Development of sugar chain-binding single-chain variable fragment antibody to adult T-cell leukemia cells using glyco-nanotechnology and phage display method

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Adult T-cell leukemia (ATL) is an intractable blood cancer caused by the infection of human T-cell leukemia virus type-1, and effective medical treatment is required. It is known that the structure and expression levels of cell surface sugar chains vary depending on cell states such as inflammation and cancer. Thus, it is expected that the antibody specific for ATL cell surface sugar chain would be an effective diagnostic tool and a strong candidate for the development of an anti-ATL drug. Here, we developed a stable sugar chain-binding single-chain variable fragment antibody (scFv) that can bind to ATL cells using a fibre-type Sugar Chip and phage display method. The fiber-type Sugar Chips were prepared using O-glycans released from ATL cell lines. The scFv-displaying phages derived from human B cells (diversity: 1.04 × 10^8) were then screened using the fiber-type Sugar Chips, and an O-glycan-binding scFv was obtained. The flow cytometry analysis revealed that the scFv predominantly bound to ATL cell lines. The sugar chain-binding properties of the scFv was evaluated by array-type Sugar Chip immobilized with a library of synthetic glycosaminoglycan-disaccharide structures. Highly sulphated disaccharide structures were found to have high affinity to scFv.

Keywords: adult T-cell leukemia; anti-sugar chain antibody; phage display; scFv; Sugar Chip.

Abbreviations: arSC, array-type Sugar Chip; ATL, adult T-cell leukemia; CS, chondroitin sulphate; fiSC, fiber-type Sugar Chip; GAG, glycosaminoglycan; HS, heparan sulphate; HTLV-1, human T-cell lymphotropic virus type-1; LSPR, localized surface plasmon resonance; PBMCs, peripheral blood mononuclear cells; scFv, single chain variable fragment antibody; SPR, surface plasmon resonance.

ATL is an intractable cancer caused by the infection of retrovirus, human T-cell leukemia virus type-1 (HTLV-1) (1, 2). Approximately 20 million people worldwide are estimated to be infected with HTLV-1 and around 2.5-5% of HTLV-1 carriers develop ATL after an incubation period of >40 years. ATL is divided into four types based on clinical features: acute, lymphoma, chronic and smoldering (3). The progression of the cancer varies depending on clinical subtypes but the median survival time is 8.3 months, and the 4-year overall survival rate is 11% for the most severe acute type of ATL in Japan (4). Although its prognosis is quite poor, effective clinical treatment for ATL is under development. Recently, anti-CC chemokine receptor 4 (CCR4) antibody, mogamulizumab, was clinically approved in Japan as a novel monoclonal antibody for relapsed and refractory ATL (5, 6). However, it sometimes causes severe skin lesions and the median survival time for relapsed ATL is still short (13.7 months) (7). Thus, a more effective antibody with minimum side effects is strongly desired.

Cell surface sugar chains play significant roles in various biological events including cell–cell recognition, immune response and signal transduction (8). Sugar chains also act as biomarkers because their structures and expression levels are remarkably varied in response to the cell states (9–11). To date, various tumour-associated sugar chain antigens such as Tn antigen and fucose-containing sugar chain have been found (12–14). We therefore hypothesized that the sugar chain specifically expressed on the ATL cell surface could be an attractive biomarker, and the antibody for the sugar chain could be an effective diagnostic tool and a strong candidate as an effective anti-cancer drug. However, the development of anti-sugar chain antibodies is not easy because (i) the determination of a cancer-specific sugar chain is difficult due to its complex structure, (ii) the amount of sugar chain expressed on the cell surface is low and (iii) in vivo antibody-producing cell response against sugar chains is quite poor unless using strong immunomodulator.

Single-chain fragment variable antibody (scFv) is a fusion protein that consisted of variable regions of heavy (VH) and light (VL) chains of immunoglobulin (15, 16). VH and VL domains of a scFv are joined with
a flexible peptide linker, and this forms the completely binding site of an antibody. So far, a number of scFVs have been developed and its efficacy as a molecularly targeted drug is being investigated in clinical studies (16). To obtain an antigen-specific scFV, phage display method is an effective in vitro screening technique (17, 18). From the libraries containing a huge number of different bacteriophages that display an scFv as a coat protein, an antigen-specific phage can be isolated by a series of recursive cycles of selection on antigens and then a soluble scFv can be easily produced by protein expression system in Escherichia coli.

