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A method for extracting and characterizing RNA from urine: For downstream PCR and RNAseq analysis

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ABSTRACT

Readily accessible samples such as urine or blood are seemingly ideal for differentiating and stratifying patients; however, it has proven a daunting task to identify reliable biomarkers in such samples. Noncoding RNA holds great promise as a source of biomarkers distinguishing physiologic wellbeing or illness. Current methods to isolate and characterize RNA molecules in urine are limited. In this proof of concept study, we present a method to extract and identify small noncoding RNAs in urine. Initially, quantitative reverse transcription PCR was applied to confirm the presence of microRNAs in total RNA extracted from urine. Once the presence of micro RNA in urine was confirmed, we developed a method to scale up RNA extraction to provide adequate amounts of RNA for next generation sequence analysis. The method described in this study is applicable to detecting a broad range of small noncoding RNAs in urine; thus, they have wide applicability for health and disease analyses.

Introduction

Physiologically representative and accessible samples such as saliva, blood or urine have long been expected to provide a source of biomarkers with high potential for characterizing conditions of health and disease. These types of samples are referred to as liquid biopsies and may harbor circulating cells, protein, DNA, and RNA biomarkers [1]. RNA is one component within these samples that was initially ignored due to its propensity for rapid degradation by ribonucleases (RNases). However, with the identification of microRNAs and their notable stability in physiologic samples, RNA has come to the forefront of readily accessible molecules for the discovery of novel biomarkers [2–4].

Noncoding RNAs (ncRNAs) found in liquid biopsies include, but are not limited to, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), tRNA fragments (tRFs) and microRNAs. The wide variety of functions of extracellular ncRNAs are currently under investigation. For example, the tRFs, although having the unfortunate name of tRNA fragments, are actively processed from mature tRNAs and their function is beginning to be elucidated [5]. While the microRNAs are already known to function in most if not all biological processes. Circulating microRNAs as serum biomarkers of health and disease have been robustly explored [6–8]; however, the microRNA repertoire in urine is less studied. The majority of the early studies of RNA in urine were focused on prostate, bladder and kidney disease because these tissues would directly contribute extracellular vesicles (EVs) to urine [9,10]. In many of the studies of EVs in urine, they are referred to as exosomes due to their size of approximately 40–100 nm. The term EV refers to any extracellular vesicle including but not limited to the microvesicles, ectosomes and exosomes [11]. The misconception that extracellular RNAs (exRNAs) must be associated with EVs persists despite recent studies that show exRNAs may be associated with protein and lipid

Abbreviations used: ncRNA, noncoding RNA; exRNA, extracellular RNA; EVs, extracellular vesicles; tRNA, transfer RNA; tRF, tRNA fragment; rRNA, ribosomal RNA; qRT-PCR, quantitative RT-PCR; NGS, next generation sequencing; miRNA, microRNA; RNases, ribonucleases.

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complexes independent of vesicles [12–14]. These findings suggest that exRNA in urine may provide useful biomarkers of physiological relevance to many diseases not limited to those involving urologic disease [15].

Urine based biomarkers would be ideal for many studies because of the accessible nature of urine. Urine is readily collected in many animal models, as well as in the veterinary setting and for human health assessment. A urine based RNA biomarker for ovarian cancer would be particularly useful due to the inaccessible nature of the ovaries and the ability of this cancer to metastasize with few symptoms [16–18]. Studies of microRNAs in ovarian cancer have delineated a set of differentially expressed microRNAs that are expected to regulate key tumor suppressors such as BRCA1 [19]. Such oncomirs would be expected to be over expressed in biological fluids from cancer patients and have been studied in tissue, serum, ascites fluid and urine from ovarian cancer patients [20–24]. microRNAs associated with extracellular vesicles (EVs) are also found in urine from healthy volunteers [25,26]. It is possible to isolate EVs from large amounts of urine by a combined method of filtration and ultracentrifugation [25,26]; however, a rapid method to isolate total RNA from small amounts of urine is needed. In this article, we present a proof of concept study of the isolation of total RNA from urine allowing either quantitative reverse transcription-PCR (qRT-PCR) or next generation sequencing (NGS).

Materials and methods

Participants and sample collection

The Gynecologic Tissue and Fluid Bank (GTFB), at the University of Colorado Anschutz Medical Campus, collected urine from women undergoing gynecologic surgery. Urine was obtained from women with ovarian cancer under an IRB approved protocol (COMIRB Protocol 11-0626). Samples were provided as 1 ml aliquots of urine with information on patient age, stage and histology of the ovarian cancer. Urine samples were collected during surgery and centrifuged prior to freezing at −80 °C for long term storage. All samples were de-identified and data was analyzed under a second IRB approved protocol at the University of Minnesota (study number: 1610E97724).

