[Supplementary Information]

Selective reaction monitoring approach using structure-defined synthetic glycopeptides for validating glycopeptide biomarkers pre-determined by bottom-up glycoproteomics

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## Supplementary Information

| Section | Description                                                                 | Page(s) |
|---------|------------------------------------------------------------------------------|---------|
| Experimental |                                                                               | S3-12   |
| Figure S1 | NMR spectra of compound 1                                                     | S13     |
| Figure S2 | NMR spectra of compound 2                                                     | S14     |
| Figure S3 | NMR spectra of compound 3                                                     | S15     |
| Figure S4 | NMR spectra of compound 5                                                     | S16     |
| Figure S5 | HPLC profiles to determine the concentrations                               | S17     |
| Figure S6 | XICs for the preparation of calibration curves                               | S18-19  |
| Figure S7 | XICs in the SRM assay of human serum samples                                 | S20-21  |
| Table S1  | Chemical shifts of glycopeptides 1, 2, 3, and 5                               | S22-26  |
| Table S2  | Mass parameters used in this study                                            | S27     |
| Table S3  | Amino acid analysis of glycopeptide 3                                         | S28     |
| Table S4  | Concentrations of glycopeptides 1 and 2                                       | S28     |
| Table S5  | Serum samples and the results of SRM assay                                   | S29     |
**EXPERIMENTAL**

**Materials and general methods.** This study was performed in accordance with the ethical standards of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Hirosaki University Graduate School of Medicine, Hokkaido University Graduate School of Advanced Life Science, and Showa University Department of Laboratory Medicine. Written informed consent was obtained from all serum donors. Serum samples from newly diagnosed RCC patients (n=8) were collected before treatment at Hirosaki University\(^{51}\) and healthy control samples (n=8) were collected at Showa University Hospital, respectively. After serum collection, all samples were immediately aliquoted, frozen, and stored at -80°C until pre-processing by our experimental workflow.

All commercially available solvents and reagents were used without further purification. Trityl-OH-ChemMatrix® resin was purchased from Biotage Japan Ltd. \(N\)-\(\alpha\)-Fluorenylmethoxycarbonyl-amino acid (Fmoc-amino acid) derivatives except for glycosylated amino acids were purchased from Merck Millipore. \(N\)-\(\alpha\)-Fluorenylmethoxycarbonyl-N-\(\beta\)-(2-\(N\)-acetylamido-2-deoxy-\(\beta\)-D-glucopyranosyl)-L-asparagine [Fmoc-Asn(Ac3\(\beta\)-GlcNAc)-OH] was synthesized by the procedure reported previously\(^{52}\) and purchased partly from Medicinal Chemistry Pharmaceuticals, Co. Ltd.
The α2,3-sialyltransferase from *Pasteurella multocida* recombinant, expressed in *E. coli* BL21 were purchased from Sigma-Aldrich. Sialylglycopeptide (SGP) derived from egg yolk and *endo-β-N*-acetylglucosaminidase from *Mucor hiemalis* recombinant, expressed in *Candida boidinii* (*endo-M*) were purchased from Tokyo Chemical Industry Co., Ltd. Cytidine-5′-monophospho-N-acetylneuraminic acid sodium salt (CMP-Neu5Ac) was purchased from Yamasa Co., Ltd. Sequencing Grade Modified Trypsin was purchased from Promega Corporation.

Mixing operation in peptide synthesis was performed by a vortex mixer. The microwave was irradiated during coupling reactions and Fmoc removal using temperature control at 50°C. MALDI-TOFMS spectra was obtained with Bruker ultraflex III mass spectrometer in reflector positive or linear negative mode using matrix as 2,5-dihydroxybenzoic acid (DHB). Typically, the sample were dissolved in 1 μL of 50% aqueous acetonitrile (ACN) and mixed with the same volume of 10 mg/mL DHB in 50% aqueous ACN containing 0.1% 2,2,2-trifluoroacetic acid (TFA). RP-HPLC analysis was conducted with Hitachi system. Separation was performed with L-6250 intelligent pump, L-7400 UV detector and C18 column: Intersil ODS-3 250×20 mm I.D. (GL Sciences Inc.). Analysis (purity check) was performed with L-7100 pump, L-7405 UV detector, and C18 column: Intersil ODS-3 250×4.6 mm I.D. (GL Sciences Inc.). Solvents used for RP-HPLC
in this study were (a) 0.1% TFA aq. and (b) 0.1% TFA in ACN. All 1H- and 13C-NMR spectra for the structural identification of synthetic glycopeptides were collected with 600 MHz Bruker AVANCE (Bruker Biospin Co.). NMR spectra was recorded at 298 K with $^1$H: 600 MHz and $^{13}$C: 125 MHz. Chemical shift is given in ppm and based on internal tetramethylsilane (TMS): $\delta$H 0.00 in CDCl$_3$. NMR spectra is assigned with ACD/NMR processor (Advanced Chemistry Development, Inc.).

