Characterization of tumor-associated MUC1 and its glycans expressed in mucoepidermoid carcinoma

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Abstract. Mucoepidermoid carcinoma (MEC) is one of the most frequently misdiagnosed tumors. Glycans are modulated by malignant transformation. Mucin 1 (MUC1) is a mucin whose expression is upregulated in various tumors, including MEC, and it has previously been investigated as a diagnostic and prognostic tumor marker. The present study aimed to reveal the differences in the mucin glycans between MEC and normal salivary glands (NSGs) to discover novel diagnostic markers. Soluble fractions of salivary gland homogenate prepared from three MEC salivary glands and 7 NSGs were evaluated. Mucins in MEC and NSGs were separated using supported molecular matrix electrophoresis, and stained with Alcian blue and monoclonal antibodies. The glycans of the separated mucins were analyzed by mass spectrometry. MUC1 was found in MEC but not in NSGs, and almost all glycans of MUC1 in MEC were sialylated, whereas the glycans of mucins in NSGs were less sialylated. The core 2 type glycans, $(Hex)_2(HexNAc)_2(NeuAc)_1$ and $(Hex)_2(HexNAc)_2(NeuAc)_2$, were found to be significantly abundant glycans of MUC1 in MEC. MEC markedly produced MUC1 modified with sialylated core 2 glycans. These data were obtained from the soluble fractions of salivary gland homogenates. These findings provide a basis for the utilization of MUC1 as a serum diagnostic marker for the preoperative diagnosis of MEC.

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Introduction

Salivary gland carcinomas are rare tumors that account for about 3 to 6% of head and neck cancers. The WHO classified salivary gland tumors into over 20 different tissue types in 2017 (1). Mucoepidermoid carcinoma (MEC) is the most common malignant salivary tumor and constitutes approximately 30% of all malignant tumors of the salivary gland (2). Surgery is the only definitive treatment for all salivary gland tumors, including MEC. The types and scopes of surgery vary greatly depending on the stage and grade of the tumor (3,4). Preoperative diagnosis helps to guide operative strategy and clinical management (5,6). A previous study reported that high-grade MEC may have lymph node metastasis, and when high-grade MEC is diagnosed before surgery, neck dissection is also considered (7).

In addition to genetic changes, epigenetic changes also play an important role in neoplastic transformation (8). The glycosylation of proteins also changes with epigenetic alterations. Glycan alterations are common features of tumor cells (9,10) and have been aggressively studied as cancer biomarkers and functional molecules involved in malignant behaviors, including metastasis and cancer cell proliferation. Mucins are hyperglycosylated proteins in which the glycans attach to Ser/Thr residues in the variable number of tandem repeat (VNTR) region of the mucins. A serum tumor marker, CA19-9, is a glycan antigen found on MUC1, and is involved in the promotion of metastasis and the dissemination of cancer cells (11). MUC1, modified with certain glycans (tumor-associated MUC1) such as sialyl

Lewis a, sialyl Lewis X, and sialyl Tn, is known as a marker of various tumors (12-15). Tumor-associated MUC1 is widely distributed in both primary and metastatic tumors, including cancer stem cells, and has been widely considered a target for many diagnostic and immunological therapeutic approaches. Based on certain criteria, such as therapeutic function, immunogenicity, and cancer cell specificity, MUC1 was listed by the National Cancer Institute pilot project to prioritize cancer antigens as the second most promising target in cancer research from a list of 75 tumor-associated antigens (16). MUC1 expression has also been reported to be associated with the histological grade of MEC (17,18). In this study, mucins and their glycans expressed in MEC were analyzed and compared with normal salivary glands (NSGs). Mucins are extensively glycosylated proteins with high molecular weights of over 1,000 kDa and are difficult to analyzed using conventional proteomic techniques. In the present study, mucins were analyzed using supported molecular matrix electrophoresis (SMME), a membrane electrophoresis method, that was previously developed to characterize mucins (19). The mucins were stained on the SMME membranes using specific antibodies (20). Furthermore, we analyzed the glycans attached to the mucins by mass spectrometry (MS) and revealed that MEC specifically produced MUC1 with core 2 type glycans modified with sialic acid.

