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Detection of Drug-induced Acute Kidney Injury in Humans Using Urinary KIM-1, miR-21, -200c and -423

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ABSTRACT

Drug-induced acute kidney injury (AKI) is often encountered in hospitalized patients. Although serum creatinine (SCr) is still routinely used for assessing AKI, it is known to be insensitive and nonspecific. Therefore, our objective was to evaluate kidney injury molecule 1 (KIM-1) in conjunction with microRNA (miR)-21, -200c, and -423 as urinary biomarkers for drug-induced AKI in humans.

In a cross-sectional cohort of patients (n=135) with acetaminophen (APAP) overdose, all four biomarkers were significantly ($p<0.004$) higher not only in APAP-overdosed patients with AKI (based on SCr increase) but also in APAP-overdosed patients without clinical diagnosis of AKI compared to healthy volunteers. In a longitudinal cohort of patients with malignant mesothelioma receiving intraoperative cisplatin (Cp) therapy (n=108) the four biomarkers increased significantly ($p<0.0014$) over time after Cp administration, but could not be used to distinguish patients with or without AKI. Evidence for human proximal tubular epithelial cells (HPTECs) being the source of miRNAs in urine was obtained first, by *in situ* hybridization based confirmation of increase in miR-21 expression in the kidney sections of AKI patients and second, by increased levels of miR-21, -200c and -423 in the medium of cultured HPTECs treated with Cp and 4-aminophenol (APAP degradation product). Target prediction analysis revealed 1102 mRNA targets of miR-21, -200c and -423 that are associated with pathways perturbed in diverse pathological kidney conditions. In summary, we report non-invasive detection of AKI in humans by combining the sensitivity of KIM-1 along with mechanistic potentials of miR-21, -200c and -423.

KEYWORDS

Nephrotoxicity in Patients, Biomarker, Acute Kidney Injury, microRNAs, KIM-1

INTRODUCTION

Acute Kidney Injury (AKI) affects 1 in 5 hospitalized patients worldwide (Susantitaphong *et al.*, 2013). A substantial proportion of AKI is attributed to drug-induced kidney injury (DIKI): 18-27% in hospitalized individuals with AKI (Taber and Pasko, 2008; Uchino *et al.*, 2005). Furthermore, nephrotoxicity is a common reason for drug development failure both in the preclinical and clinical stages. In clinical settings, AKI is assessed by measurement of functional biomarkers like serum creatinine (SCr) that is known to have low sensitivity, specificity, and limited capability for early diagnosis (Vaidya *et al.*, 2008). A delayed diagnosis hinders not only timely care of AKI patients but also prevents stratification of AKI patients for clinical trials of AKI treatment; therefore, there is an urgent need for new kidney injury biomarkers with improved characteristics.

In 2008, seven urinary protein biomarkers were amongst the first batch qualified by the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA, 2009) for the assessment of DIKI in preclinical studies. Although these biomarkers, like Kidney injury molecule-1 (KIM-1), outperformed traditional biomarkers in sensitivity and specificity in preclinical studies, successful regulatory qualification and implementation into clinical practice are still awaited (Dieterle and Sistare, 2010; Jensen, 2004; Murray *et al.*, 2014). Another class of biomarkers that have recently emerged as promising candidates for detection of diverse cancer types, organ damages and other disease states are extracellular microRNAs (miRNAs) found stable in diverse body fluids and resistant to RNase-mediated degradation, pH variability and multiple freeze- thaw cycles (McDonald *et al.*, 2011; Mitchell *et al.*, 2008; Mraz *et al.*, 2009; Weber *et al.*, 2010). MiRNAs are approximately 20-25 nucleotides long, non-coding and evolutionarily conserved small RNAs that function intracellularly as post-transcriptional regulators of gene expression by binding to complementary sequences in the 3'-untranslated regions of target mRNAs (Krol *et al.*, 2010). Our group described the methodology and application for the use of urinary miRNAs to differentiate AKI patients from healthy individuals

(Ramachandran *et al.*, 2013; Saikumar *et al.*, 2012). In particular, we found urinary levels of miR-21, miR-200c and miR-423 exhibited significantly high sensitivity and specificity in differentiating AKI patients admitted in the intensive care unit vs. patients with no evidence of AKI (Ramachandran*, et al.*, 2013).

The objective here was to evaluate the performance of KIM-1, miR-21, miR-200c and miR-423 for detecting drug-induced AKI in humans. Specifically the aims were: 1) to measure urinary KIM-1, miR-21, miR-200c and miR-423 in a cross-sectional cohort of patients (n=135) with acetaminophen (APAP) overdose and in a longitudinal cohort of patients (n=108) with malignant mesothelioma receiving cytoreductive surgery with intraoperative cisplatin therapy; 2) to identify the source of the miRNAs by performing *in situ* hybridization in human kidney sections and by conducting *in vitro* experiments using human proximal tubular epithelial cells following toxicity; and 3) to computationally predict the targets for the three candidate miRNAs and highlight the possibility of urinary miRNA profiles to reflect pathological events in the kidney.

MATERIALS & METHODS

Patients and Samples

All participants were patients or healthy volunteers recruited at the Brigham and Women's Hospital Boston, MA, or at the MRC Centre for Drug Safety Science, University of Liverpool, UK. The Institutional Review Board of both institutions approved the protocols for recruitment and sample collection, which was performed with informed consent of the participants.

Acetaminophen cohort: Urine samples from healthy volunteers (n=65) and a cross-sectional study of individuals with acetaminophen (APAP) overdose (n=70) were enrolled from the MRC Centre for Drug Safety Science BIOPAR NHS portfolio study. Approximately 60% of the APAP overdosed patients (n=43) had AKI defined by serum creatinine (SCr) concentrations >1 mg/dl.

Cisplatin Mesothelioma cohort: Urine samples were collected at the Brigham and Women's Hospital as part of a longitudinal study enrolling patients with malignant mesothelioma undergoing cytoreductive surgery with intraoperative heated cisplatin chemotherapy (n=108). Sampling was performed prior therapy (Pre) and on nine subsequent time points: 4h, 8h, 12h, 24h, 48h, 72h, 96h, 120h and 144h. Approximately 40% of the patients developed AKI (AKI Stage 1, AKI Stage 2&3) defined by Acute Kidney Injury Network (AKIN) criteria (Mehta *et al.*, 2007) at any time point. From the 108 enrolled patients, two were excluded because of incomplete data sets.