Recently, based on our glyco-nanotechnology, a fiber-type Sugar Chip (fiSC) was developed as an analytical tool for the interaction of sugar chains with sugar chain-binding proteins using localized surface plasmon resonance (LSMR) (19). Only a small amount of sugar chain was necessary to prepare the fiSCs, and the analysis using the fiSC was done in a microlitre sample solution. In this study, we applied our Sugar Chip technology to the screening of scFv-displaying phages to obtain cell surface sugar chain-binding scFvs that can selectively bind to ATL cells. Using the fiSCs immobilized with cell surface O-glycans obtained from ATL cell lines, S1T, a stable scFv that binds to ATL cell lines, but not to non-ATL leukemias obtained from ATL cell lines, S1T, a stable scFv binding scFvs that can selectively bind to ATL cells. Reuse of scFv immobilized with synthetic sulphated disaccharides evaluated using our array-type Sugar Chip (arSC) to prepare complete RPMI-1640. Milli-Q water (18.2 MΩ cm−1) was used in all experiments unless otherwise noted.

**Materials and Methods**

**Reagents**

For extraction of O-glycans, Triton X-100, ammonium hydrogen carbonate, dithiothreitol and skimmed milk were purchased from Nakalai Tesque (Kyoto, Japan). 2-idoacetamide was purchased from Wako Pure Chemical Industries (Osaka, Japan). A BiotGlyco® glycan purification and labelling kit was purchased from Sumitomo Bakelite (Tokyo, Japan). Histopaque®-1077, trypsin and sodium cyanoborohydride were purchased from Sigma-Aldrich (St. Louis, MO). N-glycanase F was purchased from Roche Diagnostics (Basel, Switzerland). For preparation of the fiSC, N, N-dimethylpropylenlyleamine, sodium borohydride and triethylamine were purchased from Nakalai Tesque. (3-(trimethoxysilyl)propyl)ethylenediamine was purchased from Sigma-Aldrich. Water-soluble carbodiimide hydrochloride was purchased from Peptide Institute (Osaka, Japan). Au colloid solution-SC (40 nm) was purchased from Sumitomo Bakelite (Tokyo, Japan). Histopaque®-1077, trypsin and sodium borohydride were purchased from Peptide Institute (Osaka, Japan). Ethylenediamine was purchased from Sigma-Aldrich. Histopaque®-1077 was used for cell culture, EDifco® 2×10^8 cells) or PBMCs (1.0×10^8 cells) or PBMCs (1.0×10^8 cells) for 1 h at 80°C. After removing the supernatant, a 20 mM HEPES buffer containing 1% Triton X-100 solution (1.0 ml) was added to the pellet and incubated for 30 min at room temperature. Then, the supernatant was carefully transferred to a fresh tube, and the sample was dissolved in H2O (100 μl) and incubated with dithiothreitol (10 μl, 120 mM) and ammonium bicarbonate (10 μl, 1 M) for 30 min at 60°C. Thereafter, 2-idoacetamide (20 μl, 123 mM) was added to the reaction solution and further incubated for 1 h at room temperature in the dark. Next, the sample was treated with trypsin (20 μl, 40 μl) for 1 h at 37°C. After that, trypsin was inactivated by heating for 5 min at 90°C. The trypsinized proteins were treated with N-glycanase F (5 units) overnight at 37°C to eliminate N-glycans in the proteins. Then, the sample was removed using a vacuum concentrator, and the residue was dissolved in H2O (20 μl). N-glycans in the solution were eliminated with the BiotGlyco® glycan purification and labelling kit according to the manufacturer’s specifications. The O-glycans of the obtained proteins were released through hydrazinolysis in accordance with the previously reported method (25).

**Conjugation of f-mono linker with O-glycans released from S1T cells**

Reagent-O-glycans from S1T cells were conjugated with f-mono linker (24) after partial purification using BiotGlyco® glycan purification and labelling kit (Sumitomo Bakelite) according to the manufacturer’s specifications with slight modification to the f-mono conjugation. O-glycans released from S1T cells were dissolved in H2O (20 μl) and transferred to the tube containing BiotGlyco beads. O-Glycans were then captured on the beads by incubating for 1 h at 80°C in 2% acetic acid in acetonitrile (180 μl). Next, the beads were washed with 2 M guanidine, H2O and 1% trimethylamine in methanol. Unreacted hydrazide groups on the beads were capped in a treatment of 10% acetic anhydride in methanol. After several washings with 2 M guanidine solution, the bound O-glycans were recovered from the beads by incubating in the mixture of 2% acetic acid in acetonitrile (180 μl) and H2O (20 μl) for 1.5 h at 70°C.
The recovered O-glycans were in situ conjugated with f-mono linker (10 mg, 34 μmol) in 30% acetic acid/dimethyl sulfoxide solution (100 μL) containing sodium cyanoborohydride (6.2 mg, 164 μmol) for 12 h at 60°C. The f-mono linker conjugated O-glycans and the BlotGlyco® beads were separated by centrifugation, and the excess f-mono linker in the solution was removed, with the spin column included in the BlotGlyco® kit.

