Urine semaphorin 3A is an early diagnostic biomarker of acute kidney injury after cardiac surgery

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Abstract

Background: Semaphorin 3A is a secreted protein that regulates cell motility and attachment in axon guidance, vascular growth, immune cell regulation and tumor progression. However, nothing is known about its role in kidney pathophysiology. Here, we determined whether semaphorin3A is induced after acute kidney injury (AKI) and whether urinary semaphorin 3A can predict AKI in humans undergoing cardiopulmonary bypass (CPB).

Methods and Principal Findings: In animals, semaphorin 3A is localized in distal tubules of the kidney and excretion increased within 3 hr after reperfusion of the kidney whereas serum creatinine was significantly raised at 24hr. In humans, using serum creatinine, AKI was detected on average only 48 hours after CPB. In contrast, urine semaphorin increased at 2 hours after CPB, peaked at 6 hours (2596 ± 591 pg/mg creatinine), and was no longer significantly elevated 12 hours after CPB. The predictive power of semaphorin 3A as demonstrated by area under the receiver-operating characteristic curve for diagnosis of AKI at 2, 6, and 12 hours after CPB was 0.88, 0.81,
and 0.74, respectively. The 2-hour urine semaphorin measurement strongly correlated with duration and severity of AKI, as well as length of hospital stay. Adjusting for CPB time and gender, the 2-hour semaphorin remained an independent predictor of AKI, with an odds ratio of 2.19.

**Conclusion:** Our results suggest that semaphorin 3A is an early, predictive biomarker in experimental and pediatric AKI, and may allow for the reliable early diagnosis and prognosis of AKI after CPB, much before the rise in serum creatinine.
Introduction:

Acute kidney injury (AKI) due to ischemia is a frequent and serious complication in the hospital setting. The incidence of AKI is increasing world-wide, affecting about 6% of all hospitalized patients in whom it is an independent predictor of mortality and morbidity [1-3]. In the critical care setting, the prevalence of AKI requiring dialysis is about 6%, with a mortality rate exceeding 60% [4]. Once established, the treatment is largely supportive, at an annual cost surpassing $10 billion in the US alone (5). The diagnosis currently depends on detection of reduced kidney function by the rise in serum creatinine concentration, which is a delayed and unreliable measure in the acute setting [5]. Ironically, experimental studies have identified interventions that may prevent or treat AKI if instituted early in the disease process, well before the serum creatinine rises [6]. The lack of early predictive biomarkers has impaired our ability to translate these promising findings to human AKI.

Cardiopulmonary bypass (CPB) surgery is the most frequent major surgical procedure performed in hospitals worldwide, with well over a million operations undertaken each year. Acute kidney injury (AKI) is a frequent and serious complication encountered in 30-40% of adults and children after CPB [7-9]. AKI requiring dialysis occurs in up to 5% of these cases, in whom the mortality rate approaches 80%, and is indeed the strongest independent risk factor for death [10]. However, even a minor degree of post-operative AKI as manifest by only a 0.2-0.3 mg/dl rise in serum creatinine from baseline is also associated with a significant increase in mortality [11;12]. Additionally, AKI after cardiac surgery is associated with adverse outcomes such as prolonged intensive care and hospital stay, dialysis dependency, and increased
long-term mortality [13]. Infants and children with congenital heart diseases may be especially vulnerable to developing AKI, since many require multiple surgeries for step-by-step repair of complex congenital anomalies[14-16]. These patients comprise an important population for the initial validation of AKI biomarkers, since they do not exhibit common co-morbid variables that complicate similar studies in adults, such as diabetes, hypertension, atherosclerosis, and nephrotoxin use [17].