Biopsy samples: Paraffin embedded kidney tissue samples were obtained from Brigham and Women Hospital's Pathology department. The biopsy was performed in patients to ascertain a clinical diagnosis of acute tubular necrosis after allograft rejection (n=3). For comparison, kidney biopsy samples diagnosed as within normal limits (n=3) were also included.

Urine Collection and Analysis

Urine was collected from spontaneous voids or from indwelling Foley catheters followed by centrifugation at 3,000xg for 10 minutes and microscopic examination of the urine sediment

(Olympus microscope). The urine supernatant was aliquoted and frozen within 4h of collection at -80°C. No additives or protease inhibitors were added. Urinary creatinine concentrations were measured utilizing the commercially available Creatinine (urinary) Colorimetric Assay from Cayman Chemical (Ann Arbor, MI). Using the Magnetic Luminex® Performance Assay (Human Kidney Biomarker Base Kit in conjunction with the Human TIM-1/KIM-1/HAVCR Kit; R&D Systems, Minneapolis, MN), KIM-1 was measured in 50 µl urine supernatant according to the manufacturer's instructions on a Bio-plex 200 (Bio-Rad; Hercules, CA). KIM-1 concentrations (pg/ml) were normalized to urinary creatinine (UCr; mg/dl) to account for dilution effects of the hydration status and are reported as urinary levels in pg/mg UCr.

In vitro Experiments

Human proximal tubular epithelial cells (HPTECs) which are passaged cells derived from normal human kidney tissue were purchased from Biopredic International (Rennes, France). Previously, we have shown that HPTECs possess characteristics of differentiated epithelial cells, such as polar architecture, junctional assembly, expression and activity of transporters, ability to synthesize enzymes like glutathione and γ-glutamyl transferase all up to passage 4 (Adler*, et al.*, 2015). Thus we consider them to be not only primary cells but also better than the immortalized cells derived from human (HK2), dog (MDCK) and pig (LLCPK1) in terms of mimicking human kidney tubular epithelial structure and function. The cells were cultured in DMEM/Hams-F12 with GlutaMAX medium supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 36 ng/ml hydrocortisone, 10 ng/m epidermal growth factor, 1% insulin-transferrin-selenium and 4 pg/ml triiodothyronin on collagen coated tissue culture plates at 37°C in a humidified 5% CO_2 incubator. Cp and 4-aminophenol (Sigma-Aldrich; Saint Louis, MO) were diluted in medium with 0.5% DMSO with final concentrations of 10 µM to 1000 µM for dose-response experiments in 96-well plates. After 24h cell viability was measured by Cell-Titer Glow assays (Promega; Madison, WI) and dose-response curves were generated using GraphPad

Prism 6 (GraphPad Software Inc.; La Jolla, CA). Calculated LD_{50} values correspond to previously published for these compounds and cells (Adler*, et al.*, 2015). For measurement of miRNAs in medium and in the cells itself, HPTECs were seeded in 6-well plates and treated with 85 and 100 µM Cp and 4-aminophenol, concentrations selected based on previously published LD_{50} values. After 24h of treatment, medium was removed, centrifuged twice (10 min 1,600xg then 10 min 16,000xg) and the resulting supernatant as well as the corresponding cells were used for total RNA isolation.

RNA Isolation and Measurement of miRNAs

RNA isolation: 200 µl urinary supernatant was used for isolation with the miRNeasy Serum/Plasma Kit from Qiagen (Valencia, CA) according to manufacturer's instructions. Total RNA form 200 µl medium supernatant and HPTECs was isolated with the miRNeasy Mini Kit (Qiagen). Quality and quantity of the cellular RNA war assessed photometrically using a NanoDrop 8000 (Thermo Scientific; Wilmington, DE).

Reverse transcription (RT) and Pre-Amplification: 1.5 µl of the eluted RNA (urinary and medium supernatant) or 10 ng cellular RNA were revers transcribed into cDNA using Qiagen's miScript RTII kit. The prepared cDNA was diluted five-fold and 5 µl of the diluted cDNA was then pre-amplified with Qiagen's miScript PreAMP kit for urinary and medium samples. The pre-amplified cDNA was diluted five-fold prior to qPCR detection.

qPCR: For urine samples, candidate miRNA evaluation was performed using custom 384-well plates preloaded with specific primer probes for miR-21, -200c and -423 from Qiagen. For medium and cellular miRNAs same assays were used. This SYBR Green-based qPCR was performed according to manufacturer's instructions with 2 µl diluted and pre-amplified cDNA in a total reaction volume of 10 µl. The thermal profile was as following: activation 15 sec at 95°C; 40 cycles of annealing/elongation with 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. Finally, a melt curve analysis war included. For urine samples, the Ct of the positive qPCR control was subtracted from the Ct of the miRNA to get a ΔCt value for each sample. These ΔCt values were converted to linear scale by computing $2^{-\Delta Ct}$ and normalized to UCr to calculate arbitrary urinary levels for each miRNA per sample ($2^{-\Delta Ct}$/UCr). Medium and cellular miRNAs were normalized to the positive qPCR control and let-7f, respectively, according to the ΔΔCt method for calculating relative quantities (RQ, $2^{-\Delta\Delta Ct}$).

In situ Hybridization

Kidney biopsy samples were fixed in neutral buffered formalin, trimmed and paraffin embedded followed by sectioning of the tissue block into approximately 5 µm thick sections. Standard H&E staining was used to assess the degree of injury. *In situ* hybridization was performed using double-digoxigenin labeled miRNA probes from Exiqon (Vedbaek, Denmark) according to the manufacturer's instructions. For hybridization, miRNA probes (miR-21-5p: TCAACATCAGTCTGATAAGCTA, Tm 83°C, 60 nM; miR-200c-3p: TCCATCATTACCCGGCAGTATTA, Tm 87°C, 80 nM; miR-423-5p: AAAGTCTCGCTCTCTGCCCCTCA, Tm 94°C, 60 nM) were incubated for one hour 30°C below the RNA melting temperature which corresponds to the optimal hybridization temperature. Nuclear Fast Red™ was used for counter staining (Sigma-Aldrich). Finally, sections were analyzed by light microscopy.