**Microwave-assisted solid-phase synthesis.** Glycopeptide 3, a key intermediate as an acceptor substrate for endo-M, was synthesized according to the procedures reported previously.$^{S3-S6}$ The followings are the general protocols and conditions for the solid-phase glycopeptide synthesis assisted by microwave irradiation.$^{S6}$ The coupling reactions were performed by using Trityl-ChemMatrix resin (0.41 mmol/g, 76.5 mg, 31.4 $\mu$mol), Fmoc-amino acid (125.5 $\mu$mol, 4.0 eq.), and Fmoc-Asn(Ac$_3$β-GlcNAc)-OH (62.7 $\mu$mol, 2.0 eq.). Firstly, Trityl-ChemMatrix resin was chlorinated in the following procedure. The resin (76.5 mg) was placed on the bottom of a polypropylene tube equipped with a filter (LibraTube®, Hipep Laboratories) and swollen with dichloromethane (DCM) at room temperature for 2 h. The resin was added 2% SOCl$_2$ in DCM and shaken with vortex mixer for overnight. The resin was washed with DCM six times. Then, the first amino acid derivative, Fmoc-Arg(Pbf)-OH (94.1 $\mu$mol, 3.0 eq.) was dissolved in DCM (600 $\mu$L)
containing \(N,N\)-dimethylformamide (DMF) (70 \(\mu\)L) and \(N,N\)-diisopropylethylamine (DIEA) (6 eq., 33 \(\mu\)L). This solution was added to the resin and the tube was shaken at room temperature for overnight. After the solution was removed, the resin was added 25% DIEA/methanol and shaken at room temperature for 2 h. Then, the resin was washed with DCM, DMF, methanol, diethyl ether three times each and dried in vacuo for overnight. The resin was allowed to swell in DCM for 2 h. After filtration and washed with DMF three times, 20% piperidine in DMF was added to remove Fmoc group and the tube was shaken under microwave irradiation for 3 min. The microwave was irradiated during Fmoc removal and amino acid coupling reactions at 50°C. The resin was washed with DMF three times.

In advance, other Fmoc amino acid derivatives (125.5 \(\mu\)mol, 4.0 eq.) were dissolved in 0.4 M 1-[Bis(dimethylamino)methyl]iumyl]-1H-benzotriazole-3-oxide hexafluorophosphate (HBTU), 1-hydroxybenzotriazole monohydrate (HOBt)/DMF (4 eq.) and DIEA (188 \(\mu\)mol, 6 eq.) in DMF. Then, the solution of the corresponding Fmoc amino acid derivative was added to the Trityl-ChemMatrix resin displaying the first amino acid residue and mixed under microwave irradiation at 50°C for 9 min. Especially, in case of the reaction with sugar amino acid derivative, Fmoc-Asn(Ac\(_3\)\(\beta\)-GlcNAc)-OH (62.7 \(\mu\)mol, 2.0 eq.) was dissolved in 0.4 M HBTU, HOBt/DMF (2 eq.) and DIEA (125
μmol, 4 eq.) in DMF and allowed to react with resin at 50°C for 30 min under microwave irradiation. After washing with DMF three times, unreacted amino groups were capped with acetyl group by treating with a solution (Ac₂O/DIEA/DMF = 85/10/5). The resin was washed with DMF six times. The streamlined procedures such as Fmoc-removal, amino acid coupling, and acetyl capping as mentioned above were performed repeatedly. After completing the total synthesis, cleavage cocktail (TFA: H₂O: triisopropylsilane = 95:2.5:2.5) was added to the resin and the reaction mixture was shaken at room temperature for 2 h. The solution was filtered and TFA was removed by evaporation, and then 10 mL of t-butyl methyl ether was added. The filtrate in t-butyl methyl ether was centrifuged and supernatant was removed. The residual solid was dissolved in 50% acetonitrile/water and lyophilized. Next, the residue was dissolved in methanol and pH of the solution was adjusted to 12.5 with 1N NaOH aq. The solution was kept at room temperature for 1 h to remove acetyl groups from sugar residues and neutralized with 1 N AcOH aq., then concentrated. Crude product was purified by RP-HPLC to afford compound 3 (9.1 mg, 15% overall yield). MALDI-TOFMS: m/z calculated for C₅₈H₆₆N₁₅O₂₅, [M+H]⁺ 1886.91, found 1887.25. (See also, Figure S5).

**Synthesis of clusterin glycopeptide 2.** An acceptor substrate, compound 3 (7.9 mg, 3.5 μmol, final; 2.5 mM), was dissolved in 200 mM (final; 100 mM) potassium phosphate
buffer (pH 6.3) and added 160 mM (final; 16 mM) SGP as donor substrate and 500 mU/mL (final; 45 mU/mL) endo-M. After incubation at 37°C for 1 h, the reaction mixture was subjected to the purification by RP-HPLC (retention time, 37.59 min) to give clusterin glycopeptide 2 (2.3 mg, 15%). MALDI-TOFMS: \textit{m/z} calculated for \(\text{C}_{158}\text{H}_{250}\text{N}_{26}\text{O}_{86}, [M-H]^\cdot\) 3886.59, found 3886.831. (See also, Figure S4).

