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Using proteomics to identify preprocedural risk factors for contrast induced nephropathy

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Abstract

Contrast induced nephropathy (CIN) is the third leading cause of hospital acquired acute kidney injury (AKI). We conducted a cross-sectional study in children undergoing elective cardiac catheterization to determine if there is a distinct preprocedural urinary proteomic profile in subjects who subsequently develop CIN. Of 90 patients enrolled, AKI due to CIN (defined as a 50% or greater increase in serum creatinine) occurred in 10 participants by the 24 h postcontrast time point. Seven patients who did not develop AKI served as age and gender matched controls. SELDI-TOF-MS was performed using Protein Chips with different chromatographic surfaces. A 4480 Da biomarker displayed significantly greater peak intensities on three chromatographic surfaces ($p = 0.02$ – 0.001) in control patients at time = 0 with an area under the curve (AUC) of 0.89–0.99. This biomarker was identified as the 41 amino acid (a.a.) variant of human beta-defensin-1. Another biomarker of 4631 Da was found to have a significantly greater peak intensity ($p = 0.03$) in AKI patients at time = 0, with an AUC of 0.84. Thus, the presence of a 4631 Da peptide, as well as the absence of the 41 a.a. variant of human beta-defensin-1 in the pre-procedural urine, may prove to be useful biomarkers for the early prediction of CIN.

Keywords

Acute kidney injury; Acute renal failure; Beta-defensin-1; Biomarker; SELDI

1 Introduction

The unfortunate consequence of the rise in use of radio-contrast agents in imaging techniques and cardiac interventions is the concomitant increase in the incidence of hospital acquired acute kidney injury (AKI), formerly known as acute renal failure. Contrast induced nephropathy (CIN) is the third leading cause of hospital acquired AKI, accounting for 10–15% of AKI in hospitalized patients [1–4]. CIN is defined as any worsening of renal function after contrast administration, usually occurs within 1–7 days, and is typically marked by a 25–50% increase in serum creatinine. While AKI is generally reversible in this population, it is associated with

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numerous adverse consequences including increased length of hospital stay, dialysis requirement, major cardiovascular events, and an increased mortality rates for 6 months to 5 years after contrast administration [3,5].

Numerous risk factors for CIN have been identified and include diabetes mellitus, hypertension, congestive heart failure, and dehydration [3,6,7]. The most effective predictive measure of CIN is preprocedural kidney function. Utilizing serum creatinine levels to estimate the glomerular filtration rate can be used to determine kidney function in individuals with chronic kidney disease, but is not routinely used as a screening tool prior to contrast administration due to practicalities with the relatively low incidence of CIN in the unselected population [3,5]. Also, serum creatinine-based methods for estimation of glomerular filtration rate are not accurate in detecting acute changes in renal function and are unreliable in certain populations, including children [8].

The pathophysiology of CIN likely involves changes in renal hemodynamics that can lead to injury as well as a direct nephrotoxicity of the contrast agents themselves [9]. There is also some evidence that CIN may result in part from oxygen free radicals and cytokine production [10]. In patients identified as at risk for CIN, physicians may opt for alternate, less toxic, contrast agents such as CO₂, but these agents generate poor image quality [11]. Additional strategies that have demonstrated some limited effectiveness include prophylactic hydration with sodium bicarbonate and the use of *N*-acetyl cysteine prior to the procedure [3,5,12].

More sensitive biomarkers that could be detected through a simple urine test would make it more feasible to screen all patients undergoing these procedures to determine their risk for CIN, and allow for the commencement of prophylactic treatments and closer follow up in the days following contrast administration. Advances in clinical proteomics have greatly accelerated the discovery of novel biomarkers in renal disease [13–17]. One of the preferred methods for urine biomarker discovery, especially in response to AKI, is SELDI-TOP-MS [18,19]. In this pilot study, we used SELDI-TOP-MS to analyze urine from children undergoing contrast administration, to identify pre-procedural biomarkers associated with AKI.

2 Materials and methods

2.1 Patients

This study was approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center. All children and adolescents with congenital heart diseases who were undergoing elective cardiac catheterization with contrast administration and angiography at our institution were prospectively recruited between 2004 and 2006. Written informed consent was obtained from the legal guardian before enrollment. Exclusion criteria included pre-existing renal insufficiency, diabetes mellitus, and concomitant nephrotoxic drug use. None of the subjects were in overt congestive heart failure, and none showed any symptoms or signs of a urinary tract infection at the time of the procedure. We therefore studied a homogeneous population of pediatric patients with no major confounding variables, in whom the only obvious renal insult would be the result of contrast administration.