Materials and methods

Case selection. All cases were diagnosed and classified by experienced oral pathologists. The pathological classification was described based on TNM Classification of Malignant Tumors (8th edition) by the Union for International Cancer Control (UICC) (21). Salivary glands were surgically removed from patients with a diagnosis of MEC and other salivary gland tumors. Surplus tissues that did not contain malignant cells were used as samples of NSGs. In this study, MEC samples and NSG samples were obtained from different patients, respectively. The tissues were cut to a predetermined size (2x2 mm) and then immediately stored in a freezer at -80°C until further use. This research was approved by the Tokyo Dental College Ichikawa General Hospital Ethics Review Committee (I16-74) and the AIST Committee on Bioethics of Experiments Involving Human-derived Materials (h-213). Written informed consent was obtained for experimentation with human subjects.

Chemicals and materials. A polyvinylidene difluoride (PVDF) membrane (Immobilon-P; pore size, 0.45 μm) was purchased from EMD Millipore. Poly (vinyl alcohol) (PVA; MW, 22,000) was purchased from Wako Pure Chemical Industries. Poly (ethylene glycol) (PEG; MW: 20,000) and polyvinylpyrrolidone (PVP; MW: 20,000) were purchased from Nacalai Tesque Inc. Porcine gastric mucin (PGM) (type III, partially purified) and Alcian blue 8GX were purchased from Sigma-Aldrich; Merck KGaA. Anti-sialyl MUC1 monoclonal antibody (MY.1E12) was provided by Dr T. Irimura (Juntendo University, Japan) (22,23). Hyaluronic acid binding protein (HABP) was obtained from Cosmo Bio Co., Ltd. Other reagents and solvents were obtained from Wako Pure Chemical Industries Ltd. and Nacalai Tesque Inc. All solvents used were of analytical reagent grade.

Extraction of mucins. Mucin extraction was performed as reported in previous studies (19,20). Briefly, pieces of MEC and NSG tissue were homogenized in cold acetone. The homogenized mixtures were centrifuged at 16,000 x g at 4°C for 10 min. After discarding the supernatants, the resulting powders were homogenized in PBS (pH 7.4, 805 μl) containing 5 μl of protease inhibitor mix (GE Healthcare Bio-Sciences). A saturated calcium acetate solution was added to the supernatants at a ratio of 1:4, followed by the addition of three volumes of ethanol. The mixtures were cooled at -80°C, left to stand for 1 h, and centrifuged. The precipitates were dissolved in 2 M urea in PBS (pH 7.4, 100 μl) and centrifuged at 16,000 x g at 4°C for 10 min. The supernatants were transferred into new tubes and precipitated with ethanol, as described above. After centrifugation at 16,000 x g at 4°C for 10 min, the resulting precipitates were dissolved in 8 M urea in PBS (pH 7.4, 20 μl). The solutions were stored at 4°C until further use.

SMME. SMME membranes were prepared by immersion in a hydrophilic polymer mixture of PVA:PVP=1:3 as previously described (24). The SMME membranes (separation length, 6 cm) were wetted in methanol and then transferred into a running buffer (0.1 M pyridine-formic acid buffer, pH 4.0). After equilibration for 30 min with gentle shaking, the membranes were subjected to electrophoresis. The solutions containing mucins extracted from clinical samples were spotted at 1.5 cm from the edge of the membrane at the negative node. Electrophoresis was performed using a membrane electrophoresis chamber (EPC105AA; Advantec) in constant-current mode at 1.0 mA/cm for 30 min. The membranes were then stained with Alcian blue as previously described (19,20).

Immunostaining. Mucin immunostaining on the SMME membrane was performed as previously reported (24,25). Briefly, the electrophoresed membranes were immersed in acetone for 30 min, followed by heating at 150°C for 5 min (25,26). The membranes were then rewetted by immersing in 5% BSA in PBS containing 0.05% Tween-20 (PBS-T) for 1 h. Following washing with PBS-T (5 min, 3 times), the membranes were incubated with PBS-T containing a primary antibody (dilution of 1/1,000) at 4°C overnight. After washing with PBS-T (5 min, 3 times), the membranes were incubated with PBS-T containing secondary antibody labeled with HRP (dilution of 1/2,000) for 1 h at room temperature. Binding was visualized with ECL reagents (Western Lightning Plus-ECL; PerkinElmer). Chemiluminescence images were obtained using ChemiDoc XRS (Bio-Rad Laboratories, Inc.).