Target analysis

Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) was employed to search for target mRNAs containing sequences complementary to those present in the miRNAs (so-called miRNA target analysis for identification of mRNAs potentially regulated by miRNAs) and for pathway analysis in general. The two following filter criteria were applied for target analysis: 1.) experimentally observed and/or highly predicted target relation, i.e. sequence complementarity between mRNA and miRNA, and 2.) known expression in the kidney. The final group of identified miRNA targets was further investigated with IPA's Core Analysis to find associated pathways and diseases.

Statistical analysis

Urinary levels of miRNAs and KIM-1 are expressed as median and interquartile range with 5th and 95th percentiles as whiskers. Statistical significance was calculated with log2 urinary levels by t-test considering a p-value cut-off adjusted for multiple comparisons (significant in APAP study: $p<0.004$; significant in Cp study: $p<0.0014$) using GraphPad Prism 6 (GraphPad Software Inc., CA). Logistic regression models were used to evaluate associations of all three candidate miRNA biomarkers as well as KIM-1 with the odds of AKI, and to estimate area under the receiver operator curve (AUC-ROC). Regression models were adjusted for age and sex. Spearman correlation analysis (ρ and corresponding p-value) was performed to assess correlation between all biomarkers using data from all patients at all time points. Statistical analyses were performed using Stata 13.0 (StataCorp, College Station, TX).

RESULTS

Detection of APAP-induced AKI in a cross-sectional study using KIM-1 and candidate miRNAs

Three groups of patients, 43 with APAP overdose and AKI and 27 without a clinical diagnosis of AKI, as well as 65 healthy volunteers were enrolled in the cross-sectional study (Table 1). Since APAP is primarily a liver toxicant, all APAP-overdosed (OD) patients had liver injury diagnosed by ~100-fold increased levels of alanine aminotransferase as compared to healthy volunteers (Table 1). Urinary levels of KIM-1, miR-21, -200c, and -423 were significantly (adjusted p-value cutoff: $p<0.004$) higher in both APAP-OD patients with AKI compared to healthy controls and in APAP-OD patients without AKI diagnosis compared to healthy controls (Figure1A and B). Among patients with APAP-OD, higher urinary concentrations of each biomarker were associated with higher odds of AKI (Table 2). After adjustment for age and gender, every doubling of miR-21 concentration was associated with 1.31-fold higher odds of AKI (95%CI: 1.07, 1.60; $p<0.01$). Every doubling of KIM-1 concentration was associated with 3.2-fold higher odds of AKI, (95% CI: 1.74, 5.82; $p<0.001$). In predictive performance analyses, KIM-1 had the highest area under the ROC curve (AUC=0.84, 95%CI: 0.74, 0.94) while miR-21, -200c and -423 had ROC-AUC's between 0.64-0.71. A combination of miRNAs with KIM-1 did not substantively increase the predictive performance, as assessed by ROC-AUCs (Table 2).

Performance of KIM-1 and candidate miRNAs in a longitudinal study of Cp-induced AKI

To evaluate early diagnostic and predictive capabilities we next measured candidate miRNAs and KIM-1 in a longitudinal cohort of patients (n=106) with mesothelioma undergoing cytoreductive surgery with intraoperative Cp before and after Cp administration (Table 3). MiR-21, -200c, and 423 were high in mesothelioma patients at baseline before Cp-treatment as compared to levels from healthy, non-cancer patients from the APAP study (Suppl. Figure 1). After Cp treatment, we found that miR-21, -200c, -423 as well as KIM-1 significantly increased (adjusted p-value cutoff: $p<0.0014$) in urine compared to levels before the treatment with each biomarker being high in patients with AKI diagnosis but also in patients without clinically proven AKI (Figure 2). At any given time point, however, none of the biomarkers were significantly different between patients with and without AKI and concentrations of biomarkers were not associated with the odds of AKI (Table 4). All miRNAs correlated highly with each other, whereas the correlation of miRNAs was weak with KIM-1 SCr. The correlation of KIM-1 with SCr was also weak (Table 5).

Expression patterns of miR-21, -200c and -423 in the human kidney

In an attempt to investigate the expression patterns of the candidate miRNAs in human kidney we conducted *in situ* hybridization based miRNA localization in kidney biopsy samples from patients with clinical diagnosis of acute tubular necrosis (ATN) – pathologically characterized by tubular dilatation, cellular debris in tubular lumen and descendent tubular epithelia (Figure 3). Biopsy samples from patients without evidence of kidney damage served as controls (normal). miR-21 was not detectable in normal tissue, but found to increase significantly and co-localize with injured areas (Figure 3, black arrows). miR-200c was neither seen in controls nor in ATN kidneys, whereas miR-423 showed a very strong expression in both (Figure 3).

Release of miR-21, -200c and -423 by human proximal tubular epithelial cells in response to toxicity

Within the nephron the primary target of Cp and APAP toxicity are the proximal tubules and therefore miRNA expressions were measured in human proximal tubular epithelial cells (HPTECs) after treatment with Cp and 4-aminophenol (4-AP; degradation product of APAP). Following 24h of exposure to 85 μM of Cp and 100 μM of 4-AP the viability of the cells was decreased by approximately 50% and all three miRNAs significantly ($p<0.05$) increased in the cell culture media (Figure 4A and B). In the cells itself, the three miRNAs were minimal decreased after Cp treatment (Figure 4C). The increase in medium not only mimics the *in vivo* findings and strengthens the hypothesis of kidney proximal tubular epithelial cells to be the source for miR-21, -200c and -423 releases in urine after kidney toxicity but also demonstrates the utility of these candidate miRNAs for screening nephrotoxic agents *in vitro*.

Mechanistic implication of miR-21, -200c and -423

MiRNAs function as intracellular regulators of gene expression, thus we hypothesized that the urinary miRNA profile might reflect affected pathways in the injured kidney. To test this hypothesis, Ingenuity Pathway Analysis (IPA) was used to find mRNA targets for miR-21, -200c and -423. In total, 1102 mRNA targets were identified mostly associated with pathways also known to be perturbed in different pathological conditions in the kidney (Figure 5A). The top pathway and associated pathological condition was found to be MYC-mediated apoptosis signaling and renal necrosis/cell death, respectively. In addition, a deeper insight into the targets associated with renal necrosis/cell death as major feature of AKI, revealed that miR-21, -200c and -423 have several overlapping targets including genes well-known in apoptosis like cyclin-dependent kinase inhibitor 1 (CDKN1A, p21) or B-cell lymphoma 2 (Bcl-2) (Figure 5B).