\textbf{Synthesis of clusterin glycopeptide 1}. Clusterin glycopeptide 2 (5.26 mg, 1.35 \(\mu\)mole) was incubated in 225.5 \(\mu\)L of 0.1 M HCl aq. at 80°C for 30 min according to the condition reported previously.\textsuperscript{S7} The reaction mixture was neutralized and purified by RP-HPLC (retention time, 37.90 min) to give asialo glycopeptide 5 (2.0 mg, 45%). MALDI-TOFMS: \textit{m/z} calculated for \(\text{C}_{136}\text{H}_{216}\text{N}_{24}\text{O}_{70}, [M-H]^\cdot\) 3304.4002, found 3304.802. (See also, Figure S6).

Asialo product 5 (3.40 mg, 1.03 \(\mu\)mole, final 1.0 mM) was dissolved in 500 mM (final; 250 nM) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0). This solution was added to a 100 mM (final; 15 mM) CMP-Neu5Ac and 1000 U/mL (final; 75 mU/mL) recombinant \(\alpha_2,3\)-sialyltransferase, and the mixture was incubated at 37°C for 8 h. The reaction mixture was subjected directly to the purification by RP-HPLC (retention time, 37.52 min) to yield pure clusterin glycopeptide 1 (1.8 mg, 45%). MALDI-
TOFMS: $m/z$ calculated for C$_{158}$H$_{250}$N$_{26}$O$_{86}$, [M-H]- 3886.59, found 3886.633. (See also, Figure S3).

1H NMR and HSQC spectra for the structural characterization of the synthetic glycopeptides 1, 2, 3, and 5 were measured with 600 MHz and recorded at 298 K (Figure S3-S6). Based on the obtained data, the structures of all new compounds were confirmed by attributing each proton and carbon as summarized in Table S2.

**Concentration of the synthetic clusterin glycopeptides.** Concentrations of the standard solution of clusterin glycopeptides 1 and 2 was determined by combining amino acid analysis and RP-HPLC analysis because of the limited amounts of the glycopeptides 1 and 2 to determine the correct concentrations from their weights. Firstly, amino acid analysis of the glycopeptide 3 was carried out and the result was used for the determination of the concentration to be 590 μM (Table S3). Then, the standard solution was subjected to spike with the measurement of RP-HPLC profiles of glycopeptides 1 and 2. By using the RP-HPLC profiles of glycopeptides 1 and 2 in the presence of concentration-defined glycopeptide 3, the concentrations were estimated to be 168 μM and 146 μM, respectively (Figure S7 and Table S4). These solutions were employed by dilution to adjust 10 μM before the SRM experiments.
**Immunoaffinity depletion of high-abundant proteins from human serum samples.** Before injection onto the multiple affinity removal system (MARS), human serum (15 μL) was diluted with 200 μL of Equil/Lad/Wash Buffer (Agilent Technology, Cat. No. 5185-5987). The sample was transferred to a 0.22 μm spin tube and centrifuged for 1 min at 10,000 g to remove particulates. The filtered sample was applied to Multi Affinity Removal Spin Cartridge (Agilent Technology, Cat. No. 5188-6560) and centrifuged at 100 g for 1 min. Flow-through was removed and the cartridge was kept for 5 min at room temperature. Then, Equil/Load/Wash Buffer (400 μL) was added to the cartridge and centrifuged at 100 g for 2 min (x 2 times). Flow-through fractions were collected and washed with MilliQ through an Amicon Ultra-0.5 centrifugal Filter Unit with Ultrace-50 membrane (Merck Millipore, Cat. No. UFC5050) at 12,000 g for 5 min. The residual solution was dried by centrifugal evaporator.

**Reductive alkylation and tryptic digestion of the depleted serum glycoproteins.**

Dried sample was dissolved in 50 mM ammonium bicarbonate (100 μL) and the solution was subjected to the reduction with 1,4-dithiothreitol (DTT, 10 μL, 100 mM in MilliQ) at 60°C for 1 h, followed by alkylation with iodoacetamide (IAA, 7 μL, 123 mM in MilliQ), and incubated in dark at room temperature for 30 min. Then, the mixture was treated with sequencing grade trypsin [10 μL, trypsin/serum proteins=1/50 (w/w)] at 37°C.
for 16 h.

Finally, tryptic digests were desalinated using GL-tip GC (GL Science, Cat. No. 7820-11201) as follows: The tryptic digests were applied to GL-Tip GC equilibrated with 20 μL of 5% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) and centrifuged at 3,000 g for 5 min. The column was washed with 20 μL of 5% acetonitrile in water containing 0.1% TFA and glycopeptides/peptides were eluted by centrifugation at 3,000 g for 5 min with 80 μL of 80% acetonitrile in water containing 0.1% TFA. The residual sample was dried up by centrifugal evaporator and dissolved in 15 μL of MilliQ containing 0.1% formic acid. The solution was used directly to LC-MS/MS analysis.