**Fractionation of f-mono linker conjugated O-glycans with HPLC**

The f-mono linker conjugated O-glycans were fractionated by HPLC using ODS column (250 mm × 4.6 mm, SP-120-5-ODS-BP, DAISSO, Japan) in a column oven at 40°C. The f-mono linker labelled O-glycans were eluted from the column using 50% methanol at a flow rate of 0.5 mL/min, and detected with UV (absorbance at 231 nm) and fluorescence (excitation at 338 nm and emission at 380 nm).

**Removal of PBMC-binding phages**

To remove PBMC-binding scFv-displaying phages, BlotGlyco® beads immobilized with O-glycans released from PBMCs of a healthy volunteer were prepared. O-glycans from PBMCs (1 × 10⁶ cells) were released through the treatment of hydrazine, and reacted with BlotGlyco® beads according to the manufacturer’s specifications. Non-specific binding of the beads was blocked using 5% skim milk in PBS. Then, a phage library solution (100 μL) was added to the beads and incubated for 30 min at room temperature. The supernatant of the beads solution separated by centrifugation was used for further experiments.

**Preparation of fSC using O-glycans from ST1 cells**

To prepare fSC, a sensitive layer of optical fiber (internal diameter: 50 μm) was functionalized with O-glycans from ST1 cells via gold nanoparticles (φ = 40 nm) as described previously (19). The sensitive layer of the optical fiber was washed with ethanol and reacted with N-(3-trimethoxysilyl)propyl) ethylenediamine (17 μL, 79 μmol) in acetic acid (75 μL) for 10 min at room temperature using a magnetic stirrer. After washing with ethanol, the fiber was heated at 120°C for 30 min. The sensitive layer was then reacted with α-lipoic acid (6.7 mg, 33 μmol) using water-soluble carbodiimide hydrochloride, 3H-1, 2, 3-triazole-4, 5-b)-pyridine-3-ol (4.1 mg, 30 μmol), and N, N-disopropylethylene (11 μL, 63 μmol) in N,N-dimethylformamide (1.5 mL) using a magnetic stirrer for 3 h at room temperature. Then, the fiber was treated with 50 mM sodium borohydride aqueous solution (2.0 mL) for 2 h and subsequently reacted with gold nanoparticles (40 nm, 2.0 mL) using a magnetic stirrer for 35 min at room temperature. The fiber functionalized with gold nanoparticles was then immobilized with f-mono linker conjugated O-glycans (10 μL, 4.5, 6.6 and 0.47 nmol for SC-1, SC-2 and SC-3, respectively) for 35 min at room temperature.

**Screening of scFv-displayed phages using fSC**

O-glycan-binding scFv-displaying phages were screened using the fSCs while being monitored using LSPR (19). An fSC was soaked into phage solution (100 μL) for 20 min and was washed three times by soaking in PBS containing 0.05% Tween 20 (PBS/T, 50 μL) for 5 min. The O-glycan-binding phages were then eluted by soaking in 100 mM trichloroacetic acid (50 μL) for 5 min. After that, the eluate was immediately adjusted to pH 7.4 by mixing with 1 M Tris-HCl (100 μL) at 4°C.

**Selective expression of O-glycan-binding phages**

The O-glycan-binding phage solution (30 μL) was added to an *Escherichia coli* TG1 cell culture medium (OD₆₀₀ of 0.5, 500 μL) and incubated for 1.5 h at 37°C. Then, the TG1 cells (100 μL) were seeded on a 2YT agar plate and incubated overnight at 37°C. The resulting colonies were randomly selected, and each colony was further incubated at 37°C in 2YT agar medium (1 mL) until the cells reached an OD₆₀₀ of 0.5–0.7. Next, M13K07 helper phage (3.0 × 10¹² pfu/mL, multiplicity of infection value is 5) was co-infected into TG1 cells. After incubation for 1 h at 37°C, the TG1 cells were harvested by centrifugation for 10 min at 3,000g, and re-suspended in a 2YTAK medium (1.5 mL). The TG1 cells were further incubated overnight in a shaker at 37°C, and the TG1 cells were harvested by centrifugation. Then the phages released in the culture supernatant were precipitated in a cold 20% poly(ethylene glycol) (PEG)/NaCl (2.5 M) solution (300 μL), and the precipitates were suspended in PBS (300 μL). To express soluble scFvs, the phages were infected with *E. coli* HB2151 as described above. The resulting colonies were further incubated overnight in a shaker at 37°C in 2YTAG medium (5 mL). The culture medium was then transferred to another 2YTAG medium (45 mL) and further incubated for 1 h at 37°C. The HB2151 cells were harvested by centrifugation for 10 min at 3,000g, and the resulting pellet was suspended in a 2YTAI medium (50 mL). After incubation for 20 h in a shaker at 30°C, the cells were collected by centrifugation for 10 min at 3,000g, 4°C. The obtained pellets were suspended in a 1 × TES buffer (2 mL, 0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose) and incubated for 5 min at 4°C to disrupt the cell membrane. The solution was added to 1/5 × TES buffer (750 μL) and further incubated for 30 min at 4°C. Then the HB2151 cells were sonicated for 20 sec and cooled on ice for 40 sec, and this process was repeated 20 times. The resulting solution was centrifuged at 15,000g for 5 min at 4°C, and supernatant containing scFv was filtered with a 0.45-μm filter unit.

