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Integrated Kidney Exosome Analysis for the Detection of Kidney Transplant Rejection

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Abstract

Kidney transplant patients require life-long surveillance to detect allograft rejection. Repeated biopsy, albeit the clinical gold standard, is an invasive procedure with the risk of complications and comparatively high cost. Conversely, serum creatinine or urinary proteins are noninvasive alternatives but are late markers with low specificity. We report a urine-based platform to detect kidney transplant rejection. Termed iKEA (integrated kidney exosome analysis), the approach detects extracellular vesicles (EVs) released by immune cells into urine; we reasoned that T cells, attacking kidney allografts, would shed EVs, which in turn can be used as a surrogate marker for inflammation. We optimized iKEA to detect T-cell-derived EVs and implemented a portable sensing system. When applied to clinical urine samples, iKEA revealed high level of CD3-positive

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b05083. Tables S1 and S2 and Figures S1–S3 (PDF)

Notes

The authors declare no competing financial interest.

EVs in kidney rejection patients and achieved high detection accuracy (91.1%). Fast, noninvasive, and cost-effective, iKEA could offer new opportunities in managing transplant recipients, perhaps even in a home setting.

Graphical Abstract

Keywords

biosensor; urine exosomes; acute cellular rejection; kidney transplant; proteomics

Kidney transplantation is the preferred and most successful therapy for end-stage renal disease. Nonetheless, long-term allograft survival remains suboptimal, as transplant recipients eventually develop acute or chronic rejection.¹ While biopsy remains the gold standard for diagnosis of renal graft rejection,^{2,3} it has many drawbacks, including inherent procedural risk, complications, and comparatively high costs. In addition, histopathological analyses are often confounded by interobserver variability and sampling error.^{3,4} As a noninvasive alternative, serum creatinine (SCr) and urinary protein excretion are used, but they lack sensitivity and specificity for allograft rejection.⁵ An increase in SCr is a late marker of kidney injury and often fails to reflect the early inflammatory processes revealed by histopathology. The push for accurate noninvasive biomarkers to achieve sensitive, frequent, and cost-effective monitoring of disease activity seeks to decrease early and late clinical rejection episodes.⁶

Extracellular vesicles (EVs), including exosomes and micro-vesicles, are actively secreted by cells into biofluids.⁷ EVs carry molecular constituents of cells, including transmembrane and intracellular proteins and nucleic acids.^{8–10} A growing number of studies have shown that (i) EVs can reflect parental cells, and (ii) with their abundance and structural stability, these vesicles can be used as a surrogate biomarker.^{11–13} EVs have been extensively studied for cancer detection;^{14,15} they often reflect global tumor burden and heterogeneity, overcoming sampling biases.¹⁶ Moreover, the amount and molecular profiles of cancer-derived EVs highly correlate with tumor burden and treatment efficacy.^{17,18}

We reasoned that EVs within urine can be used to monitor kidney transplant rejection. During acute cellular rejection (ACR) of kidney allografts, T cells infiltrate the kidney tubules and in close proximity to forming urine; this increases the chance of T-cell-derived EVs entering urine (Figure 1a). Capturing T-cell-specific EVs can thus serve as a surrogate biomarker for ACR. Here, we report an EV-based diagnostic platform for kidney rejection. Termed iKEA (integrated kidney exosome assay), it detects urine EVs (uEVs) from T cell lymphocytes. We adapted a magneto-electrochemical strategy for EV detection:¹⁹ T-cell-specific EVs were immuno-magnetically enriched and detected *via* electrochemistry. Combining magnetic enrichment and enzymatic signal amplification, the iKEA was highly

sensitive ($\sim 10^4$ EVs) and fast (2 h), and the detection system could be implemented as a portable device. Applying iKEA to cell culture and clinical urine samples, we found that CD3 is a potent marker to detect T-cell-derived uEVs. In further clinical tests, the CD3-based iKEA achieved the diagnostic accuracy of 91.1% in a discovery cohort (30 patients) and 83.7% in a validation cohort (14 patients). iKEA is an effective tool to perform noninvasive, serial monitoring, offering clinicians with actionable knowledge to improve patient outcomes and reduce complications in renal transplantation.

RESULTS AND DISCUSSION

Integrated Kidney Exosome Analysis.

Kidney histology of an ACR patient shows a distinct pattern of tubulitis, wherein lymphocytes attack tubular cells (Figure 1b). Presence of T cells in tubules increases the chance of T-cell-derived EVs entering urine, which could be used as a surrogate marker for ACR. Indeed, uEVs from a rejection patient were stained positive for CD3 (Figure 1c).