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Figure S1. (A) $^1$H-NMR and (B) HSQC ($D_2O$, 600 MHz, at 298 K) of compound 1.
Figure S2. (A) $^1$H-NMR and (B) HSQC ($D_2O$, 600 MHz, at 298 K) of compound 2.
Figure S3. (A) $^1$H-NMR and (B) HSQC (DMSO, 600 MHz, at 298 K) of compound 3.
Figure S4. (A) $^1$H-NMR and (B) HSQC (D$_2$O, 600 MHz, at 298 K) of compound 5.
Figure S5. HPLC profile to determine the concentration of glycopeptide 1 (A) and 2 (B) under the gradient from acetonitrile/H$_2$O=95/5 (0 min) to acetonitrile/H$_2$O=70/30 (50 min).
Figure S6. XICs of a series of standard mixtures based on SRM channels Q1/Q3 = 1297.2/657.4; the concentrations are 0 nM (A), 100 nM (B), 250 nM (C), 500 nM (D), 1000 nM (E) and 2500 nM (F).
Figure S7. XICs in the SRM assay using 10 μL of serum samples from ccRCC patients (left A-H: entry 1-8) and healthy subjects (right A-H: entry 9-16) based on SRM channels Q1/Q3 = 1297.2/657.4.
Table S1. $^1$H and $^{13}$C-NMR chemical shifts of synthetic N-linked glycopeptides (1, 2, 3, and 5).

