Bisecting-GlcNAc on Asn388 is characteristic to ERC/mesothelin expressed on epithelioid mesothelioma cells

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Haruhiko Fujihira1,2,*, Daisuke Takakura3,4, Atsushi Matsuda5, Masaaki Abe6, Michio Miyazaki3, Tomomi Nakagawa3, Kazunori Kajino6,7, Kaori Denda-Nagai1, Miki Noji1, Okio Hino6 and Tatsuro Irimura1,†

1Division of Glycobiologics, Intractable Disease Research Center, Juntendo University Graduate School of Medicine, 2-1-1 Hongo Bunkyo-ku, Tokyo 113-8421, Japan; 2Glycometabolic Biochemistry Laboratory, Cluster for Pioneering Research, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; 3Project for Utilizing Glycans in the Development of Innovative Drug Discovery Technologies, Japan Bioindustry Association (JBA), 2-26-9 Hatchobori, Cho-ku, Tokyo 104-0032, Japan; 4Graduate School of Medical Life Science, Yokohama City University, 1-7-29 Suehiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan; 5Department of Biochemistry, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; 6Department of Pathology and Oncology, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan; and 7Department of Human Pathology, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

*Haruhiko Fujihira, Division of Glycobiologics, Intractable Disease Research Center, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan. Tel: +81-3-3830-8715, Fax: +81-3-3830-8715, email: h-fujihira@juntendo.ac.jp
†Tatsuro Irimura, Division of Glycobiologics, Intractable Disease Research Center, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan. Tel: +81-3-5802-1876, Fax: +81-3-3830-8715, email: t-irimura@juntendo.ac.jp

Mesothelioma is a highly aggressive tumour associated with asbestos exposure and is histologically classified into three types: epithelioid-type, sarcomatoid-type and biphasic-type. The prognosis of mesothelioma patients is poor and there is no effective molecular-targeting therapy as yet. ERC/mesothelin is a glycoprotein that is highly expressed on several types of cancers including epithelioid mesothelioma, but also expressed on normal mesothelial cells. This is a predicted reason why there is no clinically approved therapeutic antibody targeting ERC/mesothelin. In the present study, we focussed on the differential glycosylation between ERC/mesothelin present on epithelioid mesothelioma and that on normal mesothelial cells and aimed to reveal a distinct feature of epithelioid mesothelioma cells. Lectin microarray analysis of ERC/mesothelin using cells and patient specimens showed significantly stronger binding of PHA-E4 lectin, which recognizes complex-type N-glycans having a so-called bisecting-GlcNAc structure, to ERC/mesothelin from epithelioid mesothelioma cells than that from normal mesothelial cells. Further, liquid chromatography/mass spectrometry
analysis on ERC/mesothelin from epithelioid mesothelioma cells confirmed the presence of a bisecting-GlcNAc attached to Asn388 of ERC/mesothelin. These results suggest that this glycoproteome could serve as a potential target for the generation of a highly selective and safe therapeutic antibody for epithelioid mesothelioma.

**Keywords:** bisecting-GlcNAc; epithelioid mesothelioma; ERC/mesothelin; glycosylation.

**Abbreviations:** ACN, acetonitrile; ERC/MSLN, ERC/mesothelin; FBS, foetal bovine serum; GPI, glycosylphosphatidylinositol; LC/MS, liquid chromatography/mass spectrometry; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate

Mesothelioma is an aggressive tumour originating from pleura, peritoneum or pericardial cavity, and is usually caused by exposure to asbestos (1–3). The incubation period of asbestos-related mesothelioma is estimated to be ~30–40 years. In Japan, the incidence of mesothelioma patients has been increasing and is predicted to reach a peak in 2030 (4). In most developed countries, the use of asbestos has been prohibited, but in developing countries it is still used (3). Therefore, the number of mesothelioma cases is predicted to increase in the future worldwide. Mesothelioma is histologically classified into three types: epithelioid-type, sarcomatoid-type and biphasic-type, and the prognosis of mesothelioma patients is poor regardless of the type (5). Therefore, the development of an effective therapy including molecular-targeting therapies against mesothelioma is urgently needed, while it has not been achieved yet.