Experimental studies aimed at a better understanding of the early adaptive response of the stressed kidney have recently yielded several candidate genes and proteins that are serendipitously emerging as non-invasive candidate biomarkers of AKI [8;9;18;19]. Here we describe the identification and validation of a new early diagnostic biomarker, semaphorin 3A, for acute kidney injury. The Semaphorins make up the largest family of axon guidance cues yet described. Semaphorins are divided into 8 classes (classes 3-7 found in vertebrates). Class 3 Semaphorins are secreted, classes 4 through 6 are transmembrane proteins, and class 7 are membrane associated via glycosylphosphatidylinositol (GPI) linkage. They are characterized structurally by a conserved ~400 amino acid sema domain [20]. They are classically described as collapsing factors and mediators of axon repulsion, although they may also act as context-dependent chemoattractants. Semaphorins have been shown to have roles in cardiovascular development and in the regulation of immune cell antigen presentation. In addition semaphorins are also known to regulate cell motility and attachment in axon guidance, vascular growth, immune cell regulation and tumor progression [20-23]. Semaphorin3A is a chemorepellent with multiple guidance functions, including axon
pathfinding, cardiac and peripheral vascular patterning and branching morphogenesis. Semaphorin 3A gene deletion results in perinatal lethality [24]. Semaphorin3A signaling is mediated by a complex of the binding receptor neuropilin 1 and the signaling receptors plexinA1 or A3 [25;26]. Both Semaphorin3A and its receptor neuropilin 1 are expressed in the developing glomerulus, and Semaphorin3A remains expressed in adult podocytes and collecting tubules [27;28]. Semaphorin3A inhibits ureteric bud branching by downregulation of glial cell-line-derived neurotrophic factor [23]. The regulation of Semaphorin3A expression in kidney and pathophysiological role is unknown. Therefore, the objective of this study was to determine the expression pattern in animal models of AKI, its functional role in AKI, and whether urinary semaphorin 3A levels predict the development of AKI in animal and human acute kidney injury.

Results:

Immunolocalization of semaphorin 3A kidney from normal animals and animal which were subjected to acute and chronic kidney injury

Immunohistochemical examination revealed that semaphorin 3A was intensely stained in distal tubular epithelial cells and collecting tubules in normal and injured kidney. The intensity of staining is increased after induction of AKI (Figure 1). The identity of collecting tubules is not confirmed. It could be thick ascending limb of the loop of Henle as well.

Semaphorin 3A is an early biomarker of ischemic acute kidney injury in experimental animal models. To determine whether semaphorin 3A excretion is
enhanced after ischemia followed by reperfusion, Western analysis was carried out. As shown in figure 2A, semaphorin 3A is undetectable in normal urine whereas a large increase was seen in the first urine collected at 6 hr after reperfusion. This increase persisted even at 24hr after reperfusion. In contrast, serum creatinine level was significantly elevated only after 24hr of reperfusion (Figure 2B). To determine whether kidney tissue levels also increased after reperfusion, semaphorin 3A expression was determined by western blot analysis. A modest increase was seen at 24hr (Figure 2C). In contrast to protein expression, the mRNA expression was down regulated within 6hr after reperfusion and the downregulation was persisted even at 24hr. Semaphorin is known to be cleaved by furin like proteases to release multiple fragments (Figure 2E) [29]. Since urine semaphorin 3A molecular mass is 50 KDa, we analyzed whether proteases from urine generate 50 KDa and lower fragments. Incubation of recombinant semaphorin 3A Fc chimera with human urine for 1hr released 50, 28, 18 and 15 KDa lower sized fragments which match the predicted molecular weight of proteolytic fragment of semaphorin 3A. In addition, release of mature 95 KDa protein (which includes the signal peptide) also increased from the Fc chimera. Interestingly, addition of EDTA completely blocked the proteolytic cleavage of semaphorin 3A suggesting that metalloproteases may mediate this release of lower size fragments in urine.

To determine absolute quantity of semaphorin 3A in mouse urine and serum after AKI, we had quantified using an ELISA kit. Sema3A is significantly increased at 6 ad 24hr after reperfusion whereas serum creatinine is increased significantly only at 24hr after reperfusion (Figure 3A and B). This confirms the above observation with western blot analysis that semaphorin 3A is an early diagnostic biomarker of AKI. Similarly,
semaphorin 3A is also increased significantly at 24, 48 and 72hr after cisplatin administration where serum creatinine rise was seen 72hr after cisplatin administration (Figure 3C and D).

Unlike urine semaphorin 3A, circulating semaphorin 3A is not increased after reperfusion but rather rapidly downregulated (Figure 4B). In contrast to ischemia reperfusion, cisplatin administration significantly upregulated at 24 and 48 hr (Figure 4A) suggesting differential regulation of circulating semaphorin 3A in response to hypoxia and toxic kidney injury. Interestingly circulating semaphorin 3A is also upregulated after induction of diabetes (Figure 4C).