| Residues | $^1$H Shifts | $^{13}$C Shifts |
|----------|--------------|----------------|
|          | 1            | 2            | 3            | 5            | 1            | 2            | 3            | 5            |
| Leu372   | $^\alpha$    | 4.290        | 4.286        | 4.335        | 4.286        | $\alpha$    | 52.60        | 52.65        | 51.89        | 52.64        |
|          | $^\beta$     | 1.569        | 1.553        | 1.486        | 1.562        | $\beta$     | 39.59        | 39.62        | 41.26        | 40.11        |
|          | $^\gamma$    | 1.507        | 1.504        | 1.597        | 1.503        | $\gamma$    | 24.40        | 24.41        | 24.51        | 24.38        |
|          | $^\delta$    | 0.751        | 0.735        | 0.852        | 0.739        | $\delta$    | 22.42        | 22.44        | 22.22        | 20.87        |
|          | $^\omega$    | 0.801        | 0.797        | 0.852        | 0.801        | $\omega$    | 8.129        | 8.136        | 7.900        | 8.156        |
| Ala373   | $^\alpha$    | 4.229        | 4.225        | 4.207        | 4.188        | $\alpha$    | 49.73        | 49.73        | 51.88        | 49.70        |
|          | $^\beta$     | 1.264        | 1.261        | 1.590        | 1.265        | $\beta$     | 16.64        | 16.71        | 24.53        | 16.69        |
|          | $^\omega$    | 8.572        | 8.569        | 7.785        | 8.551        | $\omega$    | 24.50        | 24.53        | 24.50        | 24.50        |
| Asn374   | $^\alpha$    | 4.565        | 4.562        | 4.519        | 4.568        | $\alpha$    | 49.95        | 49.97        | 49.97        | 49.99        |
|          | $^\beta$     | 2.632        | 2.625        | 2.415        | 2.622        | $\beta$     | 36.28        | 36.28        | 37.29        | 36.27        |
|          | $^\gamma$    | 2.742        | 2.743        | 2.670        | 2.758        | $\gamma$    | 37.79        | 37.79        | 37.79        | 37.79        |
|          | $^\delta$    | 8.416        | 8.417        | 8.347        | 8.426        | $\delta$    | 8.417        | 8.417        | 8.417        | 8.417        |
|          | $^\omega$    | 8.544        | 8.525        | 8.242        | 8.533        | $\omega$    | 8.544        | 8.525        | 8.242        | 8.533        |
| Leu375   | $^\alpha$    | 4.290        | 4.286        | 4.335        | 4.286        | $\alpha$    | 52.60        | 52.65        | 51.89        | 52.64        |
|          | $^\beta$     | 1.569        | 1.553        | 1.486        | 1.562        | $\beta$     | 39.59        | 39.62        | 41.26        | 40.11        |
|          | $^\gamma$    | 1.507        | 1.504        | 1.597        | 1.503        | $\gamma$    | 24.40        | 24.41        | 24.51        | 24.38        |
|          | $^\delta$    | 0.751        | 0.735        | 0.852        | 0.739        | $\delta$    | 22.42        | 22.44        | 22.22        | 20.87        |
|          | $^\omega$    | 0.801        | 0.797        | 0.852        | 0.801        | $\omega$    | 8.129        | 8.136        | 7.900        | 8.156        |
| Thr376   | $^\alpha$    | 4.219        | 4.215        | 4.228        | 4.218        | $\alpha$    | 59.16        | 59.18        | 58.66        | 59.18        |
|          | $^\beta$     | 4.077        | 4.085        | 4.038        | 4.089        | $\beta$     | 66.93        | 66.95        | 67.06        | 66.92        |
|          | $^\gamma$    | 1.081        | 1.078        | 1.038        | 1.083        | $\gamma$    | 18.93        | 18.81        | 20.16        | 18.88        |
|          | $^\omega$    | 8.123        | 8.117        | 7.882        | 8.115        | $\omega$    | 8.123        | 8.117        | 7.882        | 8.115        |
| Gln377   | $^\alpha$    | 4.221        | 4.215        | 4.267        | 4.221        | $\alpha$    | 53.23        | 53.24        | 52.81        | 53.22        |
|          | $^\beta$     | 1.883        | 1.882        | 1.778        | 1.881        | $\beta$     | 26.87        | 26.86        | 28.05        | 31.08        |
|          | $^\gamma$    | 1.997        | 1.993        | 1.922        | 1.997        | $\gamma$    | 31.29        | 31.10        | 31.11        | 26.85        |
|          | $^\delta$    | 2.233        | 2.231        | 2.245        | 2.233        | $\delta$    | 6.873        | -            | -            | -            |
|          | $^\omega$    | 8.294        | 8.285        | 8.174        | 8.284        | $\omega$    | 8.294        | 8.285        | 8.174        | 8.284        |
| Gly378   | $^\alpha$    | 3.861        | 3.857        | 3.757        | 3.863        | $\alpha$    | 42.45        | 42.49        | 42.53        | 42.45        |
|          | $^\beta$     | 8.329        | 8.328        | 8.263        | 8.321        | $\beta$     | 8.329        | 8.328        | 8.263        | 8.321        |
| Residues | 1  | 2  | 3  | 5  | 1  | 2  | 3  | 5  |
|---------|----|----|----|----|----|----|----|----|
| Glu379  | Hα | 4.161 | 4.164 | 4.219 | 4.157 | Ca | 54.03 | 53.89 | 53.05 | 54.03 |
|         | Hβ | 1.841 | 1.837 | 1.800 | 1.826 | Cβ | 27.73 | 27.57 | 28.09 | 33.56 |
|         | Hy | 1.979 | 1.977 | 1.930 | 1.965 |     |      |      |      |      |
|         | Hγ | 2.152 | 2.162 | 2.121 | 2.154 | Cγ | 33.62 | 33.46 | 31.77 | 27.76 |
|         | HNα| 8.262 | 8.071 | 8.111 |       |     |      |      |      |      |
| Asp380  | Hα | 4.393 | 4.393 | 4.484 | 4.393 | Ca | 51.86 | 51.81 | 50.71 | 51.85 |
|         | Hβ | 2.550 | 2.552 | 2.502 | 2.547 | Cβ | 38.35 | 38.30 | 38.46 | 38.86 |
|         | Hγ | 2.550 | 2.552 | 2.642 | 2.547 |     |      |      |      |      |
|         | HNα| 8.346 | 8.344 | 8.331 | 8.343 |     |      |      |      |      |
| Gln381  | Hα | 4.075 | 4.074 | 4.062 | 4.072 | Ca | 53.26 | 53.29 | 53.21 | 53.25 |
|         | Hβ | 1.738 | 1.738 | 1.668 | 1.739 | Cβ | 26.31 | 26.35 | 27.90 | 30.75 |
|         | Hy | 1.803 | 1.805 | 1.838 | 1.800 |     |      |      |      |      |
|         | Hγ | 2.012 | 2.010 | 2.041 | 2.014 | Cγ | 30.77 | 30.77 | 31.93 | 26.33 |
|         | HNα| 8.116 | 8.113 | 8.120 | 8.223 |     |      |      |      |      |
| Tyr382  | Hα | 4.336 | 4.333 | 4.243 | 4.337 | Ca | 55.44 | 55.39 | 55.53 | 55.45 |
|         | Hβ | 2.782 | 2.779 | 2.648 | 2.784 | Cβ | 35.84 | 35.74 | 37.21 | 35.69 |
|         | Hy | 2.870 | 2.867 | 2.744 | 2.871 |     |      |      |      |      |
|         | Hγ | 6.903 | 6.900 | 6.883 | 6.914 | C(2,6) | 130.3 | 130.3 | 130.5 | 130.3 |
|         | HNα| 8.003 | 7.999 | 8.019 | 8.004 |     |      |      |      |      |
| Tyr383  | Hα | 4.337 | 4.375 | 4.360 | 4.380 | Ca | 54.94 | 54.94 | 55.07 | 54.91 |
|         | Hβ | 2.814 | 2.811 | 2.774 | 2.815 | Cβ | 35.93 | 35.93 | 37.05 | 35.87 |
|         | Hy | 2.907 | 2.905 | 2.922 | 2.908 |     |      |      |      |      |
|         | Hγ | 6.976 | 6.971 | 7.069 | 6.977 | C(2,6) | 130.5 | 130.5 | 130.7 | 130.5 |
|         | HNα| 7.645 | 7.647 | 8.069 | 7.646 |     |      |      |      |      |
| Leu384  | Hα | 4.173 | 4.171 | 4.264 | 4.175 | Ca | 52.34 | 52.35 | 51.79 | 52.35 |
|         | Hβ | 1.457 | 1.454 | 1.511 | 1.458 | Cβ | 39.37 | 39.33 | 41.14 | 39.36 |
|         | Hy | 1.340 | 1.338 | 1.584 | 1.341 | Cγ | 24.12 | 24.16 | 24.51 | 24.14 |
|         | HNα| 0.739 | 0.735 | 0.879 | 0.741 | Cδ | 20.80 | 20.82 | 22.31 | 22.43 |
|         | HNα| 0.789 | 0.785 | 0.879 | 0.789 |     |      |      |      |      |
|         | HNα| 7.842 | 7.842 | 8.017 | 7.841 |     |      |      |      |      |