ERC/mesothelin (ERC/MSLN; ERC is a name from “expressed in renal carcinoma”) is a glycosylphosphatidylinositol (GPI)-anchored cell surface glycoprotein highly expressed in epithelioid mesothelioma, pancreatic cancer and ovarian cancer (Fig. 1A) (6). ERC/MSLN is synthesized as a ~71 kDa precursor form and is later cleaved into two products (a 40 kDa C-terminal product and a 31 kDa N-terminal product) by furin-like protease. The C-terminal product, which is anchored on the cell surface, is called ERC/MSLN (6). The N-terminal product, which is secreted into the blood, is reported to be a good serum biomarker for mesothelioma diagnosis (7). ERC/MSLN has been a potential drug target molecule for several cancers; however, there is no clinically applied therapy targeting ERC/MSLN. One reason is the wide expression of ERC/MSLN on normal mesothelial cells that might cause adverse effects.

Glycosylation is one of the major co- and post-translational modifications of proteins (8). It is well known that cellular glycosylation drastically changes upon malignant transformation of mammalian cells (9). Also, protein glycosylation is known to be involved in the malignant behaviours of cancer cells (10–13), which are often acquired during cancer progression. Alterations in the glycosylation of cancer cells result from various intrinsic factors affecting glycosylation, including differential expression of glycosyltransferases and enzymes responsible for the supply of precursor molecules. The changes include altered regulation at the transcriptional level (14–17), alterations of chaperon functions (18, 19) and altered glycosidases (20). However, little is known about the differential glycosylation of ERC/MSLN and other cell surface molecules expressed by mesothelioma.

Attempts to utilize such differential glycosylation in the diagnosis and therapy of mesothelioma have not been previously made.

In the present study, we aimed to reveal a characteristic glycoform of ERC/MSLN on epithelioid mesothelioma. The characteristic ‘glycoproteome (= glycoform + peptide sequence)’ of ERC/MSLN on epithelioid mesothelioma cells may be useful to overcome adverse effects in the therapeutic use of anti-ERC/MSLN antibodies against epithelioid mesothelioma. To this end, we performed comprehensive glycan analysis using lectin microarray. A characteristic glycan structure and its attachment site distinctive to epithelioid mesothelioma cells were further confirmed by liquid chromatography/mass spectrometry (LC/MS). The identified structure may represent a characteristic glycoproteome of ERC/MSLN on epithelioid mesothelioma: bisecting-GlcNAc containing glycans on Asn388 of ERC/MSLN. This glycoproteome could be the key for the development of specific therapeutic antibodies for epithelioid mesothelioma. Our results shed some light on the development of a therapy for several types of cancers focussing on differential glycosylation of a molecule expressed on both normal and cancer cells.

**Materials and Methods**

**Ethics statement**

This study was approved by the Institutional Review Board of Juntendo University Graduate School of Medicine (#201713). Patient specimens (tissue sections) were obtained from archived paraffin-embedded tumour blocks from biopsies or surgeries.

**Cell culture**

Epithelioid mesothelioma cell lines (H226 [NCI-H226], MESO-4 [ACC-MESO4]), immortalized non-tumorous mesothelial cell line (McT-5A), and primary normal mesothelial cells (MES-F) were cultivated in RPMI1640 (Gibco) supplemented with 10% foetal bovine serum (FBS), 10 mM HEPES, Medium 199 (Sigma) supplemented with 10% FBS, 3.3 mM epidermal growth factor, 400 mM hydrocortisone, 870 mM zinc-free bovine insulin, 20 mM HEPES, trace elements), Medium 199 (Sigma) supplemented with 10% FBS, penicillin streptomycin), respectively, at 37°C in a 5% CO2 atmosphere.