Release and permethylation of O-glycans. O-glycans were released from mucins on the SMME membranes by reductive β-elimination, followed by analysis using mass spectrometry, as per previous studies (19,20). Briefly, the stained mucin spots were excised from the SMME membrane, transferred to a 500 μl microcentrifuge tube, and incubated with 40 μl of 500 mM NaBH$_4$ in 50 mM NaOH at 45°C for 16 h. The reaction mixtures were quenched by adding 4 μl of glacial acetic acid until the hydrogen gas ceased. The mixtures were desalted using a cation-exchange-solid-phase extraction cartridge (3 ml)

Table I. Summary of salivary glands used in the present study.

Sample number	Age, years	Sex	Location	Tissue type	Histological grade (TNM classification)
1	38	F	Minor salivary gland	Mucoepidermoid carcinoma	Low grade (T1N0M0)
2	39	F	Minor salivary gland	Mucoepidermoid carcinoma	Low grade (T1N0M0)
3	52	M	Minor salivary gland	Mucoepidermoid carcinoma	Low grade (T2N0M0)
4	90	F	Submandibular gland	Normal	Not applicable[a]
5	68	F	Submandibular gland	Normal	Not applicable[a]
6	57	F	Submandibular gland	Normal	Not applicable[a]
7	41	M	Submandibular gland	Normal	Not applicable[a]
8	55	M	Submandibular gland	Normal	Not applicable[a]
9	51	M	Sublingual gland	Normal	Not applicable[a]
10	87	M	Minor salivary gland	Normal	Not applicable[a]

[a]TNM classification is not applicable to normal tissues; F, female; M, male.

(Oasis MCX; Waters Corp.) and concentrated. The obtained residues were dissolved in 100 μl of 1% (v/v) acetic acid in methanol and dried again using a centrifugal evaporator. The dissolving and drying procedures were repeated.

A slurry of 50 μl (80 mg/ml) of NaOH in dimethyl sulfoxide (DMSO) containing 1% (v/v) distilled water was added to the dried samples, followed by 12.5 μl of methyl iodide. The mixtures were then vigorously shaken for 30 min until the sodium iodide precipitated. After adding 50 μl of 10% acetic acid, the mixtures were diluted to 1 ml with distilled water. The diluted solutions were subjected to a solid-phase extraction cartridge (50 mg) (Sep-Pak C18; Waters Corp.), washed with 1 ml of water three times, and then dried. Finally, permethylated glycans were eluted with 1 ml of 50% acetonitrile and concentrated using a centrifugal evaporator.

MS. The MS spectra of the permethylated glycans were acquired in reflectron positive ion mode using a matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) mass spectrometer (UltraFlex; Bruker-Daltonics) equipped with a 337 nm pulsed nitrogen gas laser. 2,5-Dihydroxybenzoic acid (2,5-DHB) was used as the matrix. For sample preparation, 0.5 μl of 2,5-DHB solution (10 mg/ml in 30% ethanol) was deposited onto a MALDI target plate and dried. Then 0.5 μl of the sample solution was deposited over the dried spot of 2,5-DHB and dried. Tandem mass spectrometry was performed using a MALDI-quadrupole ion trap (QIT)-TOF mass spectrometer (AXIMA-QIT; Shimadzu Corp.) in a positive ion mode. Argon gas was used as the collision gas for collision-induced dissociation.

Statistical analysis. The relative intensities of the glycan signals in the MS spectra were calculated as a percentage of the intensity in the sum of the intensities of all observed 50 glycans. Unpaired Student's t-tests for each glycan between MEC and NSGs were performed using GraphPad Prism 8.4.2 (GraphPad Software). The MS/MS spectra of the permethylated glycans were assigned using GlycoWorkBench 2.1 (http://www.eurocarbdb.org/).

Glycan cartoon. The glycan structures were depicted using CFG graphical notation for glycans (http://www.functionalglycomics.org/static/consortium/CFGnomenclature.pdf).

