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A Rapid Noninvasive Assay for the Detection of Renal Transplant Injury

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Abstract

Background—The copy number of donor-derived cell-free DNA (dd-cfDNA) in blood correlates with acute rejection (AR) in heart transplantation. We analyzed urinary dd-cfDNA as a surrogate marker of kidney transplant injury.

Methods—Sixty-three biopsy-matched urine samples (41 stable and 22 allograft injury) were analyzed from female recipients of male donors for chromosome Y (donor)–specific dd-cfDNA. All biopsies were semiquantitatively scored by a single pathologist. Standard statistical measures of correlation and significance were used.

Results—There was baseline scatter for urinary dd-cfDNA/μg urine creatinine across different patients, even at the time of stable graft (STA) function (undetected to 12.26 copies). The mean urinary dd-cfDNA in AR (20.5±13.9) was significantly greater compared with STA (2.4±3.3; $P<0.0001$) or those with chronic allograft injury (CAI; 2.4±2.4; $P=0.001$) but no different from BK virus nephropathy (BKVN; 20.3±15.7; $P=0.98$). In AR and BKVN, the intrapatient drift was highly significant versus STA or CAI patients (10.3±7.4 in AR; 12.3±8.4 in BKVN vs. −0.5±3.5 in STA and 2.3±2.6 in CAI; $P<0.05$). Urinary dd-cfDNA correlated with protein/creatinine ratio ($r=0.48$; $P<0.014$) and calculated glomerular filtration rate ($r=−0.52$; $P<0.007$) but was most sensitive for acute allograft injury (area under the curve=0.80; $P<0.0006$; 95% confidence interval, 0.67–0.93).

Conclusion—Urinary dd-cfDNA after renal transplantation has patient specific thresholds, reflecting the apoptotic injury load of the donor organ. Serial monitoring of urinary dd-cfDNA can be a surrogate sensitive biomarker of acute injury in the donor organ but lacks the specificity to distinguish between AR and BKVN injury.

Keywords

Kidney transplantation; Biomarkers; Urinary cell-free DNA; Noninvasive biomarker; Allograft injury

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Sensitive noninvasive diagnostic tools for early detection of transplant injury remain an unmet clinical need in solid organ transplantation, as the only reliable method of detection of specific causes of allograft injury is the invasive tissue biopsy. This method uses histopathology to describe and define the type and degree of injury present in the renal architecture (1). Although the tissue biopsy is the current "gold standard," it is not optimal in explaining mixed pathology (multiple diseases/injuries present), basis upon irreversible changes (fibrosis and sclerosis), and its descriptive nature that is hard to attribute to causation (2). One of the initial challenges that faces the transplant community is to detect that there is injury to the donor organ, in its early stages, by a noninvasive assay, which could become a longterm model of allograft health monitoring. We and others have reported potential mRNA and protein biomarkers in the blood and urine that could serve as surrogate biomarker for detection of transplant injury (3–7). In this context, a urine-based biomarker would provide noninvasive means to monitor the renal graft more frequently without any morbidity to the patient. In this study, we performed digital polymerase chain reaction (dPCR) measurements to assess if donor-derived cell-free DNA (dd-cfDNA) in the urine of kidney transplant patients could be diagnostic noninvasive biomarker for donor organ health, by detecting any injury in the organ, and to assess if it could be used to ascertain acute or chronic injury or for a specific causality of the injury mechanism. We report in this study that there is a potential utility of measuring dd-cfDNA to monitor nonspecific renal transplant injury, but because this screening assay cannot determine the tempo or cause of this injury, it could provide a trigger for increased transplant health surveillance in gender-mismatched transplant patients receiving a male donor kidney.

RESULTS

Patients and Samples

The patients included in this study were on a combination of tacrolimus, mycophenolic acid, and with or without steroids. As described earlier, all recipients were female engrafted with male donor kidneys, of which 80% were from living sources. All donors had engraftment biopsies, which showed pristine histology as exhibited by an implantation Remuzzi score of 0, and no delayed graft function (8). The mean recipient age was 10.8±4.5 years and the mean donor age was 24.2±7.9 years, with a mean cold ischemia time of 494±480 min. All recipients were of low immunologic risk with a peak panel-reactive antibody level of 20% or more and a mean human leukocyte antigen mismatch of 4.8±1.4. There were no intervening clinical infections during the course of the study sampling. None of the chronic allograft injury (CAI) samples presented with concurrent tubulitis or inflammation. The BK virus nephropathy (BKVN) samples all appeared to be stage A with minimal tubular atrophy and presented with concurrent inflammation. All rejections were cellular in nature. Details of the Banff and chronic allograft damage index (CADI) pathology for each phenotype group studied are presented in Table 1.

