Biomarkers Associated with Nephropathy

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Abstract

Use of urine biomarkers for diagnosing nephropathy, monitoring nephropathy progress, and assessing efficacy of a nephropathy treatment. These urine biomarkers include leukocyte-associated Ig-like receptor-2, alpha-1 acid glycoprotein, their fragments, and combinations thereof.
Fig. 2

Two Combine Peak (log10)

Legend:
- Health
- CHN

Sample sizes:
- Health: 44
- CHN: 36
BIOMARKERS ASSOCIATED WITH NEPHROPATHY

RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Application Ser. No. 12/694,639, filed Jan. 27, 2010, which claims priority to U.S. Provisional Application No. 61/147,785, filed on Jan. 28, 2009. The contents of both prior applications are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Nephropathy, commonly known as kidney damage, is caused by, among others, diabetes, high blood pressure, drug toxicity, and inflammation.

[0003] Typically, nephropathy is diagnosed by determining the level of proteinuria (e.g., the level of urine albumin), or by examining the glomerular filtration rate (GFR), an indicator of renal function. Both approaches are not suitable for detecting early stage nephropathy, which typically displays no symptoms. While nephropathy can also be detected by renal biopsy, this invasive procedure is not an ideal diagnostic approach.

[0004] It is of great importance to develop a method for detecting early stage nephropathy. The key to achieving this goal is to identify reliable biomarkers associated with incipient nephropathy.

SUMMARY OF THE INVENTION

[0005] The present invention is based on unexpected discoveries that the urine levels of leukocyte-associate Ig-like receptor-2, alpha-1 acid glycoprotein, and fragments of these two proteins are significantly higher in a nephropathy patient than in a nephropathy-free patient. These protein molecules are therefore reliable biomarkers for diagnosis of early stage nephropathy.

[0006] Accordingly, one aspect of this invention features a nephropathy diagnostic method. This method includes at least the following steps: (a) obtaining a urine sample from a subject suspected of having nephropathy, (b) determining in the urine sample a level of a biomarker, and (c) assessing whether the subject has nephropathy based on the level of the biomarker. The biomarker used in the just-described method is one of the following: (i) leukocyte-associated Ig-like receptor-2 or a fragment thereof having at least ten amino acid residues, such as DFLELIVKGTVPGTEASGF/1DAP (SEQ ID NO:1), (ii) a fragment of alpha-1 acid glycoprotein having at least ten amino acid residues, such as GQEFAHLLLIRDTKT/YMLAFDVN3EKNWGLS (SEQ ID NO:2), (iii) a combination of (i) and (ii), or (iv) a combination of (i) and alpha-1 acid glycoprotein.

[0007] An increase in the level of one of the four biomarkers, as compared to that in a nephropathy-free subject, indicates that the subject has nephropathy. In one example, the biomarker level is determined by a mass spectrometry assay (e.g., MALDI-MS, LC-MS, and LC-MS/MS). In another example, it is determined by an immune assay (e.g., ELISA, Western-blot, RIA, FIA and LIA).

[0008] Another aspect of this invention features a method for monitoring nephropathy progress in a subject. This method includes (a) obtaining a first urine sample from a subject suffering from nephropathy (e.g., a human or a laboratory animal), (b) determining in the first urine sample a level of one of the four biomarkers listed above, (c) obtaining a second urine sample from the subject 2 weeks to 12 months after the first urine sample is obtained, (d) determining in the second urine sample a level of the biomarker, and (e) assessing nephropathy progress in the subject. An increase in the level of the biomarker in the second urine sample, as compared to that in the first urine sample, indicates that nephropathy is exacerbated in the subject. When the subject is a human in early stage nephropathy, the second urine sample is obtained 6 to 12 months after the first urine sample is obtained. For a human subject in late stage nephropathy, the second urine sample can be obtained 3 to 6 months later than the first urine sample. When this method is applied to a laboratory animal, the second urine sample can be obtained 2 to 24 weeks after the first urine sample is obtained.

[0009] In still another aspect, the present invention provides a method for monitoring efficacy of a nephropathy treatment in a nephropathy patient, including (a) determining a level of one of the biomarkers listed above in a urine sample from the nephropathy patient before the treatment, (b) determining a level of the biomarker in a urine sample from the patient after the treatment, and (c) assessing efficacy of the treatment based on a change in the level of the biomarker after the treatment. The treatment is found to be effective when the post-treatment biomarker level remains the same or decreases as compared with the pre-treatment biomarker level.