Results

SMME analyses of MEC and NSGs. The clinical information of the salivary glands of MEC and NSGs used in this study are summarized in Table I. The mucins extracted from the salivary glands were separated by SMME and stained with Alcian blue (Fig. 1A). A commercially obtained PGM containing chondroitin sulfate, hyaluronic acid, acidic mucin, and neutral mucin was used as the reference material in SMME. We estimated the stained bands by comparing the migrating positions with those of the corresponding constituents in the reference PGM. Additionally, the mucin-enrichment fraction of cultured cells (HPAF-II) was also used as a reference material containing MUC1 (25). All MEC samples 1-3 and NSGs of the minor salivary gland 10 clearly showed bands at the migrating position of the acidic mucin in the reference PGM. Another dark band was observed at the position of neutral mucin only from the NSG sample 7. Other samples showed a pale band at a lower position of the neutral mucin and/or the same position of hyaluronic acid in the reference PGM.

We also stained the SMME membranes with specific probes, such as a monoclonal antibody. Anti-MUC1 antibody (MY.1E12) revealed that all three MEC samples contained MUC1 but not NSGs, and the migrating positions of these MUC1 bands were just below that of MUC1 in HPAF-II (Fig. 1B). On the other hand, the mucins of the minor salivary gland 10 were not stained even by increasing the exposure time of chemiluminescence reaction (Fig. S1A). MUC1 and hyaluronic acid appeared close in the SMME. To confirm hyaluronic acid, we also stained the SMME membrane with HABP (Fig. 1C). Hyaluronic acid was detected in all samples at the same migration position as the reference hyaluronic acid in PGM.
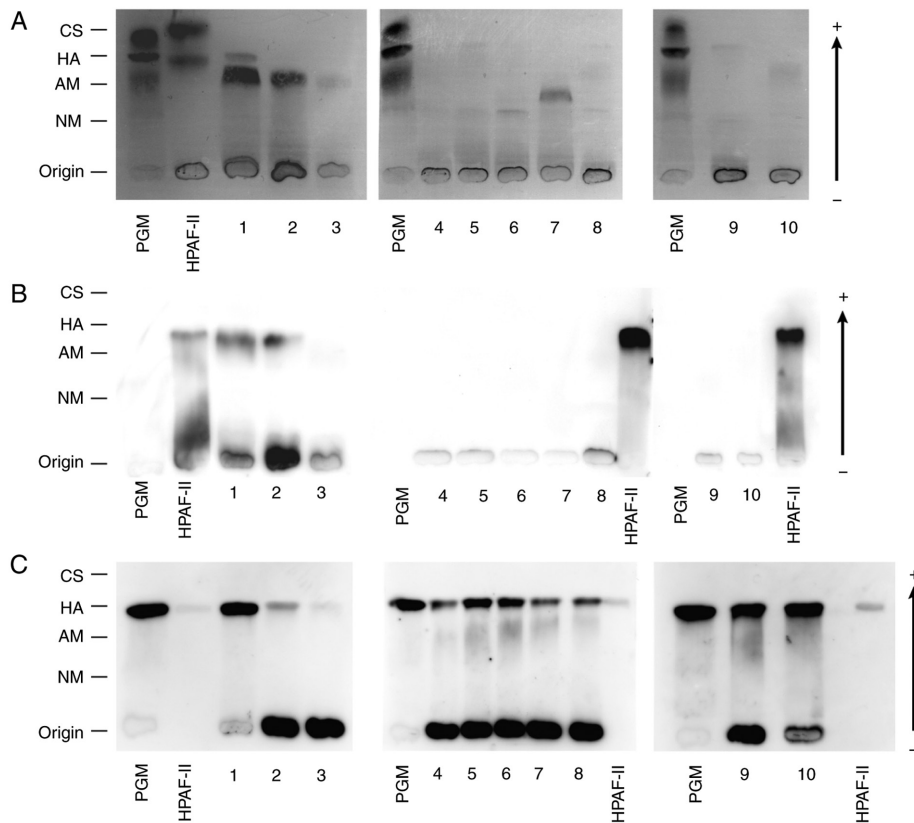
Figure 1. Mucins of mucoepidermoid carcinoma and normal salivary glands were separated by SMME. Arabic numerals indicate the sample numbers shown in Table I. PGM was used as a reference mixture of CS, HA, AM and NM. The migrating position of the neutral mucin of PGM was estimated from our previous study (18). HPAF-II lysate was used as a mixture containing MUC1. (A) Alcian blue staining of the SMME membranes. (B) Staining with anti-MUC1 antibody (MY.1E12). (C) Staining with hyaluronan binding protein (BC-40). AM, acidic mucin; CS, chondroitin sulfate; HA, hyaluronic acid; MUC1, mucin 1; NM, neutral mucin; PGM, porcine gastric mucin; SMME, supported molecular matrix electrophoresis.
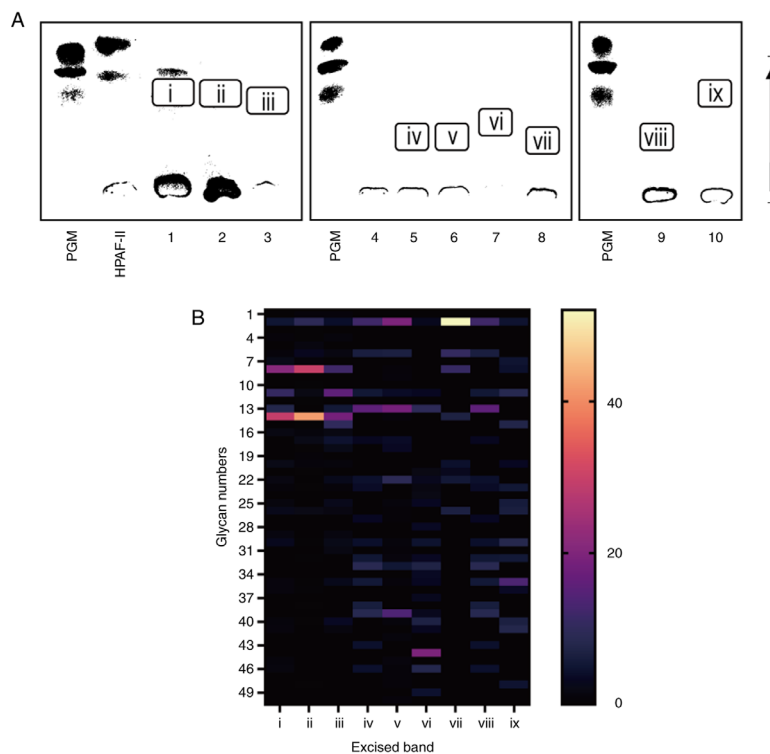