Detection of dd-cfDNA with dPCR

There was wide intrapatient variation of copy number of chromosome Y (ChrY) in the urine, even in all 41 samples of stable graft (STA) phenotypes with a range of 0 to 12.26 copies/μg urine creatinine (mean±SD=2.67±3.69), suggesting that patient-specific levels of urinary ChrY dd-cfDNA should be evaluated. There was no correlation of higher urine ChrY dd-cfDNA with donor age, length of cold ischemia time, or donor source. At the time of any graft injury event, acute rejection (AR) or CAI or BKVN, there was an increase in ChrY copy number; the mean ChrY copy number increased to a mean of 13.10±13.95/μg urine creatinine (range, 0.14–47.00) and this increase for the allograft injury group overall was significantly higher than the STA group copy numbers (P=0.001).

There was baseline scatter for urinary ChrY dd-cfDNA/μg urine creatinine across different patients, even at the time of STA function (undetected to 12.26 copies). Even with the scatter, the increase in ChrY dd-cfDNA was significantly increased in the case of acute injury (AR and BKVN) when compared with nonacute injury (STA and CAI) (Fig. 1). The mean urinary ChrY dd-cfDNA in AR (20.5±13.9) was significantly greater compared with STA (2.4±3.3; P<0.0001) or those with CAI (2.4±2.4; P=0.001) but no different from BKVN (20.3±15.7; P=0.98) (Fig. 2A). Urinary dd-cfDNA correlated with protein/creatinine ratio (r=0.48; P<0.014) and calculated glomerular filtration rate (r=−0.52; P<0.007) but was most sensitive for acute allograft injury (area under the curve=0.80; P<0.0006; 95% confidence interval, 0.67–0.93). Within the allograft injury, sample reads an absolute value of three or more copies of urine ChrY/μg creatinine, segregated injury from STA samples with an area under the curve of 0.80, sensitivity of 81%, and specificity of 75%. Using logistic regression model to evaluate ChrY and the ability of serum creatinine to associate with a binary phenotype of injury versus no injury, we observed that ChrY is more likely associated with the outcome more than serum creatinine (P=0.08 vs. P=0.37). However, due to the small sample number, this comparison would need to be re-evaluated in a larger cohort.

The overall number of urine ChrY overall positively correlated with protein/creatinine ratio (r=0.48; P<0.014), which is another biomarker of nonspecific renal injury. Additionally, the urine ChrY copy number also negatively correlated with calculated glomerular filtration rate (r=−0.52; P<0.007) (9). There was a correlation with the severity of injury as indicated by the Banff (1, 10) grade of AR (Banff i: r=0.81; P<0.05 and Banff t: r=0.71; P<0.05); however, there were no correlations with other grades of Banff injury scores of the CADI (11) grade. Given the intrapatient variability, we evaluated within each patient the delta change in urine ChrY copy numbers in serial samples (two to three per patient) from STA patients and in patients with multiple samples collected before and at the time of biopsy-confirmed graft injury. In AR and BKVN, the intrapatient drift was highly significant versus STA or CAI patients (10.27±7.44 in AR; 12.27±8.36 in BKVN vs. −0.48±3.47 in STA and 2.28±2.57 in CAI; P=0.045) (Fig. 2B).

DISCUSSION

The reliance on a drift of the serum creatinine in renal transplantation as an indicator of advanced graft injury and the utilization of this marker as the trigger for a diagnostic renal

biopsy result in a late diagnosis of AR, with resultant significant injury to the organ, which is often irreversible. For these very reasons, a rapid and noninvasive way of renal graft monitoring would be a welcome entity in the field of kidney transplantation. Presence of donor-derived cells in the circulation is believed to be a result of microchimerism. Microchimerism is a phenomenon that is reported to be present in different tissues of transplant recipients including the graft and in body fluids. Because the graft is the "event site" for any transplant-related injury, PCR-based assessment of circulating cfDNA in the urine could be valuable to evaluate for renal transplant injury after transplantation.