DISCUSSION

Using a multi-dimensional approach to examine the association of candidate biomarkers with drug-induced AKI, we evaluated urinary KIM-1, miR-21, -200c and -423 among AKI patients, enrolled in a cross-sectional as well as longitudinal study. All four biomarkers were higher in patients with APAP overdose, relative to healthy subjects and were highest among patients with APAP overdose and diagnosed AKI. In longitudinal analyses, all biomarkers were elevated post Cp treatment, regardless of future AKI status.

The poor performance of urinary miRNAs and KIM-1 to predict AKI may reflect the inadequacy of a SCr-based definition for AKI (Waikar *et al.*, 2012). Although in preclinical studies renal histopathological examination is the gold standard for AKI diagnosis, in clinical assessments SCr remains widely used. In fact, moderate performances of new AKI biomarker candidates are frequently seen in clinical studies, where AKI is mostly defined based on increased SCr levels (Vanmassenhove *et al.*, 2013). Several studies have demonstrated that patients who were SCr negative but biomarker positive are at risk for short- as well as long term morbidity and mortality (Coca *et al.*, 2014; Haase *et al.*, 2011). Haase et al. (2012) suggested to term this condition subclinical AKI, because it is not clinically detectable with existing routine diagnostics (SCr, blood urea nitrogen) however, tubular damage markers such as KIM-1 and NGAL suggest injury (Haase *et al.*, 2012).

In a preclinical study, both miR-21 and KIM-1 accurately reflected AKI diagnosed by histopathology but not when diagnosed by SCr (Suppl. Figure 2; Pavkovic *et al.*, 2014 and 2015). In clinical settings renal biopsies are not readily available, thus the evaluation of novel biomarkers becomes hindered by the inadequacy of SCr-based definitions of the outcome. Finding a solution to this paradox in clinical AKI biomarker evaluation is challenging. However, in the case of drug-induced AKI, the treatment with the nephrotoxic drug per se can be used for comparison.

Our results confirmed previous reports that KIM-1 has high sensitivity and specificity for tubular injury. A meta-analysis including data from 2979 patients concluded that urinary KIM-1 may be a promising biomarker for early detection of AKI also in clinical settings (Shao *et al.*, 2014). A recently published study using a very small number of patients (n=22) with solid tumors receiving Cp treatment showed a comparable increase of KIM-1 in urine after treatment, as seen here, whereas SCr was not increased (Tekce *et al.*, 2015). The exploration of KIM-1's function revealed interesting features involved in phagocytosis and regeneration (Ichimura *et al.*, 2008; Yang *et al.*, 2015), but limited information was added to the mechanism of initiation of AKI.

In contrast, miRNAs bear the potential to fill this gap since it is estimated that over 50% of all protein-coding genes are regulated by miRNAs (Krol*, et al.*, 2010). Applying Ingenuity Pathway Analysis for the three candidate miRNAs studied here, the top pathological kidney conditions found to be associated with the targets was renal necrosis highlighting the previously mentioned possibility of urinary miRNA profiles to mirror molecular perturbations in the kidney. MiR-21 has been extensively explored since it is ubiquitously expressed in mammalian organs; it is enriched in the kidney where it is involved in diverse physiological as well as pathophysiological processes (Landgraf *et al.*, 2007; Ma and Qu, 2013). In the context of AKI, miR-21 is described as a negative regulator in the apoptosis of tubular epithelial cells but also as involved in progression of fibrosis via SMADs after TGFβ activation (Li *et al.*, 2013). In a mouse model of Alport nephropathy it was shown that since miR-21 is further involved in metabolism and FA oxidation, inhibition of miR-21 probably enhanced PPARα/RXR activity and improved mitochondrial function. Thus it was deemed protective against TGF-β-induced fibrogenesis and inflammation in kidneys (Gomez *et al.*, 2015). MiR-200c has been mainly investigated in the context of cancer where it was found to regulate epithelial–mesenchymal transition via downregulation of ZEB1 and AKT resulting in an upregulation of E-cadherin (Bracken *et al.*, 2015; Wang *et al.*, 2013). In addition, miR-200c is involved in cell growth and cell cycle progression by suppressing the expression of CDK2 in renal carcinoma cell lines and xenografts (Wang *et al.*, 2015). MiR-423 has been less well-studied but has been shown to increase proliferation and cell growth by targeting Trefoil factor 1 and p21 in gastric and hepatocellular cancer, respectively (Lin *et al.*, 2011; Liu *et al.*, 2014). Furthermore, miR-423 is part of a miRNA signature associated with lupus nephritis (Te *et al.*, 2010). Overall, target prediction analysis and current knowledge about the function of the three miRNAs support our hypothesis of urinary miRNAs profiles as reflection of intrarenal processes.

Using human kidney biopsy samples, miR-423 was found expressed in the whole kidney cortex i.e. in tubular and glomerular structures whereas miR-200c could not be detected and miR-21 seemed to be expressed in injured areas of the kidney from patients with ATN. An expression in normal human kidneys was shown previously for miR-21 and -200c using PCR (Bao *et al.*, 2014), thus the lack of detection here could be due to the low technical sensitivity of *in situ* hybridization. Expression of all three miRNAs was detected in HPTECs. For miR-21, contrary to the *in situ* hybridization results, decreased expression was seen in HPTECs after treatment with Cp. This discrepancy could be due to the *in vitro* system per se or the different kind of AKI (ATN after allograft rejection) in the kidney biopsy samples. However, we found all three miRNAs increased in cell medium after Cp or 4-AP treatment, probably mimicking the *in vivo* situation.

Our study has several limitations. First, the longitudinal cohort consisted of patients with malignant mesothelioma. As such, an impact of concomitant cancer, rather than the Cp-treatment per se, on the miRNA profile in urine cannot be excluded. In fact, miR-21 expression in cancerous tissue was described as part of a 6-miRNA signature to predict survival in patients with malignant mesothelioma (Kirschner *et al.*, 2015). A direct comparison of all biomarker profiles in urine from both studies demonstrated high levels of miR-21, -200c and -423 in cancer patients (Suppl. Figure 1). Second, miRNA candidates were selected based on a cross-sectional discovery approach with healthy volunteers vs. AKI patients from the intensive care unit having different etiologies. Since AKI is a clinical condition with various etiologies the existence of one single, universal AKI biomarker seems unlikely. A more focused discovery approach using a case-control cohort of patients with drug-induced kidney toxicity has the potential to yield more sensitive and specific biomarkers for drug-induced AKI.