We devised the iKEA assay adopting magnetic selection with electrochemical detection.¹⁹ The assay first enriches target EVs via immunomagnetic capture. Collected EVs are then labeled with an oxidizing enzyme (horseradish peroxidase, HRP) through a second antibody. Mixing beads with a chromogenic electron mediator (3,3',5,5'-tetramethylbenzidine, TMB) generates electrical currents measured by an electrode (Figure 2a). Using magnetic beads affords several advantages: (i) large surface area of beads enhances the enrichment efficiency; (ii) assay steps are simplified with magnetic manipulation (e.g., washing); and (iii) beads can be magnetically concentrated on top of the electrode to enhance the analytical signal.

Portable Detection System.

To facilitate uEV analysis, we implemented a compact iKEA device for electrochemical readout (Figure 2b). The device contained a custom-designed potentiostat (Figure 2c and Figure S1) that measures electrical current between working (W) and counter (C) electrodes. A constant potential was applied between working and reference (R) electrodes during the measurement. The device operated standalone and communicated with external devices for data logging (Bluetooth or USB). A small magnet was placed under the electrode location to concentrate magnetic beads. When benchmarked against a benchtop system (SP-200, Bio-Logic), the iKEA device offered comparable performance (Figure 2d) despite being much cheaper ($< \$100$) and smaller ($6.4 \times 7.0 \times 3.8 \text{ cm}^3$) in size.

Assay Optimization.

We optimized iKEA to detect T-cell-derived EVs. As a model cell line, we used Jurkat T cells and collected cell-derived EVs from conditioned culture media (see Methods for details). We first measured levels of tetraspanins (CD63, CD9, CD81) in EVs. These proteins are reportedly enriched in EVs and thereby can be used as a target for EV identification. Magnetic beads (diameter = $2.7 \mu\text{m}$) specific to each marker were prepared and mixed with EV samples. Captured EVs were further labeled with a second antibody against the target marker (see Methods). We observed the highest signal with CD63

detection (Figure 3a); we thus chose CD63 for EV identification. We also tuned assay parameters (*e.g.*, bead size and concentration) to maximize analytical signal (Figure S2).

We next screened T cell EVs for lymphocyte-specific proteins (CD3, CD45, CD68, CD2, HLA-ABC, CD52). For each marker (M), we prepared magnetic beads conjugated with target-specific antibodies. Control beads were conjugated with isotype-matched antibodies. EVs were mixed with magnetic beads and labeled by antibodies against CD63. Electrical currents from targeted (I_M) and control (I_C) were measured, and the net difference I_M ($I_M - I_C$) was obtained as expression levels of each marker protein. EV samples from Jurkat cells were aliquoted, and measured by iKEA and ELISA. Both methods showed high correlation, which confirmed iKEA's quantitative analytical capacity (Figure 3b). Titration measurements further established the detection limit and dynamic range (Figure 3c); the limit of detection was $\sim 1.6 \times 10^4$ EVs with a dynamic range spanning 4 orders of magnitude. The iKEA outperformed ELISA in many aspects, including assay time (2 vs 5 h), required sample amounts (10^5 vs 10^7 EVs), and dynamic range (10^4 vs 10^1).

iKEA with Clinical Urine Samples.

We applied iKEA to detect T-cell-derived EVs in urine samples of kidney transplant patients. As candidate T cell markers, we chose CD3, CD45, CD2, HLA-ABC, and CD52, whose levels were high in EVs (Figure 3b). uEVs were first captured on magnetic beads using T cell marker antibodies and subsequently labeled with HRP-conjugated CD63 antibodies for signal generation. Our initial screening with banked samples revealed that CD3 was the most potent marker to differentiate transplant rejection status (Figure 4a).

We next conducted a clinical feasibility study. Urine samples (second void of the day) were collected from kidney transplant patients undergoing kidney biopsy for clinical indications; rejection was suspected based on high serum creatinine levels. We used 30 patient urine samples as a discovery set. Fifteen patients had ACR, while the rest did not. The rejection status was confirmed by tissue biopsy using Banff classification (Table S1). Figure 4b shows iKEA data on these samples. Overall, we observed significantly higher level of CD3+ EVs ($p = 0.0008$; unpaired two-tailed t-test) among patients undergoing cellular rejection (Figure 4c). We also observed very low CD3+ EV levels in BKV (BK virus) nephropathy ($n = 3$) and chronic antibody-mediated rejection patients ($n = 5$), which confirmed the specificity of iKEA to the acute cellular rejection.