Due to the unique absence of ChrY in the recipient in this combination of recipient–donor pairing, sequencing of donor DNA in the urine is not necessary to establish donor organ specificity of the dd-cfDNA detected in the urine. All of the ChrY detected in the urine reflects tissue breakdown and injury in the donor organ itself. Detection of donor-specific ChrY in the urine in this study was found to be an adjunct biomarker of acute, nonspecific injury in the donor organ. Earlier reports have previously demonstrated the utility of assessment of dd-cfDNA in adult transplant recipients (12, 13) and this measurement to be correlative with graft rejection, although this study did not evaluate the confounder of infection (13). A follow-up study by the same group assessed total cell-free DNA and dd-cfDNA in plasma and urine samples of renal transplant patients and found that total cfDNA in the plasma was a useful marker of AR but cfDNA from urine was not similarly correlative with AR. In this study, we have demonstrated that measuring the copy number of urinary dd-cfDNA can reflect nonspecific acute inflammatory injury in the donor kidney and likely reflects the increased burden of tissue injury and apoptosis that is filtered in the urine.

A limitation of the approach of measuring ChrY dd-cfDNA is that it cannot be applied to all renal allograft recipients due to the precise sex mismatch pairing required. In situations where this type of gender mismatch does not exist (i.e., female/female, male/male, or female/male pairings), sequencing of donor DNA will be required, as shown previously in a study of dd-cfDNA in the sera of heart transplant patients (14). Nevertheless, given the current high cost of sequencing, expanding the use of this approach from just ChrY detection to donor-specific DNA identification by sequencing and detection by customized urine dd-cfDNA assays for each transplant patient, may be too expensive an assay to move into commercialization at the present time. However, the cost of next-generation sequencing is constantly reducing and this will become available within 5 years, with this study providing the initial proof that dd-cfDNA monitoring is possible and specific for detection of renal injury. Larger sample numbers will be needed to further interrogate if urine dd-cfDNA load can allow for differentiation of other types of allograft injury, such as acute tubular necrosis or sepsis, as this method is likely measuring the overall apoptotic load of the allograft.

In conclusion, the potential of cfDNA in the diagnosis and assessment of different diseases including transplant injury is of clinical importance, but further work needs to be done on controlling for the confounding factors of infection and graft injury from other causes apart from AR, such as viral nephritis, chronic graft injury, and drug toxicity, where similar tissue apoptosis may be at play. In transplantation, the identity of cfDNA has to be established from the donor to make this type of monitoring valuable for graft injury and thus requires

the additional step of sequencing, which adds cost to what is otherwise a very simple and cheap PCR-based assay. Nevertheless, because evaluation of biopsy for organ transplantation is invasive, blood and biofluid RNA, microRNA, protein, antibody, and cfDNA are all valuable biomarkers that can be used as adjunct tools in the clinical decision-making process of managing a transplant recipient.

MATERIALS AND METHODS

Study Population and Samples

This study consisted of urine samples matched with biopsy from pediatric and young adult recipients of an adult kidney transplant from 2004 to 2006 at Lucile Packard Children's Hospital at Stanford. Patients included in the study were chosen from a biorepository of 2016 banked urine samples, of which 770 were biopsy matched. The study patients were selected to only include those patients that had more than two banked urine samples during the study period, which were matched with an allograft biopsy, with at least one urine sample each from the pool of surveillance (urine and biopsy samples banked per protocol at 0, 3, 6, 12, and 24 months after transplantation) and indication (urine and biopsy banked at the time of clinical allograft dysfunction) time points for each patient selected. The final samples selection for the study included 63 biopsy-matched urine samples collected from 21 female recipients from male donors such that ChrY (donor)–specific dd-cfDNA could be assessed in these gender-mismatched pairs as a measure of either nonspecific allograft injury or associated with a specific cause of allograft injury (such as AR, chronic rejection, or BKVN). "Allograft injury" in this study was defined as a greater than 20% increase in serum creatinine from its previous steady-state baseline value and an associated biopsy that was pathologic. The histologic diagnoses spectra for the "allograft injury" cases (n=22) were AR (n=8), chronic injury (n=10), or BKVN (n=4). There were 41 samples collected at the time of STA function and normal histology on the paired biopsy and were called "stable". All biopsies were blindly semiquantitatively scored by a single pathologist using the most recent Banff criteria for both acute and chronic injury (1, 10, 15, 16). AR was defined at minimum, as per Banff schema, a tubulitis score 1 accompanied with an interstitial inflammation score 1. CAI was defined at minimum as tubular atrophy score 1 accompanied by an interstitial fibrosis score 1. Normal (STA) allografts were defined by an absence of significant injury pathology as defined by Banff schema. The study was approved by the Ethics Committee of Stanford University Medical School and California Pacific Medical Center Research Institute, and all patients/guardians provided informed consent to participate in the research, in full adherence to the Declaration of Helsinki.