**Preparation of cell lysates**

Cultured cells were washed with phosphate buffered saline (PBS) 3 times and collected using a cell scraper. Fifty million cells were suspended with 1 ml of PBS containing 0.5% Nonidet P40 (NP-40) (FUJIFILM Wako), homogenized using a syringe and needle (gauge: 21G) on ice, and incubated on ice for at least 10 min. The samples were centrifuged at 10,000×g for 10 min at 4°C and the obtained supernatants were used as cell lysate. The protein concentration of the prepared cell lysates was measured by BCA protein assay (Pierce) with bovine serum albumin as a standard.
Fig. 1. PHA-E4 lectin strongly binds to ERC/MSLN from epithelioid mesothelioma cells compared to that from normal mesothelial cells. (A) Schematic representation of ERC/MSLN. Asn388, Asn496 and Asn523 indicate glycosylation sites. (B) Western blotting to confirm the immunoprecipitation of ERC/MSLN from cell lines. Arrow indicates ERC/MSLN. Elute x 10: 10 times amount of elutes were loaded. (C–F) Scan images and bar graphs of glycan profiling analysis of ERC/MSLN from MeT-5A (C), MES-F (D), MESO-4 (E) and H226 cells (F) using lectin microarray \( n = 3 \). White squares in scan images and arrows in bar graphs indicate the position of PHA-E4 lectin. The numbers above the arrows indicate the net intensity of PHA-E4 lectin. Error bar indicates standard deviation.
Protein extraction from patient specimens
Formalin-fixed, paraffin-embedded tissue sections from 4 epithelioid mesothelioma patients were used. The tissue sections were deparaffinized, and the parts containing epithelioid mesothelioma were microtomed and shaken at 4°C for 16 h. The supernatant was removed, and the tissue suspension was incubated on ice for 60 min and then centrifuged at 10,000×g for 2 min at 4°C. The obtained supernatants were used as tissue lysates.

Immunoprecipitation and lectin precipitation
Immunoprecipitation was performed as described previously (21). Briefly, 100 μl of cell lysates and 50 μl of tissue lysates (corresponding to about 8 mm²) were subjected to immunoprecipitation using biotinylated-mouse-anti-ERC/MSLN antibody (IBL, clone 22A31, #10357). The cell/tissue lysates were shaken with a 200 μl slurry of Dynabeads My One Streptavidin T1 (Dynal-SA) (Veritas, #DB65604) at 4°C for at least 60 min for preclearing. Two hundred and fifty nanograms of biotinylated-anti-ERC/MSLN antibody was immobilized to a 200 μl slurry of Dyna-SA by shaking at 4°C for at least 20 min. Biotinylated-mouse anti-ERC/MSLN antibody immobilized Dyna-SA (bio-anti-ERC/MSLN-beads) was washed with TBS containing 1% (w/v) Triton-X 100 (TBSTx) 3 times. Precleared lysates and washed bio-anti-ERC/MSLN-beads were mixed and shaken at 4°C for 16 h. The supernatant was removed, and the beads were washed with TBSTx 3 times. Washed beads were suspended into 10 μl of TBS containing 0.2% (w/v) sodium dodecyl sulphate (SDS) and incubated at 4°C for 5 min, respectively. The remaining glycopeptides were enriched with a 5-fold volume of cold acetone by centrifugation at 12,000×g for 10 min (25).

Antibody-overlay lectin microarray analysis
Antibody-overlay lectin microarray was performed as described previously (22) to obtain the glycan profile of IP-ERC/MSLN. Briefly, 10 μl of IP-ERC/MSLN was diluted with Probing solution (GlycoTechnica), applied to the LeeChip (GlycoTechnica) and incubated at 20°C for 16 h. After the incubation, 20 μg of human IgG (FUJIFILM Wako) was added to each well and incubated at 20°C for 30 min (22). After washing with PBS containing 1% (w/v) Triton-X 100 (PBSTx) 3 times, 60 μl of Probing solution containing 20 μg of human IgG and 200 ng of biotinylated-mouse-anti-ERC/MSLN antibody (clone 22A31, #10357) was used. After Immunoprecipitation and lectin precipitation section was added to each well and incubated at 20°C for 1 h. After washing with PBSTx 3 times, 60 μl of Probing solution containing 400 ng of Cy3-streptavidin (GE Healthcare) was added to each well and incubated at 20°C for 30 min. After washing with PBSTX 3 times, 60 μl of Probing solution was added to each well and scanned using GlycoStation Reader 1200 (GlycoTechnica). All data were analysed with GlycoStation ToolsPro 1.5 (GlycoTechnica) and GlycoStation Reader 1.5 (GlycoTechnica). The net intensity of each spot was calculated by subtracting the background value from the total signal intensity of three spots.

Western blotting
Samples (cell lysates, immunoprecipitation flow through, IP-ERC/MSLN) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) membranes. The membrane was blocked for 60 min with 4% Block Ace (KAC Co., Ltd. Kyoto, Japan) at 37°C, and incubated with primary antibody (2.5 μg/ml) in antibody diluting reagent (KIWAMI SETSYUYAKUKUN, DRC, Co., Ltd, Tokyo, Japan) at 37°C for 1 h, followed by secondary antibody in antibody diluting reagent at 37°C for 1 h. The chemiluminescence was analysed with C-DiGit membrane scanner (LI-COR, Lincoln, NE, USA) using ImmunoStar LD (FUJIFILM Wako).