In summary, we show that KIM-1 along with miR-21, -200c and -423 can be non-invasive as well as specific urinary biomarkers for the detection of drug-induced AKI in patients. Based on their kidney expression and target analysis, miR-21, -200c, and -423 could add information about the affected molecular pathways in the kidney during AKI.

SUPPLEMENTARY MATERIAL

1) Supplementary Figure 1
   - Urinary levels of miR-21, -200c, -423 and KIM-1 from both studies
2) Supplementary Figure 2
   - Literature example: inaccuracy of serum creatinine as gold standard
3) Supplementary Figure 3
   - Technical controls for qRT-PCR

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FIGURE LEGENDS

Figure 1 Urinary profiles of kidney injury molecule-1 (KIM-1), miR-21, miR-200c and miR-423 in acetaminophen-induced kidney injury. Levels of miR-21, -200c and -423 and KIM-1 were measured in urine from healthy controls (n=65) as well as in patients with an acetaminophen (APAP) overdose (OD; n=70). Within latter group approximately 62% of the patients developed clinically proven AKI (50 % increase in serum creatinine). MiRNA levels and KIM-1 concentrations were normalized to urinary creatinine. **A)** Both data sets were log2 transformed and presented as box plots (median with inter quartile range) with 5^{th} and 95^{th} percentile as whiskers. T-test was used for p-value calculation: * $p<0.004$. **B)** Table with absolute levels as medians (interquartile range) of arbitrary miRNA levels ($x10^{-2}$; $2^{-\Delta Ct}$ normalized by urinary creatinine) or absolute KIM-1 concentration (pg/mg urinary creatinine). UCr, urinary creatinine.

Figure 2 KIM-1, miR-21, -200c and -423 increase in the longitudinal cisplatin (Cp) cohort in patients with and without clinical AKI. Levels of miR-21, -200c and -423 as well as KIM-1 were measured in urine from patients (n=108) before (Pre) and on nine following time points after Cp treatment grouping patients based on their serum creatinine dependent AKI status. Data sets were normalized to urinary creatinine, log2 transformed and presented as box plots (median with 25^{th} and 75^{th} percentiles) with 5^{th} and 95^{th} percentile as whiskers. T-test was used for p-value calculation to compare to the group baseline as well as within the two groups: * $p<0.0014$. Broken lines represent the median level before treatment of No AKI developers.

Figure 3 Localization of miR-21, -200c and 423 in human kidney biopsies from patients with acute tubular necrosis. Pathological examination of the biopsy samples was performed with standard H&E stained sections. Using *in situ* hybridization, the expression patterns of miR-21, -200c and -423 were assessed in kidney biopsies from normal controls and patients diagnosed with acute tubular necrosis (ATN). *In situ* hybridization settings were proven with U6 as positive control, having an abundant nuclear expression, and a scrabbled probe, not complementary to any known miRNA sequence, used as negative control. Star, tubular casts; arrow, positive miR-21 signal. For *in situ*: blue, positive probe staining; pink, counter staining; H&E pictures taken with ×10 others with 20x objective; representative images of n=3 controls and n=3 ATN patients.

Figure 4 Human proximal tubular epithelial cells release miR-21, -200c and -423 following toxicity. Human proximal tubular epithelial cells (HPTECs) were treated with cisplatin and 4-aminophenol (APAP degradation product) for 24h followed by measurement of viability and miR-21, -200c and -423. (A) Dose-response curves after 24h treatment with 0, 10, 31.6, 100, 316 and 1000 µM cisplatin and 4-aminophenol, respectively. Data is presented as means with standard deviation of percentage viability compared to the 0.5% DMSO treated control with fitted non-linear dose-response curves (n=3 to 6 replicates). Levels of miR-21, -200c and -423 in the medium supernatant **(B)** as well as in the cells **(C)** after 24h treatment with 85 µM cisplatin and 100 µM 4-aminophenol, respectively. Data is presented as mean with standard deviation (n=3-4) of relative quantities (RQ, $2^{-\Delta\Delta Ct}$). 1-way ANOVA with Dunnett's test was used for p-value calculation: * p<0.05, ** p<0.01, and *** p<0.001.

Figure 5 Target prediction analysis of miRNA biomarker candidates. Ingenuity Pathway Analysis (IPA) was used to identify mRNA targets of the three miRNAs (experimentally verified and highly predicted based on sequence complementarity). **A)** Within the pool of 1102 identified targets, IPA's Core Analysis tool revealed associated pathways and pathological conditions. Bases on the ratio of genes found in the pool vs. genes related to the specific pathways or diseases, top 5 associated pathways and pathological condition of the kidney were listed. **B)** All

49 targets associated with Renal Necrosis/Cell Death were plotted as network with the three miRNAs revealing overlapping targets as well as a complex interaction between the targets.

Table 1 Demographic and clinical characteristics of patients from the cross-sectional APAP cohort

Characteristic	**Healthy volunteers (n = 65)**	**APAP-OD (n = 27)**	**APAP-OD with AKI (n = 43)**
Age, years	34.9 ± 9.8	39.3 ± 15.9	39.8 ± 13.4
Sex, female	37 (56.9%)	17 (62.9%)	24 (55.8%)
D/LT	N/A	3 (11.1%)	19 (44.2%)
ALT activity (U/L)	30 (24 – 33)	3597 (2251 – 6226)	4601 (2188 – 7673)
SCr (mg/dL)	N/A	0.62 (0.54 – 0.70)	2.44 (1.33 – 3.04)

Data are means ± SD, n (%) or medians (25^{th}-75^{th} interquartile range); APAP, acetaminophen; OD, overdose; ALT, alanine aminotransferase; SCr, serum creatinine; D/LT, deceased/ liver transplantation

Table 2 Cross-sectional associations of biomarkers with AKI, among participants with APAP-OD

Biomarker, per doubling	**AUC[†]** (95% CI)	**OR unadjusted** (95% CI)	**OR[a] adjusted** (95% CI)	**AUC[†] KIM-1 combined** (95% CI)
miR-21	0.71 (0.58, 0.83)	1.30 (1.07, 1.59)**	1.31 (1.07, 1.60)**	0.84 (0.73, 0.94)
miR-200c	0.64 (0.51, 0.77)	1.27 (1.04,1.55)*	1.27 (1.04, 1.57)*	0.85 (0.76, 0.95)
miR-423	0.68 (0.56, 0.81)	1.29 (1.07, 1.56)**	1.29 (1.07, 1.56)**	0.84 (0.74, 0.95)
KIM-1	0.84 (0.74, 0.94)	3.08 (1.71, 5.56)***	3.18 (1.74, 5.82)***	-/-

† Area under the curve for ROC curve; Odds ratio presented per doubling of each biomarker.