**Patient Characteristics and Renal Function Changes**

During the period of enrollment, 120 subjects underwent CPB at our institution. Of these, 60 subjects met the inclusion criteria for this study. The most common reason for excluding a subject was incomplete urine collections. AKI occurred in 26 children (43%) within a 3-day period. No significant differences were noted between the two groups with respect to age, race, need for dialysis, or mortality (Table 1). Children who developed AKI had significantly longer CPB times compared with those who did not develop AKI ($p < 0.0001$), and also experienced significantly longer hospital stays ($P = 0.0006$). Figure 1 shows the changes of serum creatinine concentrations after CPB for children who developed AKI and those who did not. During the first 24 hours after CPB, serum creatinine did not differ significantly between the two groups. Significant
differences between groups were seen by 48 hours after surgery and were maintained until 5 days after surgery.

**Associations of Semaphorin 3A with Patient Characteristics**

Semaphorin was negatively associated with patient age at baseline and at 24 hours (Table 2). Lower semaphorin levels before surgery were predictive of percent change in serum creatinine postsurgery and duration of AKI. Higher semaphorin levels at all time points between 2 and 12 hours were significantly associated with greater percent change in serum creatinine, longer CPB time, longer hospital length of stay, and longer duration of AKI. Higher semaphorin levels at 24 hours were significantly associated with longer hospital length of stay. Semaphorin levels at 48 hours were not significantly associated with any post-surgery outcomes.

**Urinary Semaphorin 3A Predicts AKI after Cardiac Surgery**

Currently used diagnostic biomarker serum creatinine start to rise significantly at 48hr after initiation of CPB and remain elevated after that in patient categorized as AKI (Figure 5). In contrast, urinary semaphorin is increased significantly in patients who developed AKI by 2 hours after the initiation of CPB, peaked at 6 hours after surgery, and was no longer significantly elevated 12 hours after surgery (Figure 6A). Patients who did not develop AKI experienced a much smaller increase shortly after surgery that resolved to baseline by 12 hours after surgery. ELISA results were confirmed by western blot analysis using two different polyclonal antibodies raised in two different species against semaphorin 3A (Figure 6B and C).
Conventional ROC curves for AKI versus no AKI were generated for urinary semaphorin at 2, 6, and 12 hours after surgery. The AUCs of the three ROC curves are 0.880 (p< 0.0001), 0.810 (p < 0.0001), and 0.737 (p = 0.0009), respectively.

After weighing the AUC, timing of measurement, and P value from the predictive logistic model, the optimal urinary semaphorin time point was selected at 2 hours after surgery. Figure 7 displays the unadjusted ROC curve for urinary semaphorin at 2 hours after cardiac surgery. The sensitivities and specificities for three semaphorin concentrations obtained at the 2-hour time point are listed in Table 3, corresponding to 95% sensitivity, optimal sensitivity and specificity, and 95% specificity. A cutoff value of 492.1 pg semaphorin per mg of urinary creatinine at 2 hours after cardiac surgery yields the optimal combination of sensitivity (81%) and specificity (94%).

During the past decade, two major classification systems for AKI have emerged (RIFLE and AKIN), based on serum creatinine and urine output criteria. A modification of the RIFLE criteria was suggested for pediatric use (pRIFLE), substituting serum creatinine values with estimated creatinine clearance. Several recent pediatric AKI studies have employed the pRIFLE criteria to report on AKI incidence and severity [30;31]. Among the 26 subjects who developed AKI, nine were classified as being in the risk (R) category, 14 in the injury (I) category, and three in the failure (F) category, on the basis of pRIFLE criteria. Analysis of semaphorin concentrations by pRIFLE classification revealed that both the risk group and the injury group differed from no AKI at 2 hours (all p< 0.004; Figure 8). No other significant differences were found among the pRIFLE groups.
Univariable logistic regression identified longer CPB time ($p < 0.0001$), male gender ($p = 0.0230$), longer hospital length of stay ($p = 0.0007$), and higher semaphorin concentrations at 2 hours ($p = 0.0015$), 6 hours ($p = 0.0025$), 12 hours ($p = 0.0038$), and 24 hours ($p = 0.0086$) as significantly associated with higher odds of AKI. A stepwise logistic regression analysis was used to determine the most parsimonious model given a set of potential variables for predicting AKI. Potential variables for this model included age, sex, CPB time, previous cardiac surgery, hospital length of stay, and semaphorin at the selected optimal time point (i.e., 2 hours after surgery). The final model revealed that sex, CPB time and semaphorin concentration at 2 hours after surgery were the only significant independent predictors of AKI in our cohort. The estimated odds ratio for every 100-pg/mg urinary creatinine increase of semaphorin at 2 hours after surgery was 2.191 (95% confidence interval: 1.001 to 4.502; $P = 0.0328$). The estimated OR for every 1-min increase of CPB time was 1.049 (95% CI: 1.012 to 1.087; $P = 0.0087$), and the estimated OR for male gender was 0.018 (95% CI: 0.001 to 0.529; $P = 0.0200$).
Reference List