In-gel digestion
An improved in-gel digestion method was used as described in a previous study (23). IP-ERC/MSLN was prepared from membrane proteins of H226 cells. SDS-PAGE was performed using 5–20% precast gradient gels (DRC, Tokyo, Japan) under a constant current of 21 mA for 90 min. The proteins were visualized by Pierce Silver Stain for Mass Spectrometry (Thermo), and the protein bands corresponding to IP-ERC/MSLN were excised. The washed gel pieces were incubated with 100 μl of reduction buffer containing 10 mM DTT in 25 mM ammonium bicarbonate (pH 8.0) in the presence of 0.2 M guanidine chloride (GuHCl) at 56°C for 1 h. The gel pieces were subsequently alkylated with an equal volume of 55 mM iodoacetamide at room temperature for 30 min in the dark. After washing with 25 mM ammonium bicarbonate (pH 8.0) 50% acetonitrile (ACN) for 1 h, 25 mM ammonium bicarbonate (pH 8.0) for 1 h, and 25 mM ammonium bicarbonate (pH 8.0) for 1 h, Tryptic digests were extracted in a stepwise manner with 20 μl of 10%, 20%, 30%, 40% and 50% ACN by shaking and sonication for 15 min, respectively. All the extracts were combined and dried using a speed-vac concentrator. A portion of the extracted glycopeptides was deglycosylated using the IGOT method (24). The remaining glycopeptides were enriched with a 5-fold volume of cold acetone by centrifugation at 12,000×g for 10 min (25).

LC/MS
Deglycosylated peptides and glycopeptides were separated on an LC20AD system (SHIMADZU) with MonoCap C18 trap column (0.2 mm × 50 mm, GL Science) and a Nano HPLC Capillary Column (75 μm × 120 mm, 3 μm, C18; Nikkyo Technos). The eluents consisted of water containing 0.1% (v/v) formic acid (pump A) and ACN containing 0.1% (v/v) formic acid (pump B). The glycopeptides were eluted at a flow rate of 0.3 μl/min with a linear gradient from 2% to 35% B over 40 min. Mass spectra were acquired on a Q Exactive (Thermo Scientific) using data-dependent acquiring method with a resolution of 70,000. Product ion mass spectra were acquired against the 10 most intense ions by SEQUEST search engine using UniprotKB database (status/2017/12). The following parameters were applied to the search: a specified trypsin enzymatic cleavage and all possible modifications (Thermo Fisher Scientific) were searched. The search was performed with Nanospray Flex Ion Source (Thermo Fisher Scientific) operated in the positive ion mode. Full mass spectra were acquired by using an m/z range of 350–2,000 for deglycosylated peptides or 700–2,000 for glycosylated peptides with a resolution of 70,000. Product ion mass spectra were acquired against the 10 most intense ions by using a data-dependent acquiring method with a resolution of 17,500 with normalized collision energy of 27%. Deglycosylated peptides were identified by the SEQUEST search engine using UniprotKB database (status/2017/12). The following parameters were applied to the search: a specified trypsin enzymatic cleavage with two possible missed cleavages, a precursor mass tolerance of 6 ppm, a fragment mass tolerance of 0.02 Da, static modification of cysteine (carbamidomethylation), and dynamic modifications of methionine (oxidation), asparagine [ delta: H(1)N(-1)180(H)], and N-term glutamine (Gln > PyroGlu).

Site-specific glycosylation analysis
Product ion spectra of glycopeptides were manually selected based on the identification of oligosaccharide oxonium ions, with a characteristic m/z such as 366.14 (HexNAc-Hex). The peptide and glycan masses of glycopeptides were deduced from the molecular mass of the peptide and glycan, respectively. The glycan masses of glycopeptides were calculated using GlycoMod tool software. The remaining glycoforms were found from mass intervals between the glycoforms, and their peak areas
we performed lectin microarray analysis using ERC/MSLN derived from epithelioid mesothelioma patient specimens. Epithelioid mesothelioma cells were obtained from surgical specimens, and ERC/MSLN was immunoprecipitated from prepared tissue extracts. IP-ERC/MSLN from the tissues were subjected to lectin microarray analysis. Our analysis revealed that PHA-E₄ binding was also high in ERC/MSLN derived from surgical specimens (Fig. 2C). Because ACG lectin showed consistently high intensities among all lectins when ERC/MSLN was subjected to lectin array analysis, we compared the relative binding intensities of PHA-E₄ versus ACG among normal mesothelial cells, mesothelioma cells and mesothelioma specimens (Fig. 2D). As a result, PHA-E₄ showed a significantly stronger binding to IP-ERC/MSLN from epithelioid mesothelioma cells than to that from normal mesothelial cells.