2.2 Study design

All subjects were maintained in a euvolemic state with intravenous fluid administration. All subjects received iover-sol injection (Optiray® 350), in doses adjusted for body weight and determined by the number and location of cardiovascular angiograms. Serial urine and blood samples were obtained at baseline (preprocedural sample), and at 2, 6, and 24 h after contrast administration. Samples were centrifuged at 2000 × *g* for 5 min and the supernatants stored at

– 80°C. Creatinine levels were measured in each serum sample using a quantitative colorimetric assay kit (Sigma, St. Louis, MO).

2.3 Urine proteomics

SELDI-TOF MS and the ProteinChip Biomarker System (PBS-IIc, BioRad Laboratories, Hercules, CA, USA) were used as described previously [16,17]. Three types of ProteinChips with different chromatographic surfaces were pre-equilibrated in the following buffers: weak cation exchange (carboxymethyl; CM10), 0.1 M sodium acetate, pH 4.0; hydrophobic/RP (H50), 10% ACN, 0.1% TFA; metal-affinity (IMAC30) arrays charged with copper sulfate, 0.1 M sodium phosphate, 0.5 M sodium chloride, pH 7.0. Equal volumes (20 µL) of urine were diluted 1:5 in a chip-specific buffer and incubated on spot for 30 min. Spots were washed with the chip specific buffer and distilled water. Bound proteins were cocrystallized on spot with 50% saturated sinapinic acid (SPA) (2×1 µL) prepared in 50% ACN, 0.5% TFA. Low mass spectra (0–20 000 Da) were obtained with a laser intensity of 185 for CM 10 and IMAC30 chips and 190 for H50 chips. High mass spectra (10000–200000 Da) were obtained with laser intensities of 195, 200, and 210 for IMAC30, CM10, and H50 chips, respectively. Resulting spectra were calibrated using All-In-One Peptide/Protein Standards (BioRad Laboratories).

2.4 Identification of the 4480 Da biomarker

A ProteinChip immunoassay (modified from O’Riordan [20]) was used to identify the 4480 Da marker that was found to be consistently up-regulated at time = 0 in the control patients. Briefly, a rabbit antibody directed against full-length Iranian beta defensin-1 (Cat#HBD14A, Alpha Diagnostic International, San Antonio, TX), or nonspecific rabbit IgG (Sigma) was bound to 2 µL of Protein A HyperD beads (Pall Corporation, East Hills, NY) for 1 h. Beads were subsequently washed three times with 200 µL PBS, pH 7.4, and 200 µL of crude urine adjusted to pH 7 with 1 M TRIS-HCL, pH 8.0 was bound to the beads for 1 h at room temperature. The beads were washed five times with 400 µL PBS and once with 400 µL of distilled water. Proteins were eluted from the beads with 10 µL of 0.1 M acetic acid for 30 min. Ten micro-liters of eluate was spotted onto a normal phase (NP)20 ProteinChip array for profiling.

2.5 Statistics

For the clinical analysis, the primary outcome variable was the development of CIN, defined as a 50% or greater increase in serum creatinine from baseline. A two-sample *t*-test or Mann-Whitney rank sum test was used to compare continuous variables. Categorical variables were compared using the Chi-square test or Fisher’s exact test, as indicated.

For the proteomic analysis, spectra were analyzed with Ciphergen Express 3.0 software (Ciphergen Biosystems, Fremont, CA). Peak intensity was normalized to TIC and those samples with normalization factors >2 SD from the mean were excluded from the analyses as these tended to be poor quality spectra. Spectra were baseline subtracted and clustered using default settings. Peaks with a S/N of > 5 in a mass window of 0.3% found in at least 20% of spectra were identified as clusters. Peaks demonstrating intensity differences between groups with a $p < 0.05$ using a Mann–Whitney rank sum analysis were considered statistically significant and were subjected to further analysis. To identify factors that could predict risk for AKI resulting from contrast administration, we focused our analysis on time = 0. To measure the sensitivity and specificity for a protein peak in the prediction of AKI, receiver-operating characteristic (ROC) curves were generated and the area under the curve (AUC) calculated. An AUC of 0.5 is no better than expected by chance, whereas a value of 1.0 signifies a perfect bio-marker (2). A $p \leq 0.05$ was considered statistically significant.