[0010] This invention also provides a method of assessing renal toxicity of an agent, including (a) obtaining a plurality of urine samples from a subject treated with an agent at various time points during treatment, (b) determining in each of the urine samples a level of one of the above-described biomarkers, and (c) assessing renal toxicity of the agent based on a change in the level of the biomarker during the treatment. An increase in the biomarker level in the course of the treatment indicates that the agent is renal toxic. The agent can be a compound (e.g., a drug or a drug candidate), an herb product, and a food product.

[0011] This invention further provides a kit useful in any of the methods described above. This kit contains at least a first antibody specifically binding to leukocyte-associated Ig-like receptor-2 and a second antibody specifically binding to alpha-1 acid glycoprotein. Both antibodies can be whole immunoglobin molecules. In one example, this kit contains only antibodies specific to antigens to be detected (e.g., biomarkers associated with nephropathy) for practicing one of the methods disclosed herein. Namely, it consists essentially of such antibodies.

[0012] Also within the scope of this invention is an isolated antibody specifically binding to DFLELIVKGTVPGTEASGF/1DAP (SEQ ID NO:1), or GQEFAHLLLIRDTKT/YMLAFDVN3EKNWGLS (SEQ ID NO:2). The term “isolated antibody” used herein refers to an antibody substantially free from naturally associated molecules. More specifically, a preparation containing the antibody is deemed as “an isolated antibody” when the naturally associated molecules in the preparation constitute at most 20% by dry weight. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, and HPLC.
Any of the antibodies described above can be used in manufacturing a kit useful in practicing any of the methods of this invention.

The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following detailed description of several examples, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings are first described.

FIG. 2 is a diagram showing boxplots for combined levels of a fragment of leukocyte-associated Ig-like receptor-2 and a fragment of alpha-1 acid glycoprotein in healthy controls and patients having various types of nephropathy. DN, IgAN, CHN, and MGN refer to diabetic nephropathy, IgA nephropathy, Chinese herb nephropathy, and membranous glomerulonephritis nephropathy. The upper and lower limits of the boxes mark the 25% and 75% values with the medians as the lines across the boxes. The upper whisker marks the largest value below the upper fence, which is the 75% value plus 1.5 interquartile range and the lower whisker marks the smallest value above the lower fence, which is the 25% value minus 1.5 interquartile range.

In another aspect, this invention relates to a method for monitoring nephropathy progress in a subject based on any of the urine biomarkers described above. To practice this method, two urine samples from a subject can be obtained within a suitable time span (e.g., 2 weeks to 12 months) and examined to determine the levels of one of the urine biomarkers described above. If the urine biomarker level in the later-obtained urine sample is greater than that in the earlier-obtained urine sample, it indicates that nephropathy progresses in the subject.

The monitoring method can be applied to a human subject suffering from or at risk for nephropathy. When the human subject is at risk for or in early stage nephropathy, the level of the urine biomarker can be determined once every 6 to 12 months to monitor nephropathy progress. When the human subject is already in late stage of nephropathy, it is preferred that the urine biomarker level be determined once every 3 to 6 months.

In yet another aspect, the present invention provides a method for assessing efficacy of a nephropathy treatment in a subject in need (i.e., a human nephropathy patient or a laboratory animal bearing renal damage). In this method, the levels of one of the urine biomarkers described above are determined before, during, and/or after the treatment. If the urine biomarker level remains the same or decreases over the course of the treatment, it indicates that the treatment is effective.
Any of the urine biomarkers can also be used to monitor renal toxicity of a target agent, i.e., whether an agent induces renal damage. The target agent can be any compound or composition for human administration. Examples include, but are not limited to, chemical compounds, which can be drugs (e.g., non-steroidal anti-inflammatory drugs) or drug candidates, food products or supplements, and herb supplements. Renal toxicity of a target agent is indicated by its ability to increase the level of a urine biomarker over time.