Figure legends:

Figure 1. Immunohistochemical localization of semaphorin 3A in kidney after different treatments. Immunohistochemical localization of semaphorin 3A was carried out as described in Materials and Methods. Immunostaining for semaphorin 3A is seen in thick ascending limb of the loop of Henle and distal tubular epithelial cells. Scale Bar: 100µM.

Figure 2. Regulation of semaphorin 3A expression and excretion after ischemia reperfusion injury of the kidney. A. Western blot analysis of semaphorin 3A excretion in mice urine before (0 hr) and at different time point after reperfusion. A large increase was detected at 6 hr and 24 hr after reperfusion. B. Serum creatinine levels before and different time after ischemia reperfusion of the kidney. *, p<0.001 vs. 0 hr. C. Semaphorin 3A expression in kidney tissue was analyzed by Western Blot. A single 95 KDa band was observed and increased expression seen at 24 hr. D. RT-PCR analysis of semaphorin 3A expression in the kidney after ischemia reperfusion. Semaphorin 3A mRNA expression is downregulated at 6 and 24hr after reperfusion of the kidney. *, p<0.001 vs. sham operated. E. Graphic representation of known protease (Furin like) cleavage site and expected band of semaphorin 3A. F. Proteolytic cleavage of semaphorin 3A in vitro. 250 ng of recombinant semaphorin 3A-Fc chimera was incubated with 10µl of human urine for 1 hr at 37° C (lane 1), in the presence of 20 mM EDTA (lane 2), human urine alone (lane 3) and recombinant semaphorin 3A alone. Proteolytic release of smaller fragments of semaphorin 3A was inhibited with addition of EDTA. Values are mean ± SEM. n=3-5.
Figure 3. Quantification of urine semaphorin 3A in different forms AKI and diabetes in mouse. Semaphorin 3A was quantified using ELISA kit as described in Methods. A. Semaphorin 3A in urine from animals subjected to sham surgery (0hr) and different time after reperfusion. Ischemia reperfusion rapidly increased urinary excretion of semaphorin 3A. *, $p<0.005$ vs. 0 hr. B. Serum creatinine at different time after reperfusion. *, $p<0.001$ vs. 0 hr. C. Semaphorin 3A excretion in urine before and different time after cisplatin administration. Cisplatin administration significantly increased the excretion of semaphorin 3A at 24, 48 and 72 hr. *, $p<0.005$ vs. 0 hr. D. Serum creatinine at different time after administration of cisplatin. *, $p<0.05$. Values are mean $\pm$ SEM. n=6-8.

Figure 4. Quantification of serum semaphorin 3A in different forms AKI and diabetes in mouse. Semaphorin 3A was quantified using ELISA kit as described in Methods. A. Circulating levels of semaphorin 3A before and different time after cisplatin administration. Cisplatin administration was significantly upregulated semaphorin 3A in the blood. *, $p<0.005$ vs. 0 hr. B. Circulating levels of semaphorin 3A in sham operated (0hr) and different time after reperfusion. Ischemia reperfusion rapidly downregulated circulating semaphorin 3A. *, $p<0.005$ vs. 0 hr. Values are mean $\pm$ SEM. n=4-6.

Figure 5. Changes in serum creatinine (LS mean±SE) at various time points after cardiac surgery in the non-AKI and AKI group. * $p\leq0.0002$ for differences between groups by repeated measures ANOVA.
Figure 6. Quantification of semaphorin 3A in urine from patients who were undergone cardiopulmonary bypass surgery (CPB). A. Changes in urinary semaphorin concentrations at various time points after CPB surgery in non-AKI and AKI patients. Error bars are LS mean ± SEM. *p ≤ 0.005 for differences between groups (non-AKI and AKI) by repeated-measures ANOVA. B. Western blot analysis of urine samples from a patient with AKI. Blot was probed with rabbit polyclonal antibody from Abcam. C. Western blot analysis of urine samples from a patient with AKI. Blot was probed with goat polyclonal antibody from Santacruz Biotechnology.