**Purification of scFv**

The obtained scFv was purified by affinity chromatography using COSMOGEL His-accept® (Nacalai Tesque) according to the manufacturer’s specifications. Briefly, a scFv solution (1 mL) was charged on His-accept. After washing the column five times with a washing buffer (20 mM imidazole, 30 mM phosphate buffer and 300 mM sodium chloride, pH 8.0). scFv was eluted with an elution buffer (500 mM imidazole, 50 mM phosphate buffer and 300 mM sodium chloride, pH 8.0). Then the scFv solution was converted to PBS by dialysis. The obtained scFvs were analysed by SDS-PAGE using a 12.5% polyacrylamide gel under reducing conditions, and stained with coomassie brilliant blue (CBB) according to the typical procedure. Typical western blotting was performed by transferring proteins from the gel to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk in PBS/T. After washing with PBS/T three times, the membrane was incubated with anti-His antibody (goat, 1:1,000 dilution with 5% skimmed milk in PBS/T, GE Healthcare) followed by anti-Mouse IgG HRP antibody (goat, 1:5,000 dilution with 5% skimmed milk in PBS/T, Novagen, Madison, WI). The gel was developed using Chemi-Lumi One.

**DNA sequence for determination of the obtained scFvs**

The nucleotide sequences of the genes expressing scFv were selectively amplified with KSB-1 (5′-ATGCGAGTGGCAGCAGGTT-3′) and KSB-340 (5′-GTAATGATTTTCGATGGAG-3′) using PCR. The obtained nucleotides were separated by electrophoresis using 1% agarose gel, and purified with the PureLink Quick Gel Extraction Kit. The purified nucleotide was determined using the BigDye Terminator v3.1 Cycle Sequencing Kit with primers KSB-1 and KSB-340 and purified with the BigDye X Terminator Purification Kit. Then the DNA sequence of the scFvs was analysed.

**Evaluation of cell-binding properties of scFvs using flow cytometry analysis**

For flow cytometry analysis, SIT, oh13T, K3T and Su9T01 cells were used for ATL cell lines. Oh13T, K3T and Su9T01 cells were established from the peripheral blood mononuclear cells of patients with ATL (25). MT-2 cells were derived from umbilical cord blood lymphocytes after cocultivation with leukemia cells from AT/IL patients (26). CEM, Jurkat, HL60 and K562 cells were used for non-ATL leukemia cell lines. CEM cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Jurkat cells were kindly provided by Dr Warner C. Greene at Duke University Medical Center (Durham, NC). HL60 and K562 cells were obtained from Hayashibara cell bank (Okayama, Japan). Those cells were cultured in complete RPMI-1640 at 37°C, 5% CO₂. PBMCs were obtained from healthy donor’s blood (40 mL) using Histopaque-1077. For flow cytometry analysis, those cells (1.0 × 10⁶ cells) were incubated with the purified scFv (10 μg/mL in PBS) for 30 min on ice. After washing the cells with cold PBS, the scFv bound to the cells were stained with anti-His antibody (1:500 dilution with PBS; GE Healthcare) followed by anti-mouse IgG-FITC (1:500 dilution with PBS; Beckman Coulter). Then the cells were washed with cold PBS and analysed by flow cytometry.

**Sugar chain-binding analysis of scFvs using an arSC and SPR imaging**

Binding interaction between the scFv and sugar chain was analysed with an SPR imaging sensor as described before (27). An arSC was...
prepared using 48 kinds of sugar chain conjugated with monovalent linker. A sugar chain ligand conjugate solution (56 μM) was mixed with 2-glucose ligand conjugate solution (Glc2-4Glc-mono, 444 μM) to control the sugar chain ligand conjugate density. To prepare the arSC, a gold chip surface was initially cleaned with UV/O3. A ligand conjugate solution (1 μl, total 500 μM) in H2O containing 10% glycerol was spotted on the chip with a spotter and left to stand overnight at room temperature. An scFv solution in PBS/T (113 μl, total 2,400 nM) was injected over the chip surface at a flow rate of 150 μl/min. The binding interaction was monitored at 25 °C for the change in luminescence intensity.