Also disclosed herein is a kit useful in practicing any of the above-described methods. This kit contains at least two antibodies, one specific to Ig-like receptor-2, e.g., capable of binding to its fragment DFLELLVKTVPGEASGFADP (SEQ ID NO:1) or any epitope contained therein, and the other specific to alpha-1 acid glycoprotein, e.g., capable of binding to its fragment IQHEHFAHLIRTDKTYSMLAFDYNDEKNWGLS (SEQ ID NO:2) or any epitope contained therein. In one example, the kit includes two different antibodies (i.e., a coating antibody and a detecting antibody) that bind to the same biomarker. Typically, the detecting antibody is conjugated to a molecule which emits a detectable signal either on its own or via binding to another agent.

The term “antibody” used herein refers to a whole immunoglobulin or a fragment thereof, such as Fab or F(ab')2 that retains antigen-binding activity. It can be naturally occurring or genetically engineered (e.g., single-chain antibody, chimeric antibody, or humanized antibody).

The antibodies included in the kit of this invention can be obtained from commercial vendors. Alternatively, they can be prepared by conventional methods. See, for example, Harlow and Lane, (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. To produce antibodies against a particular biomarker as listed above, the marker, optionally coupled to a carrier protein (e.g., KLH), can be mixed with an adjuvant, and injected into a host animal. Antibodies produced in the animal can then be purified by affinity chromatography. Commonly employed host animals include rabbits, mice, guinea pigs, and rats. Various adjuvants that can be used to increase the immunological response depend on the host species and include Freund’s adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, CpG, surface-active substances such as lyssolecithin, phoronic polyls, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Useful human adjuvants include BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Polyclonal antibodies, i.e., heterogeneous populations of antibody molecules, are present in the sera of the immunized animal.

Monoclonal antibodies, i.e., homogeneous populations of antibody molecules, can be prepared using standard hybridoma technology (see, for example, Kohler et al. (1975) Nature 256, 495; Kohler et al. (1976) Eur. J. Immunol. 6, 511; Kohler et al. (1976) Eur J Immunol 6, 292; and Hammerling et al. (1981) Monoclonal Antibodies and T Cell Hybridomas, Elsevier, N.Y.). In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described in Kohler et al. (1975) Nature 256, 495 and U.S. Pat. No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al. (1983) Immunol Today 4, 72; Cole et al. (1983) Proc. Natl. Acad. Sci. USA 80, 2026, and the EBV-hybridoma technique (Cole et al. (1983) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridomas producing the monoclonal antibodies of the invention may be cultivated in vitro or in vivo. The ability to produce high titers of monoclonal antibodies in vivo makes it a particularly useful method of production.

Moreover, antibody fragments can be generated by known techniques. For example, such fragments include, but are not limited to, F(ab')2 fragments that can be produced by papain digestion of an antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of F(ab')2 fragments.

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limiting of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference.

Example 1

Diagnosing Nephropathy Using Urine Leukocyte-Associated Ig-like Receptor-2 or Alpha-1 Acid Glycoprotein as a Biomarker

Material and Methods

(i) Subjects

The following groups of human subjects were participated in this study:

(a) healthy donors: free of diabetic mellitus with normal renal functions,
(b) DM patients: having type 2 diabetic mellitus, but free from nephropathy,
(c) DN patients: having diabetic nephropathy,
(d) DN uremia patients: having DN associated with uremia,
(e) IgAN patients: having IgA nephropathy,
(f) MGN patients: having membranous glomerulonephritis,
(g) CHN patients: having nephropathy induced by Chinese herb, and
(h) CIN patients: having chronic interstitial nephritis.

Clinical characteristics of the healthy donors and the patients are summarized in Table 1 below:

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Healthy</th>
<th>DM</th>
<th>DN</th>
<th>DN uremia</th>
<th>IgAN</th>
<th>MGN</th>
<th>CHN</th>
<th>CIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>67.94</td>
<td>57.33</td>
<td>72.38</td>
<td>58.14</td>
<td>28.33</td>
<td>38.00</td>
<td>47.42</td>
<td>59.88</td>
</tr>
<tr>
<td></td>
<td>(12.30)</td>
<td>(10.52)</td>
<td>(7.44)</td>
<td>(12.79)</td>
<td>(12.31)</td>
<td>(13.11)</td>
<td>(10.43)</td>
<td>(7.62)</td>
</tr>
</tbody>
</table>
TABLE 1-continued