| Residues | 1  | 2  | 3  | 5  | 1  | 2  | 3  | 5  |
|----------|----|----|----|----|----|----|----|----|
| Arg385   |    |    |    |    |    |    |    |    |
| Hα       | 4.030 | 4.028 | 4.130 | 4.030 | Cα | 54.60 | 54.58 | 52.46 | 54.61 |
|          | 1.590 | 1.587 | 1.578 | 1.587 | Cβ | 28.90 | 28.93 | 28.98 | 28.92 |
|          | 1.726 | 1.723 | 1.773 | 1.728 |    |    |    |    |
| Hγ       | 1.456 | 1.454 | 1.492 | 1.453 | Cy | 24.51 | 24.50 | 25.80 | 24.52 |
|          | 3.035 | 3.036 | 3.064 | 3.038 | Cε | 40.76 | 40.77 | 40.70 | 40.77 |
| HNα      | 7.653 | 7.652 | 7.925 | 7.650 |    |    |    |    |
| HNδ      | 7.044 | 7.041 | 8.247 | 7.041 |    |    |    |    |
| GlcNAc1  |    |    |    |    |    |    |    |    |
| H1       | 4.926 | 4.927 | 4.815 | 4.933 | C1 | 78.16 | 78.16 | 79.35 | 78.12 |
|          | 3.739 | 3.734 | 3.518 | 3.736 | C2 | 53.64 | 53.66 | 55.24 | 53.64 |
|          | 3.634 | 3.637 | 3.337 | 3.569 | C3 | 73.18 | 72.94 | 74.88 | 72.70 |
|          | 3.553 | 3.548 | 3.099 | 3.626 | C4 | 78.49 | 78.53 | 70.84 | 78.38 |
|          | 3.421 | 3.419 | 3.451 | 3.415 | C5 | 76.11 | 76.03 | 74.50 | 76.11 |
|          | 3.535 | 3.532 | 3.448 | 3.652 | C6 | 59.78 | 59.83 | 61.27 | 61.01 |
| H6       | 3.712 | 3.698 | 3.650 | 3.721 |    |    |    |    |
| HN       | 8.100 | 8.103 | 7.838 | 8.097 |    |    |    |    |
| GlcNAc2  |    |    |    |    |    |    |    |    |
| H1       | 4.497 | 4.496 | 4.502 | 4.933 | C1 | 101.1 | 101.1 | 101.1 |    |
|          | 3.656 | 3.648 | 3.670 | 3.670 | C2 | 54.87 | 54.68 | 54.90 |    |
|          | 3.663 | 3.661 | 3.631 | 3.631 | C3 | 72.00 | 72.01 | 72.04 |    |
|          | 3.625 | 3.617 | 3.627 | 3.627 | C4 | 79.35 | 79.61 | 79.34 |    |
|          | 3.493 | 3.487 | 3.496 | 3.496 | C5 | 74.41 | 74.36 | 74.38 |    |
|          | 3.757 | 3.733 | 3.652 | 3.652 | C6 | 59.93 | 60.23 | 59.83 |    |
| H6       | 3.881 | 3.867 | 3.721 |    |    |    |    |    |
| HN       | 8.334 | 8.330 | 8.340 |    |    |    |    |    |
| Man3     |    |    |    |    |    |    |    |    |
| H1       | 4.655 | 4.665 | 4.658 | 4.658 | C1 | 100.3 | 100.3 | 100.3 |    |
|          | 4.142 | 4.150 | 4.146 | 4.146 | C2 | 70.11 | 70.11 | 70.1  |    |
|          | 3.667 | 3.672 | 3.667 | 3.667 | C3 | 80.36 | 80.48 | 80.39 |    |
|          | 3.506 | 3.530 | 3.511 | 3.511 | C4 | 74.33 | 74.24 | 72.81 |    |
|          | 3.697 | 3.673 | 3.705 | 3.705 | C5 | 65.41 | 65.49 | 65.51 |    |
|          | 3.696 | 3.670 | 3.659 | 3.659 | C6 | 65.74 | 65.86 | 65.75 |    |
| H6       | 3.856 | 3.850 | 3.859 |    |    |    |    |    |
| Residues | 1     | 2     | 3     | 5     | 1   | 2   | 3   | 5   |
|----------|-------|-------|-------|-------|-----|-----|-----|-----|
| Man4     | 5.009 | 5.025 | 5.016 | C1   | 99.40 | 99.38 | 99.38 |
|          | 4.085 | 4.090 | 4.088 | C2   | 76.37 | 76.31 | 76.32 |
|          | 3.787 | 3.789 | 3.799 | C3   | 69.41 | 69.46 | 69.16 |
|          | 3.386 | 3.389 | 3.401 | C4   | 67.28 | 67.33 | 67.15 |
|          | 3.522 | 3.509 | 3.639 | C5   | 72.82 | 72.82 | 73.39 |
|          | 3.504 | 3.513 | 3.508 | C6   | 61.72 | 61.67 | 61.65 |
|          |       |       |       |      | 3.806 | 3.803 | 3.809 |
| Man4'    | 4.819 | 4.839 | 4.824 | C1   | 96.94 | 96.77 | 96.87 |
|          | 4.011 | 4.009 | 4.007 | C2   | 75.55 | 76.12 | 76.23 |
|          | 3.787 | 3.789 | 3.789 | C3   | 69.41 | 69.46 | 69.39 |
|          | 3.386 | 3.399 | 3.388 | C4   | 67.28 | 67.36 | 67.30 |
|          | 3.522 | 3.509 | 3.510 | C5   | 72.82 | 72.82 | 72.86 |
|          | 3.504 | 3.513 | 3.508 | C6   | 61.72 | 61.67 | 61.65 |
|          |       |       |       |      | 3.806 | 3.803 | 3.809 |
| GlcNAc5  | 4.467 | 4.495 | 4.477 | C1   | 99.36 | 99.20 | 99.33 |
|          | 3.642 | 3.689 | 3.643 | C2   | 54.84 | 54.73 | 54.86 |
|          | 3.663 | 3.661 | 3.667 | C3   | 72.00 | 72.01 | 71.99 |
|          | 3.638 | 3.548 | 3.554 | C4   | 78.19 | 80.56 | 78.48 |
|          | 3.455 | 3.487 | 3.473 | C5   | 74.71 | 74.36 | 74.58 |
|          | 3.757 | 3.740 | 3.743 | C6   | 59.93 | 60.02 | 59.94 |
|          |       |       |       |      | 3.881 | 3.859 | 3.873 |
| GlcNAc5' | 4.467 | 4.495 | 4.477 | C1   | 99.36 | 99.20 | 99.33 |
|          | 3.642 | 3.689 | 3.643 | C2   | 54.84 | 54.73 | 54.86 |
|          | 3.663 | 3.661 | 3.667 | C3   | 72.00 | 72.01 | 71.99 |
|          | 3.638 | 3.548 | 3.554 | C4   | 78.19 | 80.56 | 78.48 |
|          | 3.455 | 3.487 | 3.473 | C5   | 74.71 | 74.36 | 74.58 |
|          | 3.757 | 3.740 | 3.743 | C6   | 59.93 | 60.02 | 59.94 |
|          |       |       |       |      | 3.881 | 3.859 | 3.873 |
| HN       | 8.109 | 8.120 | 8.110 |       |       |       |       |
| GlcNAc5' | 4.467 | 4.495 | 4.477 | C1   | 99.36 | 99.20 | 99.33 |
|          | 3.642 | 3.689 | 3.643 | C2   | 54.84 | 54.73 | 54.86 |
|          | 3.663 | 3.661 | 3.667 | C3   | 72.00 | 72.01 | 71.99 |
|          | 3.638 | 3.548 | 3.554 | C4   | 78.19 | 80.56 | 78.48 |
|          | 3.455 | 3.487 | 3.473 | C5   | 74.71 | 74.36 | 74.58 |
|          | 3.757 | 3.740 | 3.743 | C6   | 59.93 | 60.02 | 59.94 |
|          |       |       |       |      | 3.881 | 3.859 | 3.873 |
| HN       | 8.157 | 8.120 | 8.156 |       |       |       |       |
| Residues | 1  | 2  | 3  | 5  | 1  | 2  | 3  | 5  |
|----------|----|----|----|----|----|----|----|----|
| Gal6     | 4.441 | 4.336 | 4.634 | C1 | 102.5 | 103.4 | 102.8 |
| H2       | 3.463 | 3.427 | 3.453 | C2 | 69.33 | 70.66 | 70.94 |
| H3       | 3.632 | 3.560 | 3.565 | C3 | 71.99 | 72.23 | 72.42 |
| H4       | 3.850 | 3.818 | 3.820 | C4 | 67.40 | 68.34 | 68.51 |
| H5       | 3.606 | 3.714 | 3.623 | C5 | 75.10 | 73.62 | 75.24 |
| H6       | 3.448 | 3.434 | 3.449 | C6 | 62.40 | 63.28 | 62.41 |
| Gal6'    | 4.441 | 4.336 | 4.634 | C1 | 102.5 | 103.4 | 102.8 |
| H2       | 3.463 | 3.427 | 3.453 | C2 | 69.33 | 70.66 | 70.94 |
| H3       | 3.632 | 3.560 | 3.565 | C3 | 71.99 | 72.23 | 72.42 |
| H4       | 3.850 | 3.818 | 3.820 | C4 | 67.40 | 68.34 | 68.51 |
| H5       | 3.606 | 3.714 | 3.623 | C5 | 75.10 | 73.62 | 75.24 |
| H6       | 3.448 | 3.434 | 3.449 | C6 | 62.40 | 63.28 | 62.41 |
| Neu5Ac7  | 1.697 | 1.599 |       |    | 39.57 | 40.09 |       |
| H3(ax)   | 2.653 | 2.552 |       |    |       |       |       |
| H3(eq)   | 2.653 | 2.552 |       |    |       |       |       |
| H4       | 3.579 | 3.546 |       |    | 68.39 | 68.18 |       |
| H5       | 3.743 | 3.700 |       |    | 51.68 | 51.86 |       |
| H6       | 3.586 | 3.585 |       |    | 72.04 | 72.45 |       |
| H7       | 3.488 | 3.453 |       |    | 68.03 | 68.32 |       |
| H8       | 3.788 | 3.783 |       |    | 71.74 | 71.62 |       |
| H9       | 3.534 | 3.533 |       |    | 62.47 | 62.62 |       |
| HN       | 7.955 | 7.933 |       |    |       |       |       |
| Neu5Ac7' | 1.697 | 1.599 |       |    | 39.57 | 40.09 |       |
| H3(ax)   | 2.653 | 2.552 |       |    |       |       |       |
| H3(eq)   | 2.653 | 2.552 |       |    |       |       |       |
| H4       | 3.579 | 3.546 |       |    | 68.39 | 68.18 |       |
| H5       | 3.743 | 3.700 |       |    | 51.68 | 51.86 |       |
| H6       | 3.586 | 3.585 |       |    | 72.04 | 72.45 |       |
| H7       | 3.488 | 3.453 |       |    | 68.03 | 68.32 |       |
| H8       | 3.788 | 3.783 |       |    | 71.74 | 71.62 |       |
| H9       | 3.534 | 3.533 |       |    | 62.47 | 62.62 |       |
| HN       | 7.972 | 7.933 |       |    |       |       |       |
Table S2. Recommended default mass parameters used in this study