Docking study of the scFv and O-glycan partial disaccharide structures using MOE

Molecular docking simulations of the scFv and synthetic O-glycan partial disaccharide structures were performed using a Molecular Operating Environment (MOE, ver. 2015.10; Chemical Computing Group Inc., Canada). All the synthetic O-glycan structures were modelled using ‘Molecular Builder’ in MOE. Those structures were subjected to energy minimization up to a gradient of 0.01 using the Amber 10 force field, and the energy minimized structures were selected using ‘Systematic Conformation Search’ in MOE. The structure was then subjected to energy minimization using the Amber 10 force field. Molecular docking simulations of the scFv and O-glycan partial structures were carried out using ‘Structure-Based Drug Design’ in MOE according to the standard procedures. The docking site was defined using ‘Site Finder’, and the geometries of the resulting complexes were analysed using MOE’s ‘Pose Viewer’.

Results and Discussion

Comparison of expression levels of glycosyltransferases gene between S1T cells and non-ATL leukemia cell line

The expression levels of glycosyltransferase genes were investigated using Gene-chip (28) and qPCR, respectively, to predict the difference between sugar chains expressed on ATL cells and other leukemia cells. In this study, SIT cells, which are HTLV-1-infected CD4+ T cell clones established from a patient with ATL, and MOLT-4 cells for non-ATL T-cell leukemia were used. The Gene-chip and PCR analysis revealed that alpha1, 3-fucosyltransferase 7 (FUT7), which is correlated to the synthesis of sialyl-LewisX found in O-glycan, is much more abundantly expressed on ATL cells than on MOLT4 cells (Table I and Supplementary Table S1). It is consistent with the previous report by Kanno et al. (29), and sialyl-LewisX is well known as a cancer-specific sugar chain (10, 30).

In addition, glycosaminoglycan (GAG) synthases such as CS beta-1, 4-N-acetylgalactosaminyltransferase (ChGn1) and N-acetylgalactosamine-4-sulfate-6-O-sulfotransferase (GALNAC4S-6ST) were highly expressed in ATL cells. Consequently, we hypothesize that O-glycans are more abundant on the ATL cell surface and can be effectively targeted to prepare an ATL cell-specific sugar chain-binding antibody.

Release and conjugation of cell surface O-glycans from S1T cells

The strategy for developing an O-glycan-binding antibody to ATL cells is shown in Fig. 1. To develop a sugar chain-binding antibody, phage display method was used in the study (16, 20). Phage display method can produce a scFv, which is a fragment of the Fab region of the antibody. The scFv-displaying phages derived from human B cells (diversity: 1.04 × 10⁵) were screened using a fSC immobilized with O-glycans from SIT cells.

To prepare fSCs, cell surface O-glycans on SIT cells were released through hydrazine treatment and conjugated with our unique linker molecule, f-mono (24). Based on fluorescence, the 2, 6-diaminopyridine moiety of f-mono was useful for monitoring during the purification. Cell membrane proteins of 7.6 × 10⁸ of SIT cells were extracted using Triton X-100 and treated with N-glycanase F to eliminate N-glycans. Released N-glycans were removed using BlotGlyco® beads and further used for another experiment. The O-glycans were then released from the resultant proteins through hydrazinolysis (23). After the partial purification of the O-glycans using BlotGlyco® beads, the O-glycans were conjugated with f-mono linker using reductive amination reaction (24). The f-mono linker conjugated O-glycans were separated into three fractions with HPLC using an ODS column (Fig. 2) with a yield of 4.5, 6.6 and 0.47 nmol, respectively.

Preparation of fSCs using f-mono linker conjugated O-glycans from S1T cells

For screening of sugar chain-binding phages, fSCs were prepared according to the described method previously (20). Each HPLC fraction of f-mono linker conjugated O-glycans were immobilized on a fiber surface via gold nanoparticles, and three kinds of fSCs (designated as SC-1, SC-2 and SC-3) were prepared. For the negative control, a β-galactose immobilized chip (βGal-chip) was prepared using f-mono linker conjugated lactose. The immobilization of O-glycans was monitored by LSPR analysis in real time. Then, the fSCs were soaked in solutions of fibronectin (known to bind to GAG) or BSA (not bind to GAG) (Fig. 3). In the case of fibronectin, gradual signal change was observed for all three fSCs, but no signal change was observed for BSA.