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Healthy</th>
<th>DM</th>
<th>DN</th>
<th>DN + Uremia</th>
<th>IgAN</th>
<th>MGN</th>
<th>CHN</th>
<th>CIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, n (%)</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>14</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(37.5)</td>
<td>(33.33)</td>
<td>(12.5)</td>
<td>(28.57)</td>
<td>(44.44)</td>
<td>(66.67)</td>
<td>(73.68)</td>
<td>(50.00)</td>
<td></td>
</tr>
<tr>
<td>Serum Creatinine (mg/dL), mean (SD)</td>
<td>0.86</td>
<td>0.85</td>
<td>1.39</td>
<td>4.23</td>
<td>1.03</td>
<td>0.60</td>
<td>5.39</td>
<td>3.54</td>
</tr>
<tr>
<td>(0.14)</td>
<td>(0.24)</td>
<td>(0.49)</td>
<td>(4.65)</td>
<td>(0.48)</td>
<td>(0.20)</td>
<td>(5.29)</td>
<td>(2.28)</td>
<td></td>
</tr>
<tr>
<td>MDRD S GFR, mean (SD)</td>
<td>86.28</td>
<td>106.86</td>
<td>58.60</td>
<td>58.02</td>
<td>104.29</td>
<td>144.96</td>
<td>22.70</td>
<td>24.32</td>
</tr>
<tr>
<td>(13.19)</td>
<td>(70.47)</td>
<td>(20.75)</td>
<td>(61.53)</td>
<td>(55.19)</td>
<td>(55.19)</td>
<td>(15.01)</td>
<td>(15.01)</td>
<td></td>
</tr>
</tbody>
</table>

(ii) MALDI-MS Assay

Midstream urinary samples were collected from the groups of human subjects listed above in early morning. These urine samples from both healthy donors and patients, mixed with protease inhibitors, were analyzed by MALDI-TOF-MS. Peptide candidates that were differentially presented in the healthy donor group and the various patient groups were identified upon comparison of polypeptide patterns between each patient group and the healthy donor group, taking into consideration statistical evaluation of demographic and sample parameters. These peptides were purified, their amino acid sequences determined via routine technology.

(iii) Western blot assay

Western blotting analysis was performed using antibodies specific to leukocyte-associated Ig-like receptor-2 fragment, DFLELLVKGTVPGTEASFGFDAP (SEQ ID NO:1) and alpha-1 acid glycoprotein fragment, GQEHFAHLILRDTKYMLAFDNDEKWNWGLS (SEQ ID NO:2), following routine technology. The results were normalized against the level of creatinine or protein in the same sample.

(iv) ELISA

Urine samples were mixed with protease inhibitors and diluted at 1:100 with a dilution buffer and serum samples were diluted at 1:10. The diluted samples were placed in ELISA plates in triplicates. The concentrations of leukocyte-associated Ig-like receptor-2 and alpha-1 acid glycoprotein were determined via the standard sandwich ELISA method and normalized against the level of creatinine or protein in the same sample.

Results

Via the MALDI-MS assay described above, peptides DFLELLVKGTVPGTEASFGFDAP (SEQ ID NO:1) and GQEHFAHLILRDTKYMLAFDNDEKWNWGLS (SEQ ID NO:2) were detected in urine samples from nephropathy patients at much higher levels as compared to urine samples from healthy controls. The levels of these two peptides in the healthy donor group and in the various patient groups are shown in Table 2 below:

<table>
<thead>
<tr>
<th>Positive rates of Positive rates of Patient SEQID NO: 2 SEQ ID NO: 1</th>
<th>Categories</th>
<th>Groups</th>
<th>Patient Numbers</th>
<th>Positive rates of</th>
<th>Positive rates of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SEQ ID NO: 2 N (%)</td>
<td>SEQ ID NO: 1 N (%)</td>
</tr>
<tr>
<td>Healthy</td>
<td>Health</td>
<td>19</td>
<td>0 (0%)</td>
<td>1 (5.3%)</td>
<td></td>
</tr>
<tr>
<td>Diabetic Nephropathy</td>
<td>DM</td>
<td>7</td>
<td>0 (0%)</td>
<td>2 (28.6%)</td>
<td></td>
</tr>
<tr>
<td>DN</td>
<td>8</td>
<td>2 (25%)</td>
<td>8 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DN + Uremia</td>
<td>11</td>
<td>6 (54.5%)</td>
<td>8 (72.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune-mediated IgAN</td>
<td>IgAN</td>
<td>12</td>
<td>0 (0%)</td>
<td>6 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Nephropathy</td>
<td>MGN</td>
<td>3</td>
<td>0 (0%)</td>
<td>2 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Interstitial</td>
<td>CHN</td>
<td>18</td>
<td>10 (55.6%)</td>
<td>17 (94.4%)</td>
<td></td>
</tr>
<tr>
<td>Nephritis</td>
<td>CIN</td>
<td>7</td>
<td>3 (42.9%)</td>
<td>7 (100%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>85</td>
<td>21</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>

