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LETTER TO THE EDITOR

Evaluation of EPI-X4 as a urinary peptide biomarker for diagnosis and prognosis of late acute GvHD

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Today, malignant and nonmalignant hematologic diseases can be treated with allogeneic hematopoietic cell transplantation (HCT). Following transplantation, many patients acquire life-threatening complications such as severe acute GvHD and require additional immunosuppressive therapy. Diagnosis and prognosis of acute GvHD relies on clinical parameters such as diarrhea, elevated liver tests and skin rash, and is confirmed by organ biopsies to distinguish GvHD from other complications that show similar symptoms. Currently, no method is available to predict the risk of acquiring GvHD or to diagnose GvHD without invasive procedures, although accumulating evidence suggests that plasma-derived biomarkers will be incorporated in the future.[1–4] Acute GvHD can be the 'classic' form occurring <100 days after HCT, or 'late acute GvHD' characterized by persistent, recurrent or new-onset acute GvHD present after day 100.[5]

A noninvasive method to identify specific biomarkers would allow impending GvHD to be recognized before the onset of clinical symptoms and might improve patients' outcomes by allowing pre-emptive treatment with immunosuppressive medications before GvHD is established. An ideal biomarker would also provide information about severity and likely treatment responsiveness. To identify a prognostic marker for GvHD, Kaiser *et al*. determined polypeptide patterns in urine from patients that developed GvHD using a liquid chromatography–mass spectrometry-based technique.[6] Among the 16 identified GvHD-specific peptides was a human serum albumin (hSA)-derived peptide encompassing amino-acid residues 408–423 (LVRYTKKVPQVSTPTL). This albumin fragment was also the most prominently excreted peptide and could be detected in urine up to 15 days before GvHD development.[6] Interestingly, the very same peptide was recently identified as natural antagonist of the CXC chemokine receptor 4 (CXCR4).[7] CXCR4 signaling has a key role in immune responses, organogenesis and inflammation, and its deregulation is associated with various diseases. The hSA (408–423) peptide binds to the extracellular region of CXCR4 and inhibits chemokine-induced receptor signaling, prompting the name EPI-X4 (endogenous peptide inhibitor of CXCR4). EPI-X4 mobilizes hematopoietic stem cells and has anti-inflammatory properties *in vivo*.[7] Moreover, the peptide reaches concentrations in the μg/mL range in the urine of patients with renal inflammation, suggesting its use as diagnostic marker for chronic kidney diseases.[7] Given the accepted role of inflammation in the development of GvHD[8] and the occurrence of EPI-X4 in the urine of classic acute GvHD samples,[6] we tested whether EPI-X4 might serve as novel biomarker for diagnosis of late acute GvHD.

To evaluate this, we determined EPI-X4 concentrations in urine obtained from 46 patients with recurrent or *de novo* late acute GvHD and 44 patients without late acute GvHD, all studied in the Chronic GvHD Consortium.[9] The median time from onset of classic acute to onset of late acute was 3.4 months (1.1–9.8) for 17 patients, with recurrent late acute GvHD. Persistent late acute GvHD was not included in this study. The study was Institutional Review Board approved, and all patients provided informed consent. Some of the late acute GvHD patients also had from day-100-before-onset samples available ($n=20$), and these samples were also tested. Cases and controls were initially selected to match for age at sample acquisition, HLA, transplant center, month from transplant to sample draw and prior classic acute GvHD. Clinical characteristics are presented in Table 1. Frozen urine aliquots were rapidly thawed and immediately analyzed using an EPI-X4-specific sandwich ELISA.[7,10] This validated ELISA is highly specific for EPI-X4 as it does not cross-react with other albumin fragments or the albumin precursor. Furthermore, it has a low intra-assay variation, a linear range between 0.078 and 5 ng/mL and a limit of detection for EPI-X4 in urine of ~0.154 ng/mL.[10]

Of the 110 urine samples tested, only 3 had EPI-X4 concentrations that were above the detection limit. Two came from one patient whose day 100-before-onset and late-acute-onset samples (obtained ~2 months later) had EPI-X4 levels of 71 and 8 ng/mL, respectively. The third sample had 7 ng/mL of EPI-X4, and came from a day-100-before-onset sample; however, this peptide was not detected in the late-acute-onset sample obtained 2.5 months later. Multivariate analyses were planned, but not conducted because of the limited number of patients with positive values. It is unlikely that external factors such as sample processing or technical problems with the ELISA affected our results because EPI-X4 is stable for 24 h at room temperature in urine and through multiple freeze–thaws.[10] The urine samples used in this study were frozen without processing unless they were obviously turbid, in which case they were first centrifuged. Neither processing method affects EPI-X4 levels.[7,10] Finally, the positive controls for each run were detected at appropriate levels and there were no technical difficulties with the assay. In summary, our results suggest that urinary levels of EPI-X4 are for the most part not detectable by ELISA in the patients we studied. We conclude that urinary EPI-X4 is unlikely to serve as a useful biomarker to diagnose late acute GvHD.