Figure 7. ROC curve analysis for urinary semaphorin at 2 hours after cardiac surgery. The values 109.8, 492.1, and 910.0 are urinary semaphorin concentrations (in picograms per milligram urine creatinine) at 2 hours after CPB, which correspond to 96% sensitivity, optimal sensitivity and specificity, and 97% specificity, respectively.

Figure 8. Changes in urinary semaphorin 3A concentrations at various time points after CPB surgery in non-AKI and AKI patients, stratified by pRIFLE categories. *p ≤ 0.004 for differences between groups (non-AKI and each of the pRIFLE categories) by repeated-measures ANOVA.
Table 1. Descriptive statistics of patient characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AKI</th>
<th>No AKI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>26</td>
<td>34</td>
<td>–</td>
</tr>
<tr>
<td>Age, yr</td>
<td>4.3 ± 4.5</td>
<td>4.0 ± 4.6</td>
<td>0.81(^a)</td>
</tr>
<tr>
<td>Male, %</td>
<td>35</td>
<td>65</td>
<td>0.04(^b)</td>
</tr>
<tr>
<td>White, %</td>
<td>81</td>
<td>91</td>
<td>0.28(^b)</td>
</tr>
<tr>
<td>Prior surgery, %</td>
<td>38</td>
<td>35</td>
<td>1.0(^b)</td>
</tr>
<tr>
<td>Bypass time, min</td>
<td>188.4 ± 62.6</td>
<td>91.4 ± 47.8</td>
<td>&lt; 0.0001(^a)</td>
</tr>
<tr>
<td>Creatinine change, %</td>
<td>171.3 ± 133.9</td>
<td>11.6 ± 12.2</td>
<td>&lt; 0.0001(^a)</td>
</tr>
<tr>
<td>Duration of AKI, d</td>
<td>4.8 ± 4.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hospital stay, d</td>
<td>13.8 ± 11.6</td>
<td>4.8 ± 2.9</td>
<td>0.0006(^a)</td>
</tr>
<tr>
<td>Dialysis, %</td>
<td>8</td>
<td>0</td>
<td>0.18(^b)</td>
</tr>
<tr>
<td>Death, %</td>
<td>4</td>
<td>0</td>
<td>0.43(^b)</td>
</tr>
</tbody>
</table>

Means ± standard deviation (SD) are reported for continuous measures, percentages are reported for categorical variables.

\(^a\)Welch modified two-sample \( t \) test.

\(^b\)Fisher exact test.
Table 2. Spearman correlation coefficients of semaphorin 3A with clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Percent Change in Serum Creatinine</th>
<th>CPB Time</th>
<th>Hospital Length of Stay</th>
<th>Days AKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>-0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.16</td>
<td>-0.13</td>
<td>-0.26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 h</td>
<td>0.10</td>
<td>0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 h</td>
<td>0.03</td>
<td>0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12 h</td>
<td>-0.09</td>
<td>0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 h</td>
<td>-0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.17</td>
<td>0.13</td>
<td>0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21</td>
</tr>
<tr>
<td>48 h</td>
<td>-0.24</td>
<td>0.08</td>
<td>0.10</td>
<td>0.23</td>
<td>0.11</td>
</tr>
</tbody>
</table>

<sup>a</sup><em>P ≤ 0.05.</em>  
<sup>b</sup><em>P ≤ 0.002.</em>
Table 3. Urinary semaphorin 3A test characteristics at different cutoff values

<table>
<thead>
<tr>
<th>Cutoff Value for Semaphorin 3A, pg/mg of Urine Creatinine</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>0.96</td>
<td>0.03</td>
<td>0.43</td>
<td>0.50</td>
</tr>
<tr>
<td>492</td>
<td>0.81</td>
<td>0.94</td>
<td>0.91</td>
<td>0.87</td>
</tr>
<tr>
<td>910</td>
<td>0.62</td>
<td>0.97</td>
<td>0.94</td>
<td>0.77</td>
</tr>
</tbody>
</table>

The cutoff values are urinary semaphorin concentrations at 2 h after cardiopulmonary bypass, which correspond to 95% sensitivity, optimal sensitivity and specificity, and 95% specificity, respectively.
Figure 2.
Figure 3.
Figure 4.

A

Serum senophorin 3A (pg/ml)

Hours after cisplatin administration

B

Serum senophorin 3A (pg/ml)

Hours after reperfusion
Figure 5.
Figure 6.
Figure 7.

AUC=0.880, P<0.0001
Figure 8.