Figure 2. *O*-glycan profiles of mucins separated by SMME. (A) Position of the excised bands in the illustration derived from the SMME membranes stained with Alcian blue (Fig. 1A). Roman numerals indicate the excised band numbers. No bands appeared from sample 4. (B) Heatmap of *O*-glycan profiles of the excised bands. The color scale indicates the relative intensity (%) of each glycan to the sum of intensities of all 50 glycans (Table II). PGM, porcine gastric mucin; SMME, supported molecular matrix electrophoresis.

Table II. Summary of MS signals of *O*-glycans detected from the excised band (Fig. 2A).

A, Acidic glycans

Glycan no.	Composition	Calcd, m/z^a	Obsd, m/z^b
1	$(HexNAc)_1(NeuAc)_1$	691.36	691.37
2	$(Hex)_1(HexNAc)_1(NeuAc)_1$	895.46	895.49
3	$(HexNAc)_2(NeuAc)_1$	936.49	936.54
4	$(Hex)_1(HexNAc)_1(Deoxyhexose)_1(NeuAc)_1$	1,069.55	1,069.58
5	$(Hex)_1(HexNAc)_2(NeuAc)_1$	1,140.59	1,140.64
6	$(Hex)_1(HexNAc)_1(NeuAc)_2$	1,256.64	1,256.67
7	$(Hex)_1(HexNAc)_2(Deoxyhexose)_1(NeuAc)_1$	1,314.68	1,314.71
8	$(Hex)_2(HexNAc)_2(NeuAc)_1$	1,344.69	1,344.74
9	$(Hex)_2(HexNAc)_1(Deoxyhexose)_2(NeuAc)_1$	1,447.74	1,447.79
10	$(Hex)_1(HexNAc)_2(NeuAc)_2$	1,501.76	1,501.81
11	$(Hex)_2(HexNAc)_2(Deoxyhexose)_1(NeuAc)_1$	1,518.78	1,518.82
12	$(Hex)_2(HexNAc)_3(NeuAc)_1$	1,589.82	1,589.86
13	$(Hex)_2(HexNAc)_2(Deoxyhexose)_2(NeuAc)_1$	1,692.87	1,692.89
14	$(Hex)_2(HexNAc)_2(NeuAc)_2$	1,705.86	1,705.88
15	$(Hex)_2(HexNAc)_3(Deoxyhexose)_1(NeuAc)_1$	1,763.90	1,763.94
16	$(Hex)_3(HexNAc)_3(NeuAc)_1$	1,793.92	1,793.90
17	$(Hex)_2(HexNAc)_2(Deoxyhexose)_1(NeuAc)_2$	1,879.95	1,880.01
18	$(Hex)_2(HexNAc)_3(Deoxyhexose)_2(NeuAc)_1$	1,937.99	1,938.03
19	$(Hex)_2(HexNAc)_3(NeuAc)_2$	1,950.99	1,951.06

