from urine-derived exosomes was shown to be stable across a broad range of conditions and produced accurate results when analyzed *via* qPCR assay for kidney allograft rejection. We identified the most optimal conditions for every step of the process, ensuring preanalytical sample integrity and robust qPCR results.

ARTICLE HIGHLIGHTS

Research background

Exosomes are nano-sized extracellular vesicles with nucleic acid and protein cargo, actively secreted by all cells within human body, and found in abundance in all body fluids, including urine. These extracellular vesicles have tremendous potential for next generation diagnostics, theoretically enabling noninvasive assessment of organ and tissue function *via* liquid biopsy analysis.

Research motivation

Recently, feasibility of an exosomal molecular test was demonstrated for post-organ transplant monitoring: analysis of urine-derived exosomal mRNA cargo allowed early detection of kidney allograft rejection. Taking into account the novelty of this approach, urine and in particular extracellular vesicles with their sophisticated RNA cargo have to be better characterized to ensure robustness of this molecular assay.

Research objectives

We further studied urine-derived exosomes and their mRNA content as a highly promising diagnostic modality. This included stability studies of urine samples and exosomal mRNA upon transportation from the point of collection to a centralized testing facility, short-term storage of urine at different conditions upon receipt till the point molecular assay is performed, and effects of various potentially interfering substances on the downstream qPCR assay.

Research methods

The urine specimens were stored at various temperatures and conditions and pre-processed in different ways. Next, samples were passed through the columns to capture all extracellular vesicles, the vesicles were lysed to release their content and the

exosomal RNA was purified on the mini-columns, reverse transcription was performed, next pre-amplification, followed by a qPCR analysis for a panel of mRNA markers.

Research results

To ensure exosomal RNA integrity, the harvested urine specimens should be shipped refrigerated, by overnight delivery. Urine can next be stored at the test site for up to 1 wk at 4C, and long term should be frozen at -80C. Urine specimens must be centrifuge at low G-force to deplete cells and debris, to ensure consistent top results in downstream molecular assays. All commonly used medications (tacrolimus, cyclosporin A, mycophenolic acid, everolimus, sirolimus, ascomycin, teriflunomide) were tested and confirmed that they do not cause assay interference.

Research conclusions

mRNA from the urine-derived exosomes was proven to be stable across a broad range of conditions and produce robust results in molecular post-transplant monitoring assays. We identified optimal conditions for every step of the workflow, ensuring pre-analytical sample integrity and robust downstream qPCR results.

Research perspectives

Exosomes and in particular their mRNA cargo have the potential to revolutionize post-transplant monitoring, and detect early rejection events for kidney as well as other allografts- based on molecular analysis of urine, saliva and other body fluids

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