Sample Collection and Processing

Urine samples (50–100 mL) were collected, midstream, in sterile containers and then centrifuged at $2000 \times g$ for 20 min at room temperature within 1 hr of collection. The supernatant was separated from the urine pellet containing cells and cell debris. The pH of the supernatant was adjusted to 7.0 using Tris-HCl and stored at −80°C until further analysis. Urine creatinine was measured using Quantichrom Creatinine Assay Kit (DICT-500) (BioAssay Systems, Hayward, CA). Total protein was measured for each urine sample using Coomassie Plus Bradford Assay Kit (Thermo Scientific, Rockford, IL). DNA

from 5 mL urine was extracted, which was then used in dPCR. Copy number of ChrY in dd-cfDNA was calculated and normalized against urine creatinine. Correlations were performed using a Pearson correlation coefficient, with r 0.45, and $P<0.05$ was considered significant. Data-specific thresholds were generated to set a diagnostic threshold for allograft injury by dd-cfDNA measurements in urine.

Cell-Free DNA Extraction and Quantification

dd-cfDNA from 5 mL urine was obtained using the QIAmp Circulating Nucleic Acids Kit (Qiagen, Valencia, CA). As described in the manufacturer's protocol, these samples were first treated with proteinase K (supplied) to degrade cellular debris and remove DNase and RNase. Samples were then buffered and RNA carrier was added to assist in precipitation of dd-cfDNA. This lysate was run through a DNA binding column and then washed multiple times with buffers (supplied) and 100% ethanol, with the bound DNA eluted using 50 μL buffer (supplied). This DNA containing eluent was used for DNA quantification and dPCR. Eluent (1 μL) was used to quantify the double-stranded dd-cfDNA using the Quant-iT Pico Green Kit (Invitrogen, Carlsbad, CA) in a 1:100 dilution with $1\times$ TE buffer (supplied), as described in the manufacturer's protocol. This was combined with an equal volume of a 1:200 dilution of Quant-iT Reagent in each well (black microtiter plate) with the resulting fluorescence read with a spectrofluorometer (Gemini EM; Molecular Devices, Sunnyvale, CA). The concentration (ng/mL) for each sample was calculated by comparison with a 10-fold standard curve (lambda DNA, supplied).

Cell-Free DNA Quantification Using Quantitative dPCR

dPCR was performed using 5 ng extracted dd-cfDNA from urine on 12.765 digital array chips with the Biomark real-time PCR system (Fluidigm, South San Francisco, CA). dd-cfDNA was quantified using primers and labeled probes (IDT, Coralville, IA) derived for each locus of single-copy SRY ChrY locus (forward primer: 5′-CGCTTAACATAGCAGAAGCA and reverse primer: 5′-AGTTTCGAACTCTCTGGCACCT; probe: 5′-TGTCGCACTCTCCTTGTTTTT GACA) using Taqman Universal Master Mix (ABI, Carlsbad, CA) as outlined in the manufacturer's protocol (Fluidigm) and as described previously (14).The number of copies of each locus was calculated by the manufacturer's software Digital PCR Analysis software version 3.0 (Fluidigm), which was corrected to reflect copies per milliliter of sample extracted and used for all analyses. The copy number of ChrY was $\log_2$ transformed and normalized against urine creatinine.

Statistical Analysis

Nominal clinical variables were analyzed using either an unpaired two-tailed Student's *t* test (parametric) or Mann–Whitney test (nonparametric). Categorical clinical and histopathology data (Banff score 1) were analyzed using Fisher's exact test with two-tailed *P* value. The intrapatient delta change of ChrY dd-cfDNA was calculated by taking the average of the ChrY copy number at normal events and subtracting this from the ChrY copy number at the time of event. Correlations were performed using a Pearson correlation coefficient, with r 0.3, and $P<0.05$ was considered significant. Receiver operating characteristic curves and

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logistic regression were used to model variables against a binary phenotype of injury versus noninjury. A probability of less than 0.05 was considered significant for all statistical analyses, which were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA). All values are expressed as mean±SD unless specified.