We further performed receiver operation characteristic (ROC) analyses to determine diagnostic statistics (Figure 4d). We used the patient cohorts with ACR ($n = 15$) and non-ACR ($n = 15$). The cutoff value ($I_c = 0.298 \mu A$) for classifying rejection/nonrejection status was obtained from the ROC curve (see Methods) that maximized both detection sensitivity and specificity. Applying this cutoff, detection sensitivity was 0.928, specificity 0.875, and the accuracy was 0.900 (Table 1). To validate the developed iKEA test, we analyzed an additional 14 patient samples (Figure 4e and Figure S3). The validation set comprised 7 patients with acute rejection and 7 patients without rejection, all confirmed by biopsy. The area under the ROC curve was 0.837 with a detection accuracy of 0.714.

CONCLUSIONS

We explored urinary EV analyses as a noninvasive tool for ACR detection. During kidney allograft cellular rejection, T cells infiltrate kidney tubules in close proximity to forming urine. We hypothesized that EVs shed from T cells can be found in urine during cellular rejection, serving as surrogate noninvasive biomarkers. In urine samples of kidney transplant recipients, we detected EVs that showed T-cell-specific markers. Interestingly, CD3 showed highest differential expression among graft rejection patients compared to those without signs of rejection on biopsy. This observation agreed with previous work, which showed that activated human T cells from peripheral blood release CD3-bearing EVs.²⁰ Furthermore, based on ROC analysis, iKEA's high sensitivity could position its use as a screening tool during longitudinal follow up of transplant patients and potentially increase rates of early detection of ACR.

The developed iKEA platform enables fast, streamlined urine EV analyses. A key feature is the integration of EV isolation and detection into a single platform: magnetic beads are used for EV capture and labeling, and bead-bound EVs are detected through electrochemical sensing. This strategy offers practical advantages: (i) the assay achieves high detection sensitivity, by combining the merits of magnetic enrichment with enzymatic amplification; (ii) through the electrical detection scheme, sensors can easily be miniaturized and expanded for parallel measurements. The resulting iKEA was superior to conventional methods (*e.g.*, ELISA, Western blotting) in speed and sensitivity.

In the future, we envision further improving certain aspects of this study. First, a more efficient method of preprocessing urine samples could be developed. Directly using urine for iKEA detection produces high background signal, presumably due to the presence of a large amount of organic molecules (*e.g.*, creatinine, urea, uric acid).^{21–24} EVs were thus isolated in a first step via ultracentrifugation in this feasibility study. Other portable devices for point-of-care EV isolation could be employed,^{25,26} once validated for high-volume urine processing. Second, the study will likely need to be expanded to larger prospective patient cohorts. Our initial results showed high detection rates of CD3+ exosomes with a distinct predilection for ACR patients. Using a larger sample size and including subclinical rejection subgroups will further help elucidate the specificity of the assay and validate its sensitivity and detection rate. Third, adding a genomic signature (*e.g.*, mRNA, miRNA) to iKEA in future studies may potentially increase the sensitivity and specificity of the test. With such improvements, iKEA will be a powerful clinical tool for noninvasive ACR detection, guiding optimized immunosuppressive therapies.

METHODS

Cell Culture.

Jurkat T cells were grown in RPMI-1640 medium (Cellgro). All media were supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (Cellgro). All cell lines were tested and were free of mycoplasma contamination (MycoAlert mycoplasma detection kit, Lonza, LT07–418).

EV Isolation from Cell Culture.

We used a conventional method to harvest EVs from cell culture media. Cells at passages 1–15 were cultured in vesicle-depleted medium (with 5% depleted FBS) for 48 h. Conditioned medium from 10^7 cells was collected and centrifuged at 300g for 5 min. Supernatant was filtered through a $0.2\ \mu\text{m}$ membrane filter (Millipore) and concentrated by 100000g for 1 h. After the supernatant was removed, the EV pellet was washed with PBS and centrifuged at 100000g for 1 h. The EV pellet was resuspended in PBS.

Preparation of Magnetic Beads.

Five milligrams of magnetic beads coated with epoxy groups (Dynabeads M-270 Epoxy, Invitrogen) was suspended in $100\ \mu\text{L}$ of 0.1 M sodium phosphate solution. One hundred micrograms of antibodies against CD63 (Ansell) or CD3 (Biolegend) was added and mixed thoroughly. One hundred microliters of 3 M ammonium sulfate solution was added, and the whole mixture was incubated at room temperature for 2 h and followed by overnight incubation at $4\ ^\circ\text{C}$ with slow tilt rotation. The beads were washed twice with PBS solution and finally resuspended in 2 mL of PBS with 1% bovine serum albumin (BSA).