[a]Odds ratio adjusted for age and gender; ‡ Adjusted for KIM-1 concentration and covariates in [a].

***p<0.001;**p<0.01;*p<0.05

Table 3 Baseline demographic and clinical characteristics of patients from the longitudinal Cp cohort by AKI status

Characteristic	**No clinical AKI (n = 61)**	**AKI Stage 1 (n = 30)**	**AKI Stage 2&3 (n = 15)**
Age, years	62.5 ± 10.5	64.9 ± 10.9	67.5 ± 10.6
Sex, female	18 (29.5%)	4 (13.3%)	3 (20%)
Race, White	57 (93.4%)	29 (96.7%)	15 (100%)
Race, Black	1 (1.6%)	1 (3.3%)	N/A

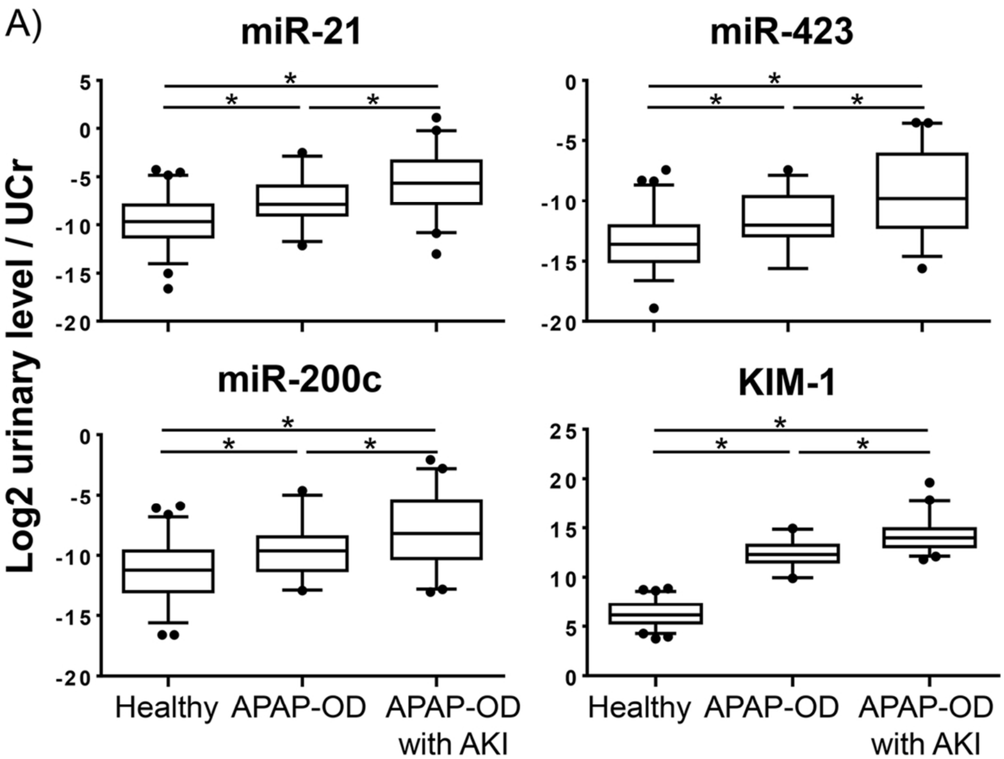
Data are mean ± SD or n (%); AKI Stage 1, 50-100% increase of SCr over baseline at any time point; AKI Stage 2&3, >100% increase of SCr over baseline at any time point.

Table 4 Association of biomarker concentration doublings at 4h, 8h, 12h and 24h with AKI status (any stage) at 48 hours

Biomarker	Time point	OR (95% CI)	p-value
miR-21	4h	0.92 (0.75, 1.12)	0.399
	8h	1.05 (0.87, 1.27)	0.612
	12h	1.20 (0.99, 1.45)	0.060
	24h	1.13 (0.94, 1.37)	0.200
miR-200	4h	0.79 (0.65, 0.97)	0.022
	8h	0.94 (0.76, 1.17)	0.575
	12h	1.18 (0.96, 1.45)	0.120
	24h	1.06 (0.87, 1.30)	0.558
miR-423	4h	0.79 (0.65, 0.97)	0.025
	8h	0.99 (0.81, 1.21)	0.929
	12h	1.13 (0.93, 1.38)	0.203
	24h	1.13 (0.91, 1.39)	0.267
KIM-1	4h	1.10 (0.98, 1.23)	0.098
	8h	1.06 (0.94, 1.20)	0.341
	12h	0.85 (0.69, 1.05)	0.140
	24h	0.89 (0.73, 1.10)	0.288
SCr	4h	1.18 (0.40, 3.54)	0.762
	8h	1.49 (0.50, 4.43)	0.478
	12h	3.35 (1.03, 10.95)	0.045
	24h	5.77 (2.01, 16.58)	0.001

Table 5 Correlation of all biomarkers over all groups and time points in the cisplatin cohort

		SCr	KIM-1	miR-21	miR-200c	miR-423
SCr	ρ	1				
	p-value					
KIM-1	ρ	0.1312	1			
	p-value	<0.001				
miR-21	ρ	-0.016	0.2388	1		
	p-value	0.6218	<0.001			
miR-200c	ρ	-0.0802	0.1381	0.8331	1	
	p-value	0.0132	<0.001	<0.001		
miR-423	ρ	-0.1381	0.1322	0.6989	0.8556	1
	p-value	<0.0001	<0.001	<0.001	<0.001	



B) Absolute miRNA and KIM-1 level

Biomarker	Healthy (n = 65)	APAP-OD (n = 27)	APAP-OD with AKI (n = 43)
miR-21	0.123 (0.042-0.387)	0.42 (0.196-1.618)	1.971 (0.463-8.427)
miR-200c	0.042 (0.015-0.123)	0.126 (0.041-0.281)	0.341 (0.082-1.991)
miR-423	0.008 (0.003-0.023)	0.024 (0.013-0.126)	0.113 (0.025-1.229)
KIM-1	70.75 (40.63-144.92)	5021.63 (3000.03-9584.97)	16152.51 (8905.3-29996)