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REFERENCES

1. Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. Kidney Int. 1999; 55:713. [PubMed: 9987096]

2. Rush D. Can protocol biopsy better inform our choices in renal transplantation? Transplant Proc. 2009; 41:S6. [PubMed: 19651297]

3. Li L, Khatri P, Sigdel TK, et al. A peripheral blood diagnostic test for acute rejection in renal transplantation. Am J Transplant. 2012; 12:2710. [PubMed: 23009139]

4. Sarwal M, Chua MS, Kambham N, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med. 2003; 349:125. [PubMed: 12853585]

5. Sigdel T, Ling B, Lau K, et al. Integrative urinary peptidomics in renal transplantation identifies novel biomarkers for acute rejection. Am J Transplant. 2010; 10:60.

6. Sigdel T, Kaushal A, Gritsenko M, et al. Novel shotgun proteomics approach identifies proteins specific for acute renal transplant rejection. Am J Transplant. 2010; 10:289.

7. Nakorchevsky A, Hewel JA, Kurian SM, et al. Molecular mechanisms of chronic kidney transplant rejection via large-scale proteogenomic analysis of tissue biopsies. J Am Soc Nephrol. 2010; 21:362. [PubMed: 20093355]

8. Remuzzi G, Grinyo J, Ruggenenti P, et al. Early experience with dual kidney transplantation in adults using expanded donor criteria. Double Kidney Transplant Group (DKG). J Am Soc Nephrol. 1999; 10:2591. [PubMed: 10589699]

9. Schwartz GJ,Munoz A, Schneider MF, et al. New equations to estimate GFR in children with CKD. J Am Soc Nephrol. 2009; 20:629. [PubMed: 19158356]

10. Solez K, Colvin RB, Racusen LC, et al. Banff 07 classification of renal allograft pathology: updates and future directions. Am J Transplant. 2008; 8:753. [PubMed: 18294345]

11. Isoniemi H, Taskinen E, Hayry P. Histological chronic allograft damage index accurately predicts chronic renal allograft rejection. Transplantation. 1994; 58:1195. [PubMed: 7992362]

12. Zhang J, Tong KL, Li PK, et al. Presence of donor- and recipientderived DNA in cell-free urine samples of renal transplantation recipients: urinary DNA chimerism. Clin Chem. 1999; 45:1741. [PubMed: 10508119]

13. Garcia Moreira V, Prieto Garcia B, Baltar Martin JM, et al. Cell-free DNA as a noninvasive acute rejection marker in renal transplantation. Clin Chem. 2009; 55:1958. [PubMed: 19729469]

14. Snyder TM, Khush KK, Valantine HA, et al. Universal noninvasive detection of solid organ transplant rejection. Proc Natl Acad Sci U S A. 2011; 108:6229. [PubMed: 21444804]

15. Racusen LC, Halloran PF, Solez K. Banff 2003 meeting report: new diagnostic insights and standards. Am J Transplant. 2004; 4:1562. [PubMed: 15367210]