Biotinylation of Labeling Antibodies.

Sulfo-NHS-biotin (10 mM, Pierce) solution in PBS was incubated with antibodies for 2 h at room temperature. Unreacted sulfo-NHS-biotin was removed using a Zeba spin desalting column, 7K MWCO (Thermo Scientific). Antibodies were kept at $4\ ^\circ\text{C}$ until use.

iKEA Detection Device.

The device consists of five components: (i) a microcontroller (ATmega328, Microchip), (ii) a digital-to-analog converter (DAC8552, Texas Instruments), (iii) a potentiostat, (iv) an analog-to-digital converter (ADC161S626, Texas Instruments), and (v) a bluetooth communication module (Bluefruit EZ-Link, Adafruit). The potentiostat consists of two operational amplifiers (AD8606, Analog Devices) and a low-pass filter (a parallel circuit of a capacitor and a resistor), which formed a transimpedance amplifier.

iKEA Procedure.

Ten microliters of EV solution was mixed with $50\ \mu\text{L}$ of the immunomagnetic bead solution for 30 min at room temperature. The bead concentration was $10^8/\text{mL}$ as previously reported.¹⁹ The magnetic beads were separated from the solution with a permanent magnet and resuspended in $80\ \mu\text{L}$ of PBS (1% BSA). After 5 s of vortexing, the beads were separated and resuspended in $80\ \mu\text{L}$ of PBS (1% BSA). Ten microliters of antibodies of interest ($20\ \mu\text{g}/\text{mL}$ in PBS) was mixed with the beads for 30 min at room temperature. The magnetic beads were separated and washed as described before, and they were resuspended in $50\ \mu\text{L}$ of PBS (1% BSA). Five microliters of streptavidin-conjugated HRP enzymes (1:100 diluted in PBS) was mixed with the beads for 15 min at room temperature. The magnetic beads were separated and washed as described before, and they were resuspended in $7\ \mu\text{L}$ of PBS. The prepared bead solution and $20\ \mu\text{L}$ of UltraTMB solution (Thermo-Fisher Scientific) were loaded on top of the screen-printed electrode. After 3 min, chronoamperometry

measurement was started with the electrochemical sensor. The current levels in the range of 40–45 s were averaged.

Clinical Samples.

The study was approved by the Institutional Review Board at Brigham and Women's Hospital (PI: Azzi), and the procedures followed were in accordance with institutional guidelines. Informed consent was obtained from all subjects (see Tables S1 and S2, $n = 44$). Urine samples were collected from renal transplant patients undergoing kidney biopsy for clinical indications. In the discovery set, the number of patients was 30 of whom 15 had ACR confirmed by biopsy using Banff classification; 15/30 had no rejection. In the validation set, the number of patients came up to 14 of whom 7 were undergoing ACR and 7 without rejection. Urine samples (~15 mL) were centrifuged at 400g for 10 min at room temperature. The supernatant was further spun at 2000g for 10 min at room temperature. The resulting supernatant was filtered with a 0.8 μm syringe filter. The filtrate was ultracentrifuged at 100000g at 4 °C for 70 min. The EV pellet was washed with PBS and ultracentrifuged at 100000g at 4 °C for 70 min. EV pellets were resuspended with PBS and kept at –80 °C until use.

Enzyme-Linked Immunosorbent Assay.

CD63 and IgG1 antibodies (Ancell) were diluted to 5 $\mu\text{g}/\text{mL}$ in PBS and added to the Maxisorp 96-well plate (Nunc), respectively, for overnight incubation at 4 °C. After being washed with PBS, a blocking solution with 2% BSA in PBS was added to the plate and incubated for 1 h at room temperature. Subsequently, $\sim 10^8$ exosomes in 100 μL of PBS were added to each well for 1 h incubation at room temperature. After the blocking solution was removed, antibodies (1 $\mu\text{g}/\text{mL}$) against various markers were added to each well and incubated at room temperature for 1 h. Unbound antibodies were triple washed with PBS. Streptavidin–horseradish peroxidase molecules were added to the each well for 1 h at room temperature. After being washed out with PBS, chemiluminescence signals were measured.

Immunogold Labeling of EVs for TEM.