Table I. Comparison of expression levels of glycosyltransferase genes between ATL cells and leukemia cells by PCR analysis

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Symbol</th>
<th>SIT</th>
<th>MOLT-4</th>
<th>Ratio</th>
<th>Gene product</th>
</tr>
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<tbody>
<tr>
<td>NM_004479.2</td>
<td>FUT7</td>
<td>7.88</td>
<td>0.006</td>
<td>1300.4</td>
<td>Alpha-1, 3-fucosyltransferase 7</td>
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<td>NM_002465.2</td>
<td>MNFG</td>
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<td>0.04</td>
<td>433.1</td>
<td>Manic fringe (Drosophilia) homolog</td>
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<td>NM_004482.2</td>
<td>pgGnTases</td>
<td>9.85</td>
<td>0.22</td>
<td>45.4</td>
<td>UDP-GalNAc: polypeptide acetylgalactosaminyltransferase</td>
</tr>
<tr>
<td>NM_018371.3</td>
<td>ChGn1</td>
<td>15.54</td>
<td>0.43</td>
<td>35.8</td>
<td>Chondroitin sulfate beta1, 4-N-acetylgalactosaminyltransferase</td>
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<tr>
<td>NM_015892.2</td>
<td>GALNAC4S-6ST</td>
<td>5.59</td>
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<td>N-acetylgalactosamine-4-sulfate-6-O-sulfotransferase</td>
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<td>NM_005329.2</td>
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<td>0.6</td>
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<td>0.9</td>
<td>20.3</td>
<td>Xylosyltransferase</td>
</tr>
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</table>
observed for the βGal-chip (Fig. 3A). In the case of BSA, no signal change was found for all chips (Fig. 3B). These results indicate that O-glycans from S1T cells were immobilized on the fSCs and non-specific interaction with protein and fSCs was negligible.

**Screening of sugar chain-binding phages using fSCs**

To screen the scFv-displaying phages that selectively bind to S1T cell surface O-glycan using the fSCs, a phage library derived from human B cells (diversity of 1.04 × 10⁸), established from the PBMCs of 20 healthy volunteers (20), were used. The phage library was first negatively screened using BlotGlyco® beads immobilized with O-glycans from the PBMCs of a healthy volunteer, to remove the phages bound to the PBMC. Approximately 30% of phages were purged through the negative screening. The resultant phage library was then screened using the fSCs immobilized with O-glycans from S1T cells while being monitored using LSPR (Supplementary Fig. S1). After several washings with PBS/T, the phages bound to O-glycan on the Sugar Chip were eluted with triethylamine.

**Expression and purification of O-glycan-binding scFv**

The O-glycan-binding scFv-displaying phages were transfected into two types of E. coli to express soluble scFv tagged with a histidine hexamer for purification as described previously (20). The phages were initially infected with E. coli TG1 to obtain a monoclonal phage solution. After overnight incubation on an agar plate, 69, 14 and 7 colonies were obtained from SC-1, SC-2 and SC-3, respectively. Among those colonies, 14 colonies were randomly selected, and the PCR analysis revealed that 2 (SC-1), 4 (SC-2) and 4 (SC-3) colonies contained the scFv gene (Supplementary Fig. S2). Each confirmed phage clone was amplified through co-infection with M13KO7 helper phage to prepare a monoclonal phage solution. Ten monoclonal phage solutions were then obtained and purified with PEG precipitation from culture supernatant (Supplementary Fig. S3). The O-glycan-binding monoclonal phages were transfected with E. coli HB2151 to

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Fig. 1 Schematic image for preparation of O-glycan binding scFv against ATL cell.

Fig. 2 Separation of f-mono linker conjugated O-glycans extracted from S1T cells. The f-mono linker conjugated O-glycans were separated into three fractions (Fr. 1 = 4.5 nmol, Fr. 2 = 6.6 nmol, Fr. 3 = 0.47 nmol) by HPLC using ODS column (φ 4.6 mm × 250 mm) in a column oven at 40 °C. The samples were eluted by 50% methanol at a flow rate of 0.5 ml/min and detected by UV absorption at 231 nm.
express soluble O-glycan-binding scFvs. After overnight incubation, the bacterial cells were disrupted using sonication in order to release the scFv from cytoplasmic domain. The obtained scFvs were purified from culture supernatant using affinity chromatography with a His-tag trap column. The purified scFvs were analysed with SDS-PAGE and western blotting (Fig. 4A). Among 10 monoclonal phage solutions, 1 (SC-1), 3 (SC-2) and 4 (SC-3) scFvs showed bands at 27 kDa, which is the expected molecular mass of scFv, on SDS-PAGE stained with CBB (Fig. 4A). Western blotting analysis with anti-His antibody showed the presence of scFv (Fig. 4B). Amino acid sequences of these scFvs were deduced from the DNA sequence of the phage, and the sequences of obtained eight scFvs were all identical (Fig. 5). In the preliminary experiments, we obtained one stable scFv from the same phage library using a similar fibronectin immobilized with O-glycans from S1T cells, but without negative screening for PBMC from a healthy volunteer (data not shown). The amino acid sequence of the scFv was identical to the scFv obtained in this study. These results suggest that the scFv-binding sugar chain might be a kind of polymer consisted of similar repeating units, and further, selective screening using fSC can be effective in obtaining scFv specific for O-glycan structures on S1T cells.