3 Results

Ninety pediatric patients were included in the study, all of whom had normal renal function and no evidence for a urinary tract infection or overt congestive heart failure prior to contrast administration. AKI due to CIN, defined as a 50% increase in serum creatinine from baseline, occurred in ten participants by the 24 h postcontrast time point (11%). Thus, the diagnosis of CIN using currently accepted practices could be achieved only several hours after the inciting event. Out of the 80 subjects who did not develop CIN, we identified seven children who were matched with the CIN group for age and gender. A comparison of demographic features between the two groups is shown in Table 1. No differences were noted between the two groups with respect to age, height, weight, body surface area, gender, ethnicity, presence of cyanosis (defined as an oxygen saturation below 90%), contrast volume (absolute or adjusted for weight), or baseline serum creatinine measurement.

Equal volumes of urine from the control and AKI groups were analyzed by SELDI-TOF MS using three chromatographic surfaces. Similar results were obtained from each chip type. Representative spectra obtained from control and AKI patients at baseline (time = 0) and 2 h postcontrast administration are shown in Fig. 1. A peak at 4480 Da was identified on all three chromatographic surfaces as consistently up-regulated at time = 0 in the control patients (Fig. 1A). This peak was up-regulated in the control patients 4-fold ($p = 0.02$) on the H50 surface, 12-fold ($p = 0.01$) on the IMAC30 surface, and 9.5-fold ($p = 0.005$) on the CM10 surface (Fig. 2). A 4631 Da peak was identified in the AKI group on the H50 surface (Fig. 1B) as being up-regulated 4.7-fold ($p = 0.03$) at time = 0 (Fig. 2B). Receiver operating characteristic curves (Table 2) revealed an AUC for prediction of CIN of 0.89–0.99 for the 4480 Da biomarker, and 0.84 for the 4631 Da biomarker in the preprocedural urine samples, indicative of excellent biomarker characteristics. No other peaks were consistently changed between the AKI and control groups at time = 0.

The spectra containing the 4480 Da prospective biomarker had a peak pattern strikingly similar to spectra characterized recently after acute renal allograft rejection (Vladimir Podust and Nathan Harris, personal communication; [20]). These authors previously identified a 4.7 kDa marker as the 44 amino acid (a.a.) variant of HBD-1. Another variant of HBD-1 that has been found in urine is the 41 a.a. variant which has a mass of 4479 Da [21]. We therefore wished to determine if the 4480 Da biomarker identified in this study was potentially a variant of HBD-1. A rabbit antibody directed against full-length HBD-1 (HBD14-A, Alpha Diagnostic International) or Rabbit IgG was used to capture HBD-1, and eluates analyzed by SBLDI-TOF-MS. The resulting spectra are shown in Fig. 3. The results indicate that the identity of the 4480 Da biomarker is the 41 a.a. variant of HBD-1. This peak is prominent in the control patients but greatly diminished in the patients that develop AKI, which may indicate a protective effect of the 41 a.a. variant of HBD-1 against CIN. The predominant 4750 Da (44 a.a. variant of HBD-1) peak is also identified in the control and AKI spectra. The immunoassay did not identify a peak 4631 Da in size.

4 Discussion

This cross-sectional pilot study of children and adolescents with congenital heart disease undergoing elective cardiac catheterization and angiography with contrast administration demonstrated that (i) CIN, defined as a 50% increase in serum creatinine, occurs in 11% of subjects, (ii) this increase in serum creatinine requires several hours to manifest, (iii) distinct preprocedural urinary proteomic profiles exist in subjects who subsequently develop CIN, and (iv) the absence of a 4480 Da biomarker (identified as the 41 a.a. variant of HBD-1) and the presence of a 4631 Da protein peak (as yet unidentified) in the preprocedural urine may prove to be useful biomarkers for the early prediction of CIN.

Human beta-defensin 1 (HBD-1) has several active forms ranging from 36 to 47 a.a. residues (3.9–5 kDa) that are formed through amino truncations, and is part of a family of antimicrobial peptides [21]. Eight variants of HBD-1 have been identified in urine and kidney tissue from healthy individuals, with the predominant variant being the 44 a.a. (4750 Da) form. Other HBD-1 variants ranging from 36 to 43 a.a. are present in much lower concentrations in normal urine. The relative abundance of these shorter variants varies between individuals [21,22]. One such form of HBD-1 that has been found in urine is the 41 a.a. variant which has a mass of 4479 Da [21]. Our results clearly indicate that the 4480 Da biomarker detected exclusively in the urine of subjects that did not develop CIN is the 41 a.a. variant of HBD-1. This peak is prominent in the control patients but greatly diminished in the patients that develop AKI, which may indicate a protective effect of the 41 a.a. variant of HBD-1 against CIN. It is worth noting that this peak is diminished after treatment to roughly equal levels in both groups. This 41 a.a. variant of HBD-1 should not be confused with the 41 a.a. variant of HBD-2. The latter is the major secreted form of HBD-2, and while it has similar antibacterial properties, it has a distinct mass of 4328 Da [23] and is not recognized by the specific HBD-1 antibody used in this study.