| Parameter                          | 5-10 μL/min | 200 μL/min |
|-----------------------------------|-------------|------------|
| Curtain Gas                       | 10          | 40         |
| Collision Gas                     | High        | High       |
| IonSpray Voltage (Positive)       | 5000        | 5500       |
| Temperature                       | 0           | 400        |
| Ion Source Gas1                   | 20          | 50         |
| Ion Source Gas2                   | 0           | 80         |
| Interface Heater                  | on          | off        |
| Declustering Potential            | 70          | 70         |
| Entrance Potential                | 10          | 10         |
Table S3. The result of amino acid analysis of glycopeptide 3

| amino acid | concentration (nmol/40 µL) |
|------------|-----------------------------|
| Asp        | 11.94631                    |
| Thr        | 5.69843                     |
| Ser        | 0.03384                     |
| Glu        | 17.87096                    |
| Gly        | 5.93202                     |
| Ala        | 5.85351                     |
| Cystine    | 0.01410                     |
| Val        | 0.01973                     |
| Leu        | 17.66651                    |
| Nle        | 1.08671                     |
| Tyr        | 11.80230                    |
| Phe        | 0.01977                     |
| Lys        | 0.01866                     |
| His        | 0.01369                     |
| NH₃        | 21.35955                    |
| Arg        | 5.98819                     |