**Evaluation of cell-binding properties of scFvs with ATL cell lines using flow cytometry analysis**

The cell-binding properties of the O-glycan-binding scFv, designated as S1TSCFR3-1, was evaluated using flow cytometry analysis. S2E-P1-13 was used for the control scFv (Supplementary Figs S4 and S5). First, S1T and CEM cell lines were used. The CEM cell is not ATL, but is a T lymphoblastic leukemia cell line. These cell lines were incubated with purified scFv (10 μg/ml) and stained with mouse anti-His antibody followed by anti-mouse IgG-FITC. The flow cytometry analysis revealed that S1TSCFR3-1 strongly binds to S1T cells but not for CEM cells, and S2E-P1-13 have minimal binding potency to both cell lines (Fig. 6A and B). To further assess the binding specificity to ATL cells, four kinds of ATL and three kinds of non-ATL leukemic cell lines were examined. As a result, S1TSCFR3-1 strongly bound to all ATL cell lines and weakly bound to some leukemia cell lines (Supplementary Fig. S4). It is known that some sugar chain antigens, such as Tn antigen and sialyl Lewis X, are broadly expressed on cancer cells (10). Thus, it is suggested that S1TSCFR3-1 recognizes specific O-glycan structure broadly expressed on cancer cells. To investigate the binding property of S1TSCFR3-1 to normal lymphocytes, human PBMCs from healthy volunteers were examined. The result showed that S1TSCFR3-1 have almost no binding potency to most of normal lymphocyte, indicating that S1TSCFR3-1 predominantly bound to ATL cells (Fig. 6). By conducting further analysis, we found that scFv had slight binding to monocyte fraction in the PBMCs (Supplementary Fig. S5).

**Sugar chain-binding analysis of scFvs using SPR imaging with an arSC**

The sugar chain-binding properties of the scFvs were analysed using SPR imaging with an arSC (27).
According to the gene analysis of glycosyltransferases, it was speculated that sialyl-LewisX and GAG were involved in the sugar chain antigens expressing on S1T cells. In the present study, we focused on GAG and performed the SPR imaging using arSC immobilized with 38 kinds of synthetic disaccharide structures of GAG including HS, CS and DS. α-glucose (maltose conjugate) was used as a non-sulphated sugar chain control. The linker conjugates of sulphated sugar chains were mixed with those of α-glucose to decrease the density of sulphated sugar chain in the spot on the arSC, to avoid non-specific electrostatic interaction between the sulphated sugar chains and scFv. The scFv solutions in PBS/T (113–2,400 nM) were applied onto the arSC at a flow rate of 150 μl/min. In the case of S2E-P1-13, it exhibited quite weak binding potency to any disaccharide structures (Supplementary Fig. S8). However, the structure and concentration-dependent interaction between sulphated disaccharides and S1TSCFR3-1 were observed. The binding potency of α-glucose or non-sulphated structures such as GlcNAc-GlcA and GlcA-GalNAc (CS-O) was quite weak (Table II and Fig. 7). Among GAG disaccharide structures, GlcNS3S6S-IdoA2S, one of the HS...
disaccharides, showed the highest affinity ($K_D = 34\text{ nM}$). GlcA3S-GalNAc4S6S structure, which is known as CS-M in CS, showed the second highest affinity ($K_D$ value = 90 nM). Tanaka et al. reported that HS is expressed abundantly on ATL cell surface (34), and it is suggested the expression level of CS in S1T cells is higher when subjected to gene analysis of glycosyltransferases (28) (Table I). Therefore, it is