Figure 1 Urinary profiles of kidney injury molecule-1 (KIM-1), miR-21, miR-200c and miR-423 in acetaminophen-induced kidney injury.
98x108mm (300 x 300 DPI)

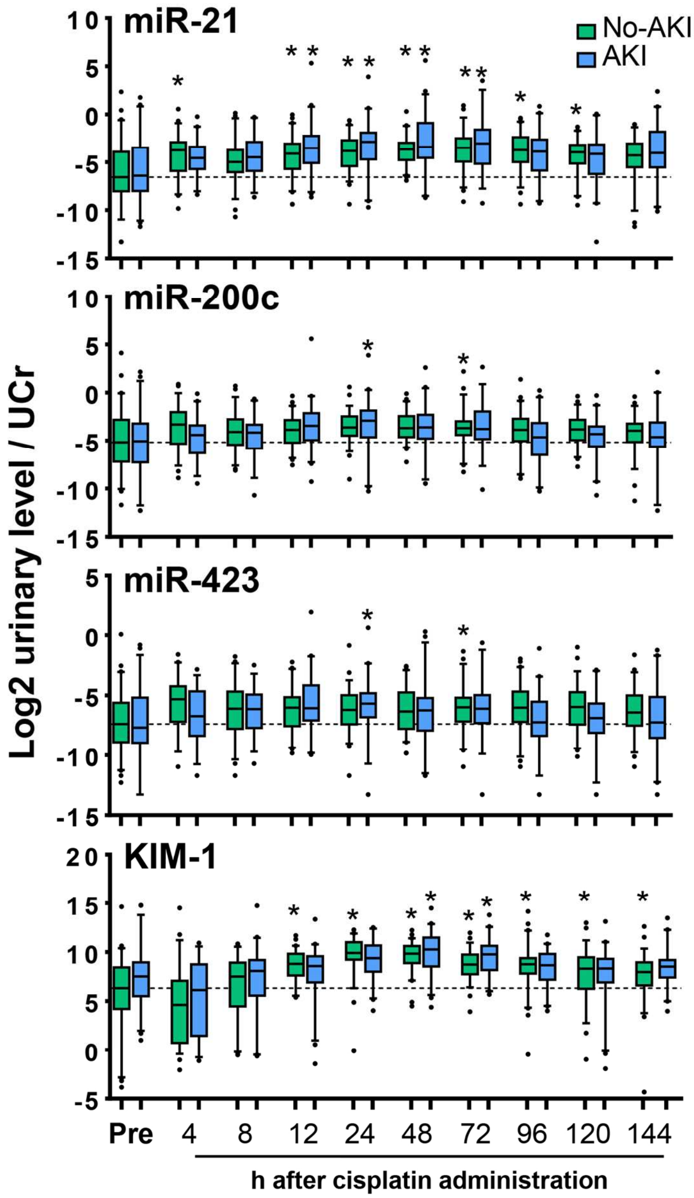


Figure 2 KIM-1, miR-21, -200c and -423 increase in the longitudinal cisplatin (Cp) cohort in patients with and without clinical AKI.
127x217mm (300 x 300 DPI)

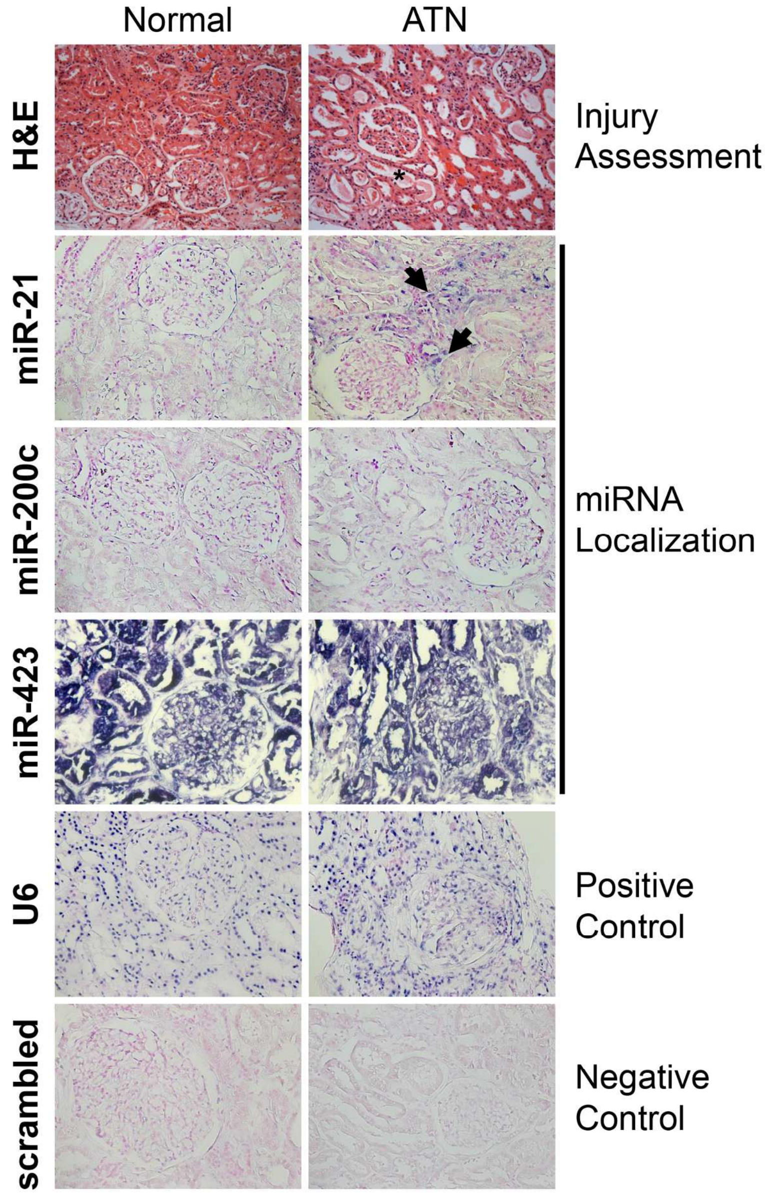


Figure 3 Localization of miR-21, -200c and 423 in human kidney biopsies from patients with acute tubular necrosis.
138x214mm (300 x 300 DPI)