16. Solez K, Colvin RB, Racusen LC, et al. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant. 2007; 7:518. [PubMed: 17352710]
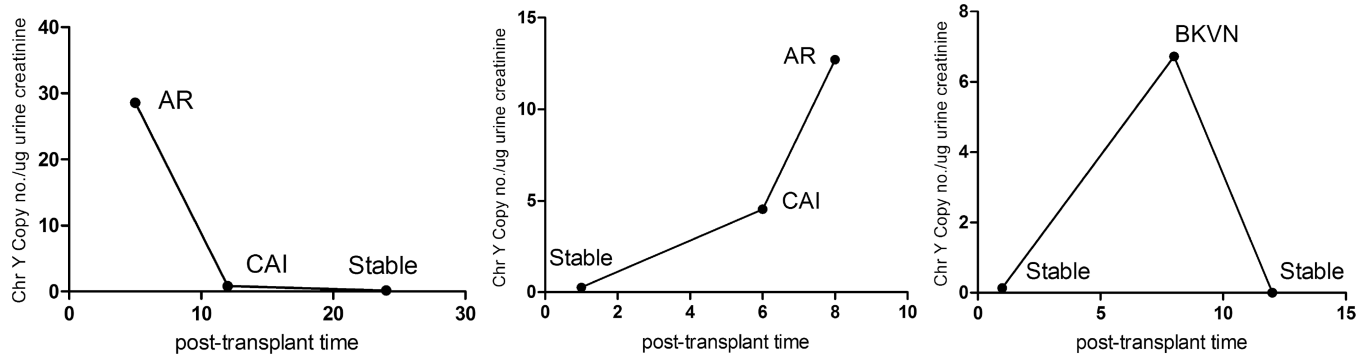
FIGURE 1.
Change in the copy number of urinary ChrY dd-cfDNA over time after transplantation is presented for three representative patients. The copy number of dd-cfDNA increases at time of injury from baseline as defined by dd-cfDNA copy number at a stable allograft tissue biopsy.
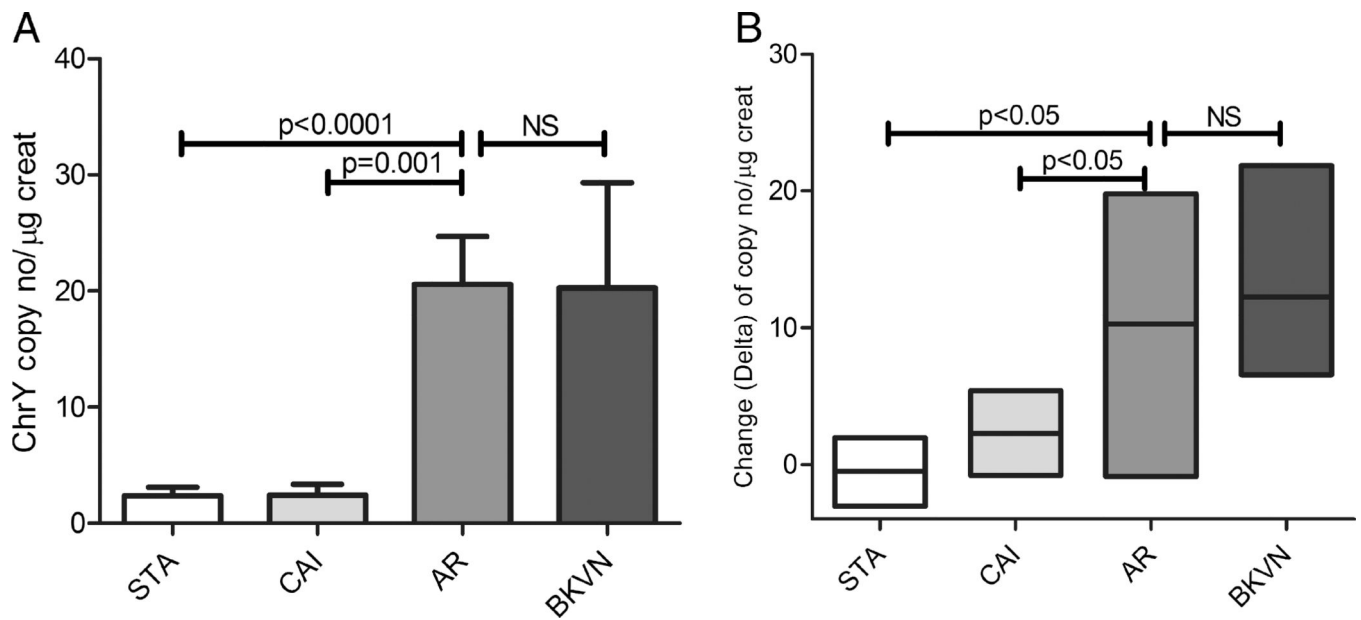
FIGURE 2.
ChrY dd-cfDNA can be a surrogate sensitive biomarker of acute injury in the donor organ. A, mean urinary ChrY dd-cfDNA in AR (20.5±13.9) was significantly greater compared with STA (2.4±3.3) or those with CAI (2.4±2.4) but no different from BKVN (20.3±15.7). In BKVN, mean levels were also statistically greater than those seen in both STA and CAI ($P<0.01$). Bar graphs present mean±SEM. B, in AR and BKVN, the intrapatient drift was highly significant vs. STA or CAI patients (10.3±7.4 in AR; 12.3±8.4 in BKVN vs. −0.5±3.5 in STA and 2.3±2.6 in CAI). This finding was also significant for BKVN when compared with either STA or CAI categories ($P<0.05$). Box plots show mean, minimum, and maximum values.

TABLE 1

Detailed Banff and CADI pathology scores for each phenotype investigated (mean±SD)

Phenotype	Banff ci	Banff ct	Banff i	Banff t	CADI
AR	0.4±0.9	0.4±0.5	1.6±1.5	1.6±1.1	3.2±2.9
CAI	1.3±1.0	1.5±0.8	0±0	0±0	3.8±2.7
BKVN	0±0	1±0	1±1.4	1.5±2.1	2±1.4
Stable/normal	0.2±0.4	0.3±0.5	0±0	0±0	0.6±1.0