<table>
<thead>
<tr>
<th>GAG type</th>
<th>Sugar chain structure</th>
<th>$k_a$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
</tr>
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<tbody>
<tr>
<td>HS</td>
<td>GlcNS3S6S-IdoA2S</td>
<td>$8.68 \times 10^4$</td>
<td>$2.88 \times 10^{-6}$</td>
<td>33</td>
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<td></td>
<td>GlcNS3S6S-IdoA</td>
<td>$1.54 \times 10^2$</td>
<td>$8.70 \times 10^{-5}$</td>
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<tr>
<td></td>
<td>GlcNS3S6S-GlcA2S</td>
<td>$1.31 \times 10^2$</td>
<td>$3.48 \times 10^{-5}$</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td>GlcNS3S6S-GlcA</td>
<td>$1.26 \times 10^2$</td>
<td>$2.78 \times 10^{-5}$</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>GlcNAc-GlcA</td>
<td>N.D.$^a$</td>
<td>N.D.$^a$</td>
<td>N.D.$^a$</td>
</tr>
<tr>
<td>CS</td>
<td>GlcA-GalNAc4S6S (CS-E)</td>
<td>$2.11 \times 10^2$</td>
<td>$7.92 \times 10^{-5}$</td>
<td>376</td>
</tr>
<tr>
<td></td>
<td>GlcA3S-GalNAc4S6S (CS-M)</td>
<td>$8.69 \times 10^1$</td>
<td>$7.76 \times 10^{-6}$</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>GlcA2S3S-GalNAc?CS-R$^a$</td>
<td>$1.80 \times 10^2$</td>
<td>$1.36 \times 10^{-4}$</td>
<td>757</td>
</tr>
<tr>
<td></td>
<td>GlcA-GalNAc (CS-O)</td>
<td>N.D.$^a$</td>
<td>N.D.$^a$</td>
<td>N.D.$^a$</td>
</tr>
<tr>
<td></td>
<td>αGlc</td>
<td>N.D.$^a$</td>
<td>N.D.$^a$</td>
<td>N.D.$^a$</td>
</tr>
<tr>
<td>DS</td>
<td>IdoA-GlcNAc4S6S (DS-E)</td>
<td>$2.33 \times 10^2$</td>
<td>$7.09 \times 10^{-5}$</td>
<td>304</td>
</tr>
<tr>
<td></td>
<td>IdoA-GIcNAc4S6S (DS-E)</td>
<td>$2.33 \times 10^2$</td>
<td>$7.09 \times 10^{-5}$</td>
<td>304</td>
</tr>
</tbody>
</table>

$^a$N.D. means not determined due to feeble interaction.
speculated that the higher sulphated GAG structures are expressed abundantly on ALT cell surface and relates to the metastasis of ATL cells. Future studies on the epitope analysis of S1TSCFR3-1 are necessary.

Molecular docking study of the scFv and synthetic O-glycan partial disaccharide structures using MOE

To predict the binding mode of the S1TSCFR3-1 and GAG partial disaccharide structures, a computational docking study was performed using MOE (ver. 2015.10) docking software. The 3D model of S1TSCFR3-1 was constructed based on the amino acid sequence, using Antibody Modeler implemented in MOE. In the present study, five kinds of HS and CS disaccharide structures were picked out as strongly, moderately and weakly binding O-glycan structures from the result of SPR analysis using arSC, and binding energies (docking scores) were compared (Fig. 8A). As a result of molecular docking simulations, highly sulphated disaccharide structures, GlcNS3S6S-IdoA2S and GlcA3S-GalNAc4S6S (CS-M), showed much lower docking scores than the non-sulphated O-glycan structure, GlcA-GalNAc (SC-O), and α-glucos as with SPR analysis. In addition, interaction between the specific amino acids, His(37), Tyr(108), Ser(109) and Tyr(110) of the VH domain on S1TSCFR3-1, and a sulfate group of highly sulphated disaccharide structures were observed (Fig. 8C and D). These results imply that a highly sulphated structure is necessary for strong binding.

Conclusion

In this study, a cell surface O-glycan-binding scFv to S1T cells (S1TSCFR3-1) was obtained using phage display method and our Sugar Chip technology. The scFv showed high affinity to highly sulphated disaccharide structures of HS and CS according to SPR analysis, and predominantly binding properties to ATL cell lines using flow cytometry analysis. Therefore, the scFv has the potential to be a diagnostic tool, and could be considered for use as an anti-cancer drug.
against ATL. A clinical investigation using the scFv is now underway. Typically, a large amount of antigens are necessary for the screening of a scFv-displaying phage library. Since a fsc can be prepared using a tiny amount of sugar chains and can be used to analyse the interaction between the sugar chain and analytes in a microliter sample solution, it is favourable for the screening of cell surface sugar chain-binding scFv-displaying phages. In addition, our Sugar Chip technology can be applied to a wide range of sugar chains, suggesting that our screening system could be a powerful method to obtain scFvs binding to the sugar chain antigens.

**Supplementary Data**

**Supplementary Data** are available at JB Online.

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**Conflict of Interest**

None declared.

**References**