B, Neutral glycans

Glycan no.	Composition	Calcd, m/z^a	Obsd, m/z^b
20	$(Hex)_1(HexNAc)_1$	534.29	534.28
21	$(HexNAc)_2$	575.32	575.18
22	$(Hex)_1(HexNAc)_1(Deoxyhexose)_1$	708.38	708.40
23	$(Hex)_1(HexNAc)_2$	779.41	779.44
24	$(Hex)_2(HexNAc)_1(Deoxyhexose)_1$	912.48	912.46
25	$(Hex)_1(HexNAc)_2(Deoxyhexose)_1$	953.50	953.54
26	$(Hex)_2(HexNAc)_2$	983.52	983.57
27	$(Hex)_1(HexNAc)_3$	1,024.54	1,024.59
28	$(Hex)_2(HexNAc)_1(Deoxyhexose)_2$	1,086.57	1,086.53
29	$(Hex)_1(HexNAc)_2(Deoxyhexose)_2$	1,127.59	1,127.63
30	$(Hex)_2(HexNAc)_2(Deoxyhexose)_1$	1,157.60	1,157.64
31	$(Hex)_1(HexNAc)_3(Deoxyhexose)_1$	1,198.63	1,198.69
32	$(Hex)_2(HexNAc)_3$	1,228.64	1,228.68
33	$(Hex)_2(HexNAc)_2(Deoxyhexose)_2$	1,331.69	1,331.75
34	$(Hex)_3(HexNAc)_2(Deoxyhexose)_1$	1,361.70	1,361.66
35	$(Hex)_2(HexNAc)_3(Deoxyhexose)_1$	1,402.73	1,402.78
36	$(Hex)_3(HexNAc)_3$	1,432.74	1,432.75
37	$(Hex)_3(HexNAc)_1(Deoxyhexose)_3$	1,464.76	1,464.69
38	$(Hex)_2(HexNAc)_4$	1,473.77	1,473.83
39	$(Hex)_2(HexNAc)_2(Deoxyhexose)_3$	1,505.78	1,505.81
40	$(Hex)_2(HexNAc)_3(Deoxyhexose)_2$	1,576.82	1,576.85
41	$(Hex)_3(HexNAc)_3(Deoxyhexose)_1$	1,606.83	1,606.82
42	$(Hex)_4(HexNAc)_3$	1,636.84	1,636.94
43	$(Hex)_2(HexNAc)_4(Deoxyhexose)_1$	1,647.86	1,647.88
44	$(Hex)_3(HexNAc)_2(Deoxyhexose)_3$	1,709.88	1,709.81
45	$(Hex)_2(HexNAc)_3(Deoxyhexose)_3$	1,750.91	1,750.96

Table II. Continued.

B, Neutral glycans

Glycan no.	Composition	Calcd, m/z^a	Obsd, m/z^b
46	$(Hex)_3(HexNAc)_3(Deoxyhexose)_2$	1,780.92	1,780.95
47	$(Hex)_2(HexNAc)_4(Deoxyhexose)_2$	1,821.95	1,822.05
48	$(Hex)_3(HexNAc)_4(Deoxyhexose)_1$	1,851.96	1,852.00
49	$(Hex)_3(HexNAc)_3(Deoxyhexose)_3$	1,955.01	1,954.90
50	$(Hex)_2(HexNAc)_4(Deoxyhexose)_3$	1,996.04	1,996.10

[a]All signals were calculated as $[M + Na]^+$ ions. [b]The observed m/z values are the averages of the corresponding signals detected in all spectra. All glycans were permethylated before MS acquisition. The m/z values are monoisotopic. Calcd, calculated; MS, mass spectrometry; Obsd, observed.