RNA extraction and quantitative RT-PCR analysis of microRNA

Urine was defrosted once and aliquoted in 100 μl aliquots and refrozen following the addition of 700 μl of Qiazol Reagent (Qiagen, Valencia, CA). Total RNA was isolated from 100 μl of urine using the miRNeasy Mini Kit (Qiagen). The isolation procedure followed the miRNeasy protocol with a few clarifications explained in more detail in Supplemental Table 1. The final elution volume for total RNA from the spin column was the minimum required, 30 μl RNase free water, to allow the maximum concentration of RNA per μl for downstream applications. The concentration and quality of extracted RNA were assessed by spectrophotometry on the NanoDrop 1000 (Thermo Scientific, Waltham, MA). However, the concentration of RNA obtained is much lower than the expected accuracy for the NanoDrop 1000; thus, in the following steps, we ignored concentration estimates and simply used the maximum template volume allowed in the protocol. For example, in the Miscript II kit (Qiagen) for a 20 μl reaction it is possible to reverse transcribe a volume of 12 μls of total RNA for cDNA preparation. Amplifiable RNA extraction and cDNA preparation were confirmed by a positive result for qRT-PCR of a small RNA compared to the cDNA water control. qRT-PCR was conducted using the miScript SYBR Green reagent (Qiagen) with a custom primer for miR-29a-3p 5′-cccTAGCACCATCTGAAATCGGTTA or miR-146a-5p 5′-ggT GAG AAC TGA ATT CCA TGG GTT. We also used the RNU6B primer available from Qiagen for qRT-PCR assays (RNU6B_13). Although in this study, we did not use an internal control for calibration of qRT-PCR results, it is important to mention that an appropriate internal control for exRNA studies is currently controversial. Several helpful studies have examined this controversy and may be of use to those working in this field [27–29].

RNA precipitation, RNA quality assessment and Illumina Mi-seq methods

Urine is a complex liquid containing EVs, protein, nucleic acids and many other metabolites [30]. Tamm-Horsfell is a protein in urine that is known to form networks that trap EVs [31]. In the study presented here we extracted RNA from six 100 μl aliquots of urine and then combined these in the following step to reduce the concentration of contaminating substances such as Tamm-Horsfell protein. Total RNA from 600 μl of urine (in 100 μl aliquots) was extracted using the above protocol. The 30 μl aliquots of purified RNA were combined into 1 tube for a total of 180 μls. This was extracted with addition of GenElute LPA as described by the manufacturer (Sigma-Aldrich, St. Louis, MO) as previously described [32]. The use of LPA as a carrier is required because alternatives such as glycogen or yeast tRNA are isolated from biological sources and maybe contaminated with small ncRNA [32]. After LPA addition, the samples were extracted with low pH phenol (Ambion 9710) and chloroform:isoamyl alcohol (49:1) with a standard ethanol precipitation using 3 M NaAcetate pH 5.2 (protocol included, in Supplemental Table 1).

Library preparation and Illumina sequencing analysis

RNA was forwarded to the UCD Genomics and Microarray Core for library construction. In the core facilities, RNA was assessed for quality on the Agilent Bioanalyzer 2100 using the Eukaryote Total RNA Pico Chip (Agilent Technol., Palo Alto, CA). RNA libraries were constructed using a volume of 5 μls of total RNA rather than the recommended concentration of RNA need to prepare the Illumina HiSeq libraries. The TruSeq Small RNA kit uses a 3′ adapter modified to target microRNAs and other small RNAs that have a 3′ hydroxyl group. Enriching for RNA with a 3'hydroxyl allows the detection of RNA that has been enzymatically cleaved by Dicer or other RNA processing enzymes. Small RNA template libraries were sequenced using NGS technology on the Illumina HiSeq2000 platform at the University of Colorado's Genomics and Sequencing Core Facility.

miR-seq data analysis

The microRNA sequence reads were identified for known and novel microRNA sequences using the program miRDeep. We calculated the expression of the microRNA variants based on normalized read counts and tested for significant differences using ANOVA in R. Galaxy cutadapt was used for each fastq file to remove adapters. Then fastq files were imported into CLC Genomics workbench to identify and count unique small RNAs using two databases as reference (miRBase Release 19 and *Homo sapiens* GRCh37.57 ncrna). The individual data files of trimmed reads have been uploaded to the publicly available data base at https://doi.org/10.5281/zenodo.801484.

Additional data sets were searched from publicly available data at NCBI on the SRA website [33].

Results

In order to identify the repertoire of ncRNAs in urine samples,