**Structural determinations of N-glycans from ERC/MSLN of epithelioid mesothelioma cells**

We performed LC/MS analysis of IP-ERC/MSLN from an epithelioid mesothelioma cell line (H226) to confirm the presence of glycans that are recognized by PHA-E₄ lectin, *i.e.* ‘bisecting-GlcNAc’. ERC/MSLN was isolated from H226 cells by immunoprecipitation followed by separation on SDS–PAGE (Fig. 3A). The protein was subjected to in-gel tryptic digestion. A portion of in-gel digested samples was analysed by the isotope-coded glycosylation site-specific tagging (IGOT) method (Fig. 3B) (24). The rest of in-gel digested samples was directly analysed by LC/MS (Fig. 3B) after glycopeptide enrichment. ERC/MSLN has three potential N-glycosylation sites (Asn388, Asn496 and Asn523) and all of these glycosylation sites were glycosylated according to the results of our analysis (Fig. 3C–E and Table I). When we estimated the glycan structures at each site from the *m/z* and MS/MS analyses (Supplementary Fig. S2A–C), glycans were highly heterogeneous. The glycans estimated to contain bisecting-GlcNAc residues were most abundant at Asn388, accounting for 11% of total N-glycans attached to this site (Fig. 3C). Glycans estimated to contain bisecting-GlcNAc were also observed at Asn496, but the relative content was very low (2.7% of total N-glycans attached to this site) (Fig. 3D). Glycans attached to Asn523 did not contain bisecting-GlcNAc (Fig. 3E). Our lectin microarray and LC/MS analyses indicate that ERC/MSLN from H226 cells carries glycans containing a bisecting-GlcNAc residue mainly at the Asn388 position.

**Discussion**

In the present study, we comparatively analysed glycoforms of ERC/MSLN derived from epithelioid mesothelioma cells versus those derived from normal mesothelial cells to identify a characteristic structure in epithelioid mesothelioma cells. Results of our lectin microarray analysis revealed that PHA-E₄ lectin showed significantly stronger binding to ERC/MSLN from epithelioid mesothelioma cells than that from normal mesothelial cells. Similarly, ERC/MSLN from

**Statistical analysis**

Statistical analysis was performed using R software. Wilcoxon rank sum test was used to evaluate differences in relative fluorescence intensities of PHA-E₄/ACG or RCA120/ACG among normal mesothelial cells, epithelioid mesothelioma cells and epithelioid mesothelioma patient specimens.

**Results**

**PHA-E₄ lectin binds more strongly to ERC/MSLN derived from epithelioid mesothelioma cells than to that from normal mesothelial cells**

To reveal a structural characteristic of ERC/MSLN on epithelioid mesothelioma cells, ERC/MSLN was immunoprecipitated from cell lysates of normal mesothelial primary culture cells (MES-F), an immortalized nontumorous mesothelial cell line (MeT-5A), and two epithelioid mesothelioma cell lines (H226 and MESO-4). Immunoprecipitation of ERC/MSLN was confirmed by western blotting (Fig. 1B). Equal amounts of immunoprecipitated-ERC/MSLN (IP-ERC/MSLN), calibrated based on western blotting band intensities, were subjected to lectin microarray analysis. As a result, we found that PHA-E₄ lectin showed a higher reactivity against IP-ERC/MSLN from epithelioid mesothelioma cell lines than that from normal mesothelial cells (Fig. 1C–F). On the other hand, RCA120 lectin showed a lower reactivity against IP-ERC/MSLN from epithelioid mesothelioma cell lines compared to that from normal mesothelial primary culture cells (Fig. 1D–F). Other lectins did not show any differential reactivity against IP-ERC/MSLN between epithelioid mesothelioma and normal mesothelial cells. A glycan structure that increases in epithelioid mesothelioma cells could be a distinct feature of epithelioid mesothelioma, therefore, we focussed on PHA-E₄ lectin binding.