The results also show that the urine levels of these two peptides in nephropathy patients were not correlated with proteinuria, indicating that they can be used to detect kidney lesions prior to the appearance of proteins, particularly albumin, in urine.

Further, the urine levels of these two peptides in nephropathy patients were found to exhibit reverse correlations with GFR, indicating that they can serve as markers for monitoring renal function changes and nephropathy progression.

Via the ELISA and Western blot assays described above, leukocyte-associated Ig-like receptor-2 and alpha-1 acid glycoprotein were found to be differentially presented in urine samples from nephropathy patients (e.g., patients having Chinese herb-induced nephropathy) versus urine samples from healthy controls. See Table 3 below. More specifically, presence of either protein in urine samples from healthy controls was barely detectable; while a higher level of the protein was found in urine samples from nephropathy patients. This result indicates that either protein can be used as a marker for diagnosing nephropathy.
TABLE 2

<table>
<thead>
<tr>
<th>Ratios of Alpha-1 Acid Glycoprotein to Creatinine in Various Patient Groups</th>
<th>Healthy</th>
<th>DM</th>
<th>DN</th>
<th>IgAN</th>
<th>MGN</th>
<th>CHN</th>
<th>CIN</th>
<th>DN uremia</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGP/Cr (ng/mg) × 1000 mean (SD)</td>
<td>2.57 (2.52)</td>
<td>2.91 (1.68)</td>
<td>29.18 (30.93)</td>
<td>15.52 (20.87)</td>
<td>139.51 (137.53)</td>
<td>19.55 (39.36)</td>
<td>51.31 (65.46)</td>
<td>97.13 (140.69)</td>
</tr>
</tbody>
</table>
What is claimed is:

1. An isolated antibody specifically binding to DFLELLVKGTVPGEASGFDAP, or GQEHFAHLLILRDRTKTYMLAFDVNEKMNGLS.
2. The antibody of claim 1, specifically binding to DFLELLVKGTVPGEASGFDAP.
3. The antibody of claim 1, specifically binding to GQEHFAHLLILRDRTKTYMLAFDVNEKMNGLS.
4. The antibody of claim 1, wherein the antibody is a monoclonal antibody.
5. The antibody of claim 1, wherein the antibody is a whole immunoglobulin molecule.
6. The antibody of claim 1, wherein the antibody is a single-chain antibody, a chimeric antibody, or a humanized antibody.
7. A kit for diagnosing nephropathy, comprising a first antibody specifically binding to leukocyte-associated Ig-like receptor-2 and a second antibody specifically binding to alpha-1 acid glycoprotein.
8. The kit of claim 7, wherein the first and second antibodies are whole immunoglobulin molecules.
9. The kit of claim 7, wherein the first and second antibodies are monoclonal antibodies.
10. The kit of claim 7, wherein the first antibody specifically binds to DFLELLVKGTVPGEASGFDAP.
11. The kit of claim 7, wherein the second antibody specifically binds to GQEHFAHLLILRDRTKTYMLAFDVNEKMNGLS.
12. The kit of claim 7, wherein the first antibody specifically binds to DFLELLVKGTVPGEASGFDAP, and the second antibody specifically binds to GQEHFAHLLILRDRTKTYMLAFDVNEKMNGLS.
13. The kit of claim 7, consisting essentially of the first antibody and the second antibody.
14. The kit of claim 7, further comprising reagents and materials for an immunoassay, the immunoassay being ELISA, Western blot, RIA, FIA or LIA.