pregnancy–associated miRNAs may clarify the molecular mechanism of CHMs and may lead to the discovery of novel therapeutic targets.

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References


Novel Circulating Isomers of Hepcidin

To the Editor:

The 25 amino acids constituting peptide hepcidin-25 (Hep25)\(^1\) are key regulators of iron metabolism (1). Two isoforms with 20 (Hep20) and 22 (Hep22) amino acids have been found in urine, whereas Hep20 has been detected in blood (2 and serum (3)). Increased concentrations of Hep25 are observed in chronic kidney disease (1). We used liquid chromatography–high-resolution mass spectrometry (LC-HRMS) to analyze plasma samples from patients with post-operative infections after gastrointestinal surgery. Analysis of these samples revealed high concentrations of C-reactive protein and several putative hepcidin isoforms. To the best of our knowledge, some of these hepcidin isoforms have not previously been reported.

Blood samples obtained by venipuncture and collected into lithium-heparin tubes were maintained at room temperature for 30 min and centrifuged at 1500g for 15 min at 4 °C, and then the plasma was transferred in 300-μL aliquots into 2-mL polypropylene tubes and stored at −80 °C until analysis. Written consent was obtained from the patients (n = 5) whose results are included in the present work.

The samples were processed according to the method described by Ikonen et al. (4), except that we used a QExactive mass spectrometer (ThermoScientific) for detection and a gradient running from 10% methanol in water (both containing 0.1% formic acid) to 100% methanol over 14.5 min to elute the hepcidins. Data were recorded by operating the QExactive in targeted single-ion–monitoring data-dependent tandem MS mode. The instrument recorded the m/z of the theoretically most abundant isotope of [M + 4H]\(^+\) of all anticipated isoforms of hepcidin, i.e., Hep25 (m/z 698.264), Hep24 (m/z 669.508), Hep23 (m/z 644.246), Hep22 (m/z 609.731), Hep21 (m/z 572.964), Hep20 (m/z 548.701), Hep19 (m/z 520.430), and the C-terminal–truncated hepcidin 1–24 (m/z 672.752) and hepcidin 1–23 (m/z 640.729), as well as the product ion spectra of the isoforms, if present. All mass spectra were interpreted with the aid of mMass 5.4.1 (5). Fig. 1 shows an extracted ion chromatogram. Hep25 eluted with a retention time (t\(_R\)) of 12.09

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Nonstandard abbreviations: Hep25, hepcidin-25; LC-HRMS, liquid chromatography-high resolution mass spectrometry.

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min and the [M + 4H]^{4+} ion was recorded with a mass accuracy (Δm) of 3.2 ppm for the isoforms Hep24 (t_R = 11.92 min, Δm = 1.7 ppm), Hep23 (t_R = 11.96 min, Δm = 1.7 ppm), Hep22 (t_R = 12.14 min, Δm = 0.2 ppm), Hep20 (t_R = 11.36 min, Δm = 1.2 ppm), and Hep19 (t_R = 10.74 min, Δm = 0.3 ppm). The fit between observed and in silico–generated isotope distributions was excellent for all recorded isoforms (data not shown).

The product ion spectra of all hepcidin isoforms shared several characteristic ions that are presented here. An internal b-ion containing the 15 amino acids CCGC CHRSDKGMCCCK was observed as its (b_{n-n+15} + H)^{2+} ion at m/z values of 799.25 and 799.75. The y_{19}^{2+} ion at m/z 1039.35 was observed in all spectra except Hep20 and Hep19, and the (y_{16} + H)^{2+} ion at m/z 858.78 and the (y_{16} + SH)^{2+} ion at m/z 874.77 were observed in all spectra except Hep20, Hep19, and an ion containing 13 amino acids was recorded at m/z 683.70 (b_{n-n+13})^{2+}, except for Hep23, and N-terminal b-ions were recorded in all spectra except for Hep20 and Hep19, whereas y_{2}^{+} (m/z 248.16) was recorded in all spectra.

On the basis of the observed signal-to-noise ratio of Hep25 in the calibration samples, we determined a limit of quantification of 0.6 ng/mL (signal-to-noise ratio = 10) for Hep25. Linear regression gave a calibration curve for Hep25 of: (Area_{Hep25}/Area_{ISTD}) = 0.0409(Hep25 ng/mL) − 0.00215 (R² = 0.9999), where ISTD is the internal standard. The isoforms were semiquantified using the equation above. Hep25 was detected in all 5 samples (range 11.9–337 ng/mL), Hep24 in 4 samples (5.0–28 ng/mL), Hep23 in 3 samples (0.7–3.7 ng/mL), Hep22 in 5 samples (1.0–11 ng/mL), Hep20 in 5 samples (4.5–27 ng/mL), and Hep19 in 1 sample (0.7 ng/mL).

This is the first described approach for the analysis of Hep25 for which LC-HRMS has been used, and our results could also explain why the isoforms have not been reported previously. Immunoassays monitor only total hepcidin, because the antibodies are not specific to Hep25 (or any of the isoforms). We provide evidence for the existence of the novel hepcidin isoforms. However, because only a few samples were included, no clinical relevance related to the observed isoforms can be deduced, except for the fact that plasma high in Hep25 tended to be relatively high in all other isoforms compared to plasma low in Hep25. The origins, target organs, and biological functions of the isoforms are not known, and further research is warranted to determine whether the isoforms are catabolites of Hep25 or whether they are excreted as final products.

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