**The strong binding of PHA-E₄ lectin to epithelioid mesothelioma derived ERC/MSLN is further validated using epithelioid mesothelioma patient specimens**

To validate the PHA-E₄ lectin binding to epithelioid mesothelioma derived ERC/MSLN, we performed lectin precipitation. Equal amounts of IP-ERC/MSLN, calibrated based on western blotting band intensities (Fig. 2A, lower panel), were subjected to PHA-E₄ lectin precipitation. PHA-E₄ lectin precipitated a greater portion of IP-ERC/MSLN from epithelioid mesothelioma cell lines (H226 and MESO-4) than from normal mesothelial primary culture cells (MES-F) (Fig. 2A, upper panel). PHA-E₄ lectin is known to bind to complex-type N-glycans with a bisecting-GlcNAc residue (26). To investigate if the PHA-E₄ binding to IP-ERC/MSLN from epithelioid mesothelioma cells is N-glycan-dependent, we treated IP-ERC/MSLN from H226 cells with PNGase F and conducted lectin microarray analysis. Upon PNGase F treatment, almost all lectin signals including PHA-E₄ disappeared (Fig. 2B), confirming that PHA-E₄ lectin binding depends on N-glycans present on ERC/MSLN. Next,

were calculated from the extracted ion chromatograms and summed across all charge states of glycoforms.
surgical specimens from mesothelioma patients also showed significantly stronger reactivity with PHA-E4 lectin. On the other hand, both ERC/MSLN from epithelioid mesothelioma cells and surgical specimens showed a significantly weaker reactivity with RCA120 lectin than that from normal mesothelial primary culture cells (Supplementary Fig. S1C). Therefore, the characteristic glycoforms of ERC/MSLN from epithelioid mesothelioma cells are clinically relevant. The structural feature of ERC/MSLN, having bisecting-GlcNAc recognized by PHA-E4 lectin, could be used as a target to develop specific therapies or as a biomarker for epithelioid mesothelioma.

PHA-E4 lectin is known to bind to complex-type N-glycans having bisecting-GlcNAc residues (27). On the other hand, RCA120 lectin is known to bind to complex-type N-glycans having terminal galactose residues (28). The insertion of bisecting-GlcNAc is thought to inhibit N-glycan branching (29–32) and might reduce terminal galactose residues. This could explain why PHA-E4 lectin binding was increased and RCA120 lectin binding was decreased in epithelioid mesothelioma.
Fig. 3. Bisecting-GlcNAc, the glycan structure recognized by PHA-E4, predominantly exists on the Asn388 residue of ERC/MSLN derived from epithelioid mesothelioma cells. (A) Silver staining result of IP-ERC/MSLN from H226 cells. Dotted line square indicates ERC/MSLN and digested position used for LC/MS analysis. (B) Flow chart showing the sample preparation of ERC/MSLN from H226 cells for glycan analysis by LC/MS. (C–E) Glycan profile of ERC/MSLN at Asn388 (C), Asn496 (D) and Asn523 (E). Black bar indicates glycan structures containing bisecting-GlcNAc, and white bar indicates glycan structures without bisecting-GlcNAc. (F) Expected glycan structure containing bisecting-GlcNAc. dHex, deoxyhexose; Hex, hexose; HexNAc, N-acetylhexosamine; NeuAc, N-acetylneuraminic acid.
mesothelioma cell-derived ERC/MSLN. In the present study, lectin microarray and LC/MS corroborate each other to show that N-glycans of ERC/MSLN synthesized by mesothelioma cells contain a bisecting-GlcNAc residue. Mass to charge signals derived from bisecting-GlcNAc structures were most abundant in N-glycans attached to Asn388, while none was present in N-glycans attached to Asn523. These results suggest that the glycopeptide which includes Asn388 and bisecting-GlcNAc could serve as a characteristic ‘glycoproteome’ to epithelioid mesothelioma cells. Molecular mechanisms regulating the extension and the peripheral modification of each glycan on a glycoprotein are not fully understood. Coordinated events involved in cell-cell adhesion (37–42). E-cadherin is a transmembrane glycoprotein involved in cell-cell adhesion (43), and bisecting-GlcNAc modification of its N-glycans altered its membrane expression (44). ERC/MSLN was also reported against this glycoproteome could be an effective therapeutic antibodies. In addition, having glycans containing a bisecting-GlcNAc pre-son levels in MGAT3 expression level is significantly higher in epithelioid mesothelioma cells (H226, Meso-4) than in normal mesothelial cells (MeT-5A) (Supplementary Fig. S3A). A role for N-glycans with bisecting-GlcNAc in modulating the expression and functions of some glycoproteins, such as E-cadherin, was previously reported (37–42). E-cadherin is a transmembrane glycoprotein involved in cell-cell adhesion (43), and bisecting-GlcNAc modification of its N-glycans altered its membrane expression (44). ERC/MSLN was also reported to be involved in cell adhesion (45), but the roles of its N-glycans and their glycoforms remain to be clarified. Our preliminary results of cell proliferation assay using MGAT3-KO Meso-4 cells, which we generated using the CRISPR/Cas9 system, showed a reduced cell proliferation rate in MGAT3-KO cells (Supplementary Fig. S3D). We confirmed that PHA-E 4 reactivity was reduced concomitant with a decrease in MGAT3 expression levels in MGAT3-KO cells (Supplementary Fig. S2B and C). Therefore, MGAT3 seems to contribute, at least in part, to the proliferation of epithelioid mesothelioma cells.