We pursued this study because EPI-X4 has been shown to be excreted in high levels in classic acute GvHD, as assessed by capillary electrophoresis and mass spectrometry.[6] Unfortunately, we did not have access to urine samples from classic acute GvHD patients, precluding a direct comparison with the data obtained by Kaiser *et al*.[6] Thus, future studies will be needed to address whether urinary levels of this CXCR4-antagonizing peptide have prognostic or diagnostic potential in classic acute GvHD. We have previously shown by ELISA that this peptide is present at concentrations of 1.73 ± 0.87 μg/mL in urine derived from 10 patients with renal failure.[7] These values are more than four orders of magnitude higher than the detection limit of the ELISA.[10]

We may also have failed to detect EPI-X4 in the present study because levels of the peptide were beyond the detection limit of the ELISA, and may require more sensitive methods of detection, such as mass spectrometry. Translating these lab- and cost-intensive methods to the clinic as a routine diagnosis method, however, is not feasible. It is also possible that EPI-X4 is not enriched in urine of patients with late acute GvHD, but is in those

Table 1. Patient characteristics

Group	*Control (N = 44)*	*Late acute (N = 46)*	*P-value*
Age at sample collection, median (range)	57.4 (28.6–70.1)	56.4 (20.4–73.3)	0.52
Gender			0.41
Female	17 (39%)	14 (30%)	
Male	27 (61%)	32 (70%)	
Months from transplant to sample collection, median (range)	5.7 (2.7–12.1)	5.1 (2.8–12.1)	0.35
Conditioning regimen			0.13
Myeloablative	15 (34%)	23 (50%)	
Not myeloablative	29 (66%)	23 (50%)	
Graft source			0.29
Bone marrow	6 (14%)	2 (4%)	
Cord	4 (9%)	4 (9%)	
Peripheral blood	34 (77%)	40 (87%)	
Donor type			0.97
Cord	4 (9%)	4 (9%)	
Related	12 (27%)	13 (30%)	
Unrelated	28 (64%)	27 (61%)	
Disease			0.84
ALL/AML	21 (48%)	26 (57%)	
CLL/CML	5 (11%)	3 (7%)	
HD/NHL	2 (5%)	3 (7%)	
MDS	6 (14%)	6 (13%)	
Others	10 (23%)	8 (17%)	
Prior classic acute grade			0.69
0	19 (48%)	25 (58%)	
1	2 (5%)	3 (7%)	
2	15 (38%)	13 (30%)	
3	3 (8%)	2 (5%)	
4	1 (3%)	0 (0%)	
Prednisone dose mg/kg per day, median (range)	0 (0–1.0)	0 (0–1.0)	0.48
Calcineurin inhibitor			0.37
No	4 (9%)	2 (4%)	
Yes	40 (91%)	44 (96%)	
Rapamycin			0.006
No	40 (91%)	31 (67%)	
Yes	4 (9%)	15 (33%)	
Mycophenolate mofetil			0.09
No	23 (52%)	32 (70%)	
Yes	21 (48%)	14 (30%)	
Creatinine mg/dL, median (range)	1.2 (0.6–2.6)	0.9 (0.5–1.7)	0.18

Abbreviations: HD = Hodgkin's disease; MDS = myelodysplastic syndrome; NHL = Non-Hodgkin's lymphoma.

with classic acute GvHD. Because late acute GvHD by definition cannot be diagnosed until 100 or more days after HCT compared with classic acute, which is usually diagnosed between 20–40 days after HCT, the differing immunologic milieu may result in unique biomarkers even if the disease manifests similarly. Numerous variables may affect biomarker levels, including timing of the sample collection relative to disease stage, and the presence or absence of concurrent immunosuppressive treatment.[11] These variables are not directly related to the underlying pathobiology, but have to be considered in biomarker studies. In our study, approximately half of the cases and controls had prior classic acute GvHD, and most were still on immunosuppressive therapy. In addition, although EPI-X4 was not enriched in urine samples from patients with late acute GvHD, it remains possible that it may be enriched in the corresponding plasma samples from these patients. In the present study, we focused on urine because it is easy and noninvasive to procure, and urinary EPI-X4 levels are stable, whereas the peptide is proteolytically degraded in serum samples,[7] which makes its reliable detection more complicated.

In summary, we did not find any meaningful elevation of the levels of EPI-X4 in the urine of late acute GvHD patients. Although the number of cases and controls was limited, the absence of EPI-X4 detection in most of the late acute GvHD samples suggests that pursuit of this peptide as a biomarker for this disease will not be fruitful. Future evaluation of this peptide in GvHD should focus on the classic acute GvHD setting, where the initial observations were made.

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CONFLICT OF INTEREST

OZ and JM declare that they filed an application for a patent to utilize the ELISA for the diagnosis and prognosis of diseases of the kidneys and GvHD. The remaining authors declare no conflict of interest.

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