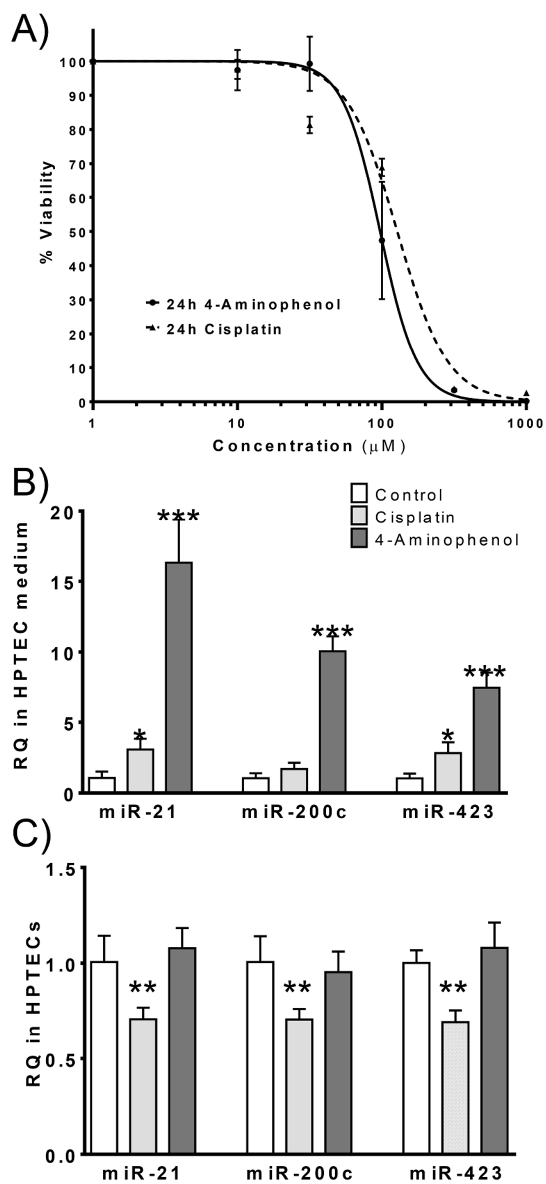


Figure 4 Human proximal tubular epithelial cells release miR-21, -200c and -423 following toxicity.
194x425mm (300 x 300 DPI)

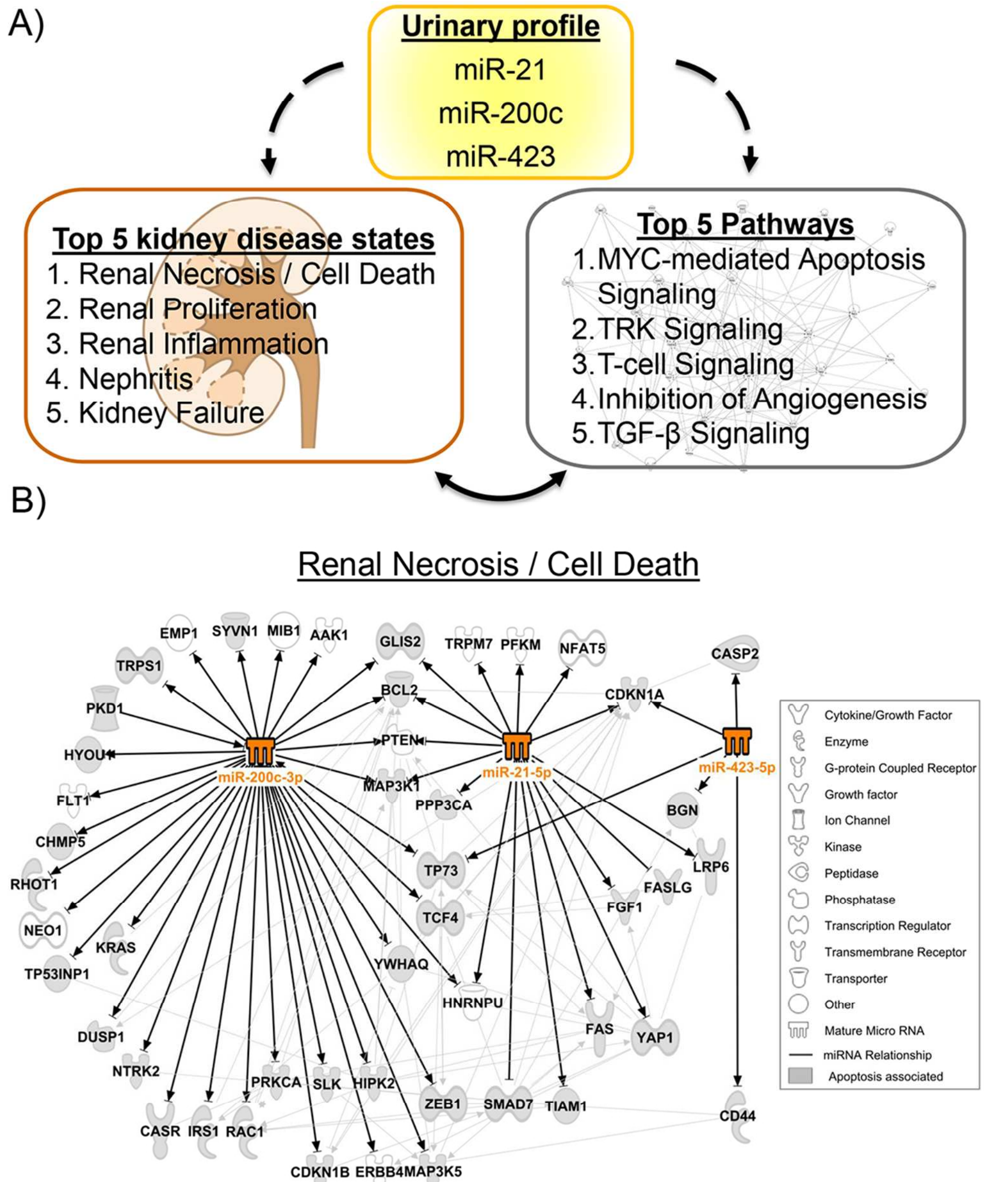


Figure 5 Target prediction analysis of miRNA biomarker candidates.
101x123mm (300 x 300 DPI)