In summary, our study provides a characteristic glycoproteome of ERC/MSLN expressed on epithelioid mesothelioma cells, with ERC/MSLN having complex-type N-glycans containing a bisecting-GlcNAc predominantly on Asn388. A specific antibody generated against this glycoproteome could be an effective therapy for epithelioid mesothelioma and would potentially show less adverse effects than currently available therapeutic antibodies. In addition, having glycans containing a bisecting-GlcNAc residue may be a prerequisite for ERC/MSLN to be involved in the malignant

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### Table I Glycopeptide sequences of ERC/MSLN from H226 cells identified by LC/MS analysis

| Glycosite | Sequence                  | MW   |
|----------|---------------------------|------|
| Asn388   | (K) WNWTSLETLK (A)        | 1189.63 |
|          | (R) KDNWTSLETLK (A)       | 1317.73 |
|          | (K) WNWTSLETKALLEVNK (G)  | 1957.09 |
|          | (R) KDNWTSLETKALLEVNK (G) | 2085.18 |
|          | (K) MSPEDIRKWNVSLETL (A)  | 2146.11 |
|          | (K) M*SPEDIRKWNVSLETL (A) | 2162.10 |
|          | (K) WNWTSLETKALLEVNKGEHMSPOQATLIDR (F) | 3491.84 |
|          | (K) WNWTSLETKALLEVNKGHEMSPQVATLIDR (F) | 3507.84 |
| Asn496   | (R) LAFQMNMGSEYFVK (I)    | 1646.78 |
|          | (R) LAFQMNMGSEYFVK (I)    | 1662.78 |
|          | (K) ARLAFQMNMGSEYFVK (I)  | 1873.91 |
|          | (K) ARLAFQNMNGSEYFVK (I)  | 1889.91 |
|          | (R) LAFQMNMGSEYFYKIQSFLGGAPEDLK (A) | 3103.53 |
|          | (R) LAFO MNMGSEYFVKIQSFLGGAPEDLK (A) | 3119.53 |
| Asn523   | (K) ALSQONVMDLATFMRK (L)  | 1782.86 |
|          | (K) ALSQONVMDLATFMRK (L)  | 1798.86 |
|          | (K) ALSQONVMDLATFMRK (L)  | 1814.85 |
|          | (K) ALSQONVMDLATFMRK (L)  | 1820.85 |
|          | (K) ALSQONVMDLATFMRK (L)  | 1826.85 |
|          | (K) IFSFLGGAPEDLKALSOQONVMDLATFMRK (L) | 3329.62 |
|          | (K) IFSFLGGAPEDLKALSOQONVMDLATFMRK (L) | 3355.61 |
|          | (K) IFSFLGGAPEDLKALSOQONVMDLATFMRK (L) | 3371.61 |

M*: methionine oxidation, N*: glycosylation site.
behaviour of certain cancers cells with high expression levels of ERC/MSLN.

Supplementary Data
Supplementary Data are available at JB Online.

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Conflict of Interest
None declared.

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