Table S4. Peak areas and calculated concentrations from HPLC spectrums in Figure S7

| glycopeptide | peak area | concentration (µM) |
|--------------|-----------|--------------------|
| 3            | 550182    | 590                |
| 1            | 147108    | 168                |
| 3            | 514549    | 590                |
| 2            | 136567    | 146                |
| Entry | Clinical diagnosis | Sex | Age | TNM classification | Conc. of clusterin with glycopeptide 2 [nM] |
|-------|-------------------|-----|-----|--------------------|------------------------------------------|
| 1     | RCC               | male| 60's| T1aN0M1            | 315.3                                    |
| 2     | RCC               | female| 70's| T1bN1M0            | 513.3                                    |
| 3     | RCC               | male| 70's| T3N0M0            | 461.8                                    |
| 4     | RCC               | male| 40's| T1aN0M0            | 461.1                                    |
| 5     | RCC               | male| 40's| T1aN0M0            | 488.2                                    |
| 6     | RCC               | male| 40's| T1aN0M0            | 538.9                                    |
| 7     | RCC               | female| 60's| T1aN0M0            | 354.9                                    |
| 8     | RCC               | male| 60's| T3bN0M1          | 363.7                                    |
| 9     | healthy           | male| 50's| —                | 465.9                                    |
| 10    | healthy           | male| 50's| —                | 440.9                                    |
| 11    | healthy           | male| 50's| —                | 574.0                                    |
| 12    | healthy           | male| 60's| —                | 601.8                                    |
| 13    | healthy           | female| 50's| —                | 697.5                                    |
| 14    | healthy           | male| 60's| —                | 458.1                                    |
| 15    | healthy           | male| 50's| —                | 503.5                                    |
| 16    | healthy           | female| 60's| —                | 561.4                                    |

Serum samples from 8 RCC patients (entry 1-8) were collected before treatment at Hirosaki University and 8 healthy subjects (entry 9-16) were collected at Showa University Hospital, respectively. Immediately after serum collection, samples were aliquoted, frozen, and stored at -80°C until pre-processing by an experimental workflow.