Ten microliter aliquots of undiluted exosome suspensions were placed onto Formvar-coated nickel mesh grids and allowed to adsorb for 15 min. Grids were transferred onto drops of primary antibody (CD3, mouse monoclonal, Biolegend 300302) diluted 1:25 in DAKO antibody diluent and allowed to incubate at least 1 h at room temperature. Grids were then rinsed on drops of PBS and incubated in an appropriate secondary gold conjugate (goat anti-mouse 10 nm IgG, Ted Pella/BBI #15751) at least 1 h at room temperature. Preparations were then rinsed on drops of distilled water and contrast stained for 10 min in droplets of chilled tylose/uranyl acetate and air-dried prior to being examined on a JEOL JEM 1011 transmission electron microscope at 80 kV. Images were collected using an AMT digital camera and imaging system with proprietary image capture software (Advanced Microscopy Techniques, Danvers, MA).

Statistical Analysis.

Receiver operation characteristic curves were generated from patient iKEA data. The optimal cutoff value for CD3 was obtained from the validation set by determining the point closest to the top-left corner (perfect sensitivity or specificity) of the ROC curve. All diagnostic metrics (*i.e.*, sensitivity, specificity, accuracy) were calculated using standard formulas. We used the R package (version 3.3) for ROC curve analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

T-cell-derived EVs in urine samples from patients under kidney transplant rejection. (a) Schematics showing how T-cell-derived EVs can be present in patient urine samples. During acute cellular rejection (ACR), T cells infiltrate into kidney and can secrete their EVs into the tubule. Released EVs are then collected in urine. (b) Histology of a kidney tissue from a ACR patient. The image shows multiple tubules where tubular cells are marked with an asterisk. The tubule in the middle has lymphocytes (arrows) causing tubulitis. (c) Electron micrograph of a urinary EV (uEV) from an ACR patient. uEVs were immunogold stained for CD3. Scale bar, 50 nm. White arrows indicate CD3 immunogold staining.

Figure 2.

Integrated kidney exosome analysis. (a) Assay procedure. EVs were immunomagnetically captured and further labeled with antibodies for signal generation (electrical current) through enzymatic reaction. HRP, horseradish peroxidase; TMB, tetramethylbenzidine. (b) Portable iKEA detector. The device was designed for point-of-use operations with a small form of factor ($6.4 \times 7.0 \times 3.8 \text{ cm}^3$). Measured data were wirelessly transferred to Bluetooth-ready devices. Scale bar, 2 cm. (c) Device housed a custom-designed potentiostat, an analog-to-digital converter (ADC), a digital-to-analog converter (DAC), and a microcontroller unit (MCU). Electrical currents from working (W) to counter (C) electrodes were measured with a constant potential applied between working and reference (R) electrodes. (d) Comparison between benchtop equipment and the portable iKEA system. Serial dilution of $\text{K}_4\text{Fe}(\text{CN})_6$ in 0.1 KCl solution was measured. A linear correlation between two devices was observed.

Figure 3. iKEA optimization for T-cell-derived EV detection. (a) EV identification marker selection. iKEA measured expression levels of three EV tetraspanins. CD63 generated the highest signal. (b) Protein profiling of Jurkat EVs were performed with ELISA and iKEA. Results from both measurements showed good agreement. Highly expressed markers were selected as candidate uEV targets. (c) Titration curve of T-cell-derived EVs detection by iKEA. CD3 was selected as a target. EVs were captured with CD3 antibody magnetic beads and then detected with CD63 antibody. Data are shown as mean $\pm$ SD from duplicate measurements. (Right) Scanning electron micrograph of EVs captured by CD3 antibody-functionalized magnetic beads. Scale bars, 300 μm and 200 nm (inset).

Figure 4.

iKEA of urinary EVs in clinical samples. (a) EV protein levels in rejection and nonrejection patients. CD3 was selected for rejection patient diagnosis. (b) Waterfall plot of CD3+ EVs in 30 patient urine samples. CD3 expression of EVs was measured with iKEA assay. (c) Scatter plot showed higher CD3 expression level of EVs in acute cellular rejection patients than other patient groups; * $p = 0.02$; ** $p = 0.006$; *** $p = 0.0008$. (d) Receiver operation characteristic (ROC) curve was used to determine the sensitivity, specificity, and accuracy of CD3 marker. AUC was 0.911. (e) Validation sets with additional 14 patients. The ROC curve has the AUC of 0.837.

Table 1.**Patient Samples Analysis Results^a**

	training set (<i>n</i> = 30)^b	validation set (<i>n</i> = 14)^b
sensitivity	92.8%	63.6%
specificity	87.5%	100%
accuracy	90.0%	71.4%
AUC	0.911 ± 0.054	0.837 ± 0.109

^aPatient samples of training and validation sets were analyzed by iKEA. Statistical analysis showed sensitivity, specificity, accuracy, and AUC.

^bFor each set, the ratio between nonrejected and rejected patients is 1:1.