The biological significance of our findings that urinary HBD-1 appears to identify subjects who may be protected from developing CIN is speculative. Human β -defensins are primarily antimicrobial peptides expressed predominantly in epithelial tissues that provide the first line of defense between the subject and the external environment [24]. HBD-1 was first isolated from the filtrate of subjects with end-stage renal disease undergoing hemodialysis [25]. It is expressed in the normal kidney, where it localizes to the distal tubules and collecting ducts [21]. This expression is enhanced after bacterial infections, indicating a prominent role in antimicrobial host defense within the urinary tract [26–28]. Emerging evidence also suggests an important role for HBD-1 as a regulator of cytotoxic and immune responses in the kidney [20,22]. It is intriguing to propose that the same properties of HBD-1 that contribute to its antimicrobial and immune activation functions also confer protection from acute kidney injuries due to other stimuli such as contrast agents. In this regard, it is interesting to point out the similarities between the present findings and a series of studies linking AKI with another primordial antimicrobial system in the kidney, namely neutrophil gelatinase-associated lipocalin (NGAL). Immediately following a variety of injurious stimuli, the gene and protein encoding for human NGAL are dramatically up-regulated in the distal nephron segments of the kidney, in a distribution similar to that reported for HBD-1 [29–31]. In these situations, NGAL ameliorates structural and functional renal damage by inhibiting apoptotic cell death and enhancing the early regenerative response [32]. Urinary NGAL is also emerging as a highly promising, early, predictive biomarker of AKI in several common clinical situations, including CIN [33–35].

Our results also indicate that the presence of a 4631 Da protein peak in the urine at baseline may provide a useful biomarker for the early prediction of CIN. The 4631 Da peak in tile AKI patients at time = 0 is increased in intensity in the same proportion to the controls as the 4480 Da peak is increased in the controls relative to the AKI group (4631 Da - up fourfold in the AKI; 4480 Da - up four-fold in the control; both on the H50 surface). We hypothesized that the 4631 Da biomarker may represent the 43 a.a. variant of HBD-1 [21] and that a simple shift in abundance of HBD-1 variants may exist in subjects destined for CIN. We were, however, unable to identify this peak in our HBD-1 immunoassay. The identity of this putative CIN biomarker is currently unknown.

This study has several strengths. First, we studied a relatively homogeneous cohort of pediatric subjects in whom the only obvious etiology for AKI would be the result of contrast administration. These patients comprise an ideal and important population for the study of AKI biomarkers, since they do not exhibit common comorbid variables such as diabetes, hypertension, atherosclerosis, and nephrotoxin use. Second, all subjects received ioversol, a

low osmolar nonionic contrast agent, thereby eliminating any variability due to type of agent used. Third, all subjects started with normal kidney function. Other variables such as patient demographics and amount of contrast agent used (both absolute as well as weight-adjusted volumes) were not predictive of CIN, and could not be used for risk assessment in this cohort. Fourth, we identified two reciprocal biomarkers that may predict the development of CIN in urine samples obtained before contrast administration. In this pilot study, both the absence of a 4480 Da protein (the 41 a.a. variant of HBD-1) and the presence of a 4631 Da protein peak (identity unknown) were predictive of CIN.

This study has limitations. First, it is a single-center pilot study of pediatric subjects with congenital heart defects receiving contrast during elective cardiac catheterization. These results will need to be validated in a prospective trial of a larger population, including adults with the usual confounding variables and comorbid conditions that normally accumulate with increasing age. Second, this was a cohort with normal kidney function to begin with, and it will be important to confirm our findings in documented high-risk settings such as pre-existing kidney dysfunction, diabetes mellitus, volume depletion, concomitant nephrotoxic drug use, and the hemodynamically compromised patient. Third, we used a definition of a 50% increase in serum creatinine for CIN, whereas others have suggested that a 25% increase in serum creatinine may be a more sensitive marker of CIN [1,36]. However, the majority of studies published in the field have utilized and recommended a 50% increase in serum creatinine for the basic definition of AKI [37], as well as for identification of novel biomarkers of AKI in general [16,33,34] and CIN in particular [35]. Fourth, it will be important in future studies to establish the identity of the 4631 Da bio-marker. Fifth, future translational studies should assay for these biomarkers by employing standard quantitative tests, since the SBLDI-TOF methods used in this study do not directly measure concentrations.

The primary take-home message of our study is that monitoring of baseline urinary biomarker levels may provide an early, simple, and noninvasive strategy for predicting CIN. Ideally, the absence of the 4480 Da biomarker and/or the presence of the 4631 Da protein would trigger the appropriate clinical response. At the very least, clinicians informed of such a situation would monitor the patient more closely for impending renal insufficiency, avoid the use of additional nephrotoxins, and optimize hydration and renal perfusion to prevent further injury. The ability to predict which patients will develop CIN may also add substantively to existing risk-stratification systems [36], and enable early initiation of interventions to change the poor outcomes associated with this common clinical problem.

Abbreviations

AKI, acute kidney injury; CIN, contrast induced nephropathy; CM10, carboxymethyl; H50, hydrophobic; HBD-1, human beta defensin-1; NGAL, neutrophil gelatinase-associated lipocalin; NP, normal phase 20; ROC, receiver operator characteristic.

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Figure 1.

Representative SELDI-TOF-MS spectra of urine from patients undergoing cardiac catheterization who either did not develop AKI (control) or subsequently developed AKI. (A) Spectra obtained on a CM10 cation exchange ProteinChip. Note the prominent 4750 Da peak present in all spectra and the 4480 Da peak that is only prominent in the control patients at time = 0, but is depleted postcontrast administration in the controls and is very weak in the patients who develop AKI at both time points. The intensity of the 3730 Da peak was highly variable and did not differ significantly between groups. (B) Spectra obtained on an H50 RP ProteinChip. Note the prominent 4750 Da peak present in all spectra as on the CM10 spectra. The 4631 Da peak is present only in the AKI patients at time = 0 and absent in the controls at both time points.

Figure 2.

Scatter plots of the 4480 and 4631 Da biomarkers. Comparisons were made between control and AKI patients at time = 0 and reported p -values calculated using the Mann-Whitney rank sum analysis. The top three panels represent the relative intensity of the 4480 Da biomarker on the H50, IMAC30, and CM10 ProteinChip. The bottom panel represents the relative intensity of the 4631 Da biomarker on the H50 ProteinChip.

Figure 3.

ProteinChip immunoassay for human beta-defensin 1 (HBD-1). The top spectrum represents neat urine spotted onto an NP20 chip for comparison of peak sizes. The second and third panels result from incubating control (2nd) and AKI patient (3rd) urine with beads bound to HBD-1 antibody and spotting the eluate onto an NP20 ProteinChip. Note the 4480 Da peak representing the 41 a.a. variant of HBD-1 appearing exclusively in the control patient sample and the 4750 Da peak representing the predominant 44 a.a. variant of HBD-1 present in both groups. The bottom panel represents control urine incubated with beads bound to rabbit IgG, followed by spotting the eluate onto an NP20 ProteinChip.

Table 1

Patient demographics and clinical outcomes

	No CIN	CIN	<i>P</i>
Number of subjects	7	10	
Age (y)	6.9 ± 3.2	7.3 ± 3.1	NS
Weight (kg)	27 ± 8.3	23.3 ± 5.8	NS
Height (cm)	109.7 ± 13	108.3 ± 12	NS
Body surface area (m ²)	0.85 ± 0.18	0.82 ± 0.14	NS
Males (%)	57	55	NS
Caucasians (%)	84	82	NS
SaO ₂ <90% (%)	17	18	NS
Contrast volume (mL)	62 ± 4	74 ± 13	NS
Contrast volume (mL/kg)	3.7 ± 0.2	4.2 ± 0.6	NS
S creatinine baseline (mg/dL)	0.74 ± 0.09	0.60 ± 0.08	NS
S creatinine 24 h (mg/dL)	0.86 ± 0.04	1.22 ± 0.04	<i>p</i> <0.001

CIN, contrast-induced nephropathy, defined as a 50% or greater increase in serum creatinine from baseline, NS, not significant.

Table 2Biostatistical analysis of increases in peak biomarker intensity in the control group *versus* AKI at time = 0

<i>m/z</i>	Chip surface	Fold increase in average peak intensity	<i>p</i> -value	ROC AUC
4480	H50	4	0.02	0.89
4480	IMAC 30	12	0.01	0.94
4480	CM10	9.5	0.005	0.99
4631	H50	0.21	0.03	0.84

For ROC curve calculations the control group has been designated "positive" for the 4480 Da biomarker, while the AKI group has been designated "positive" for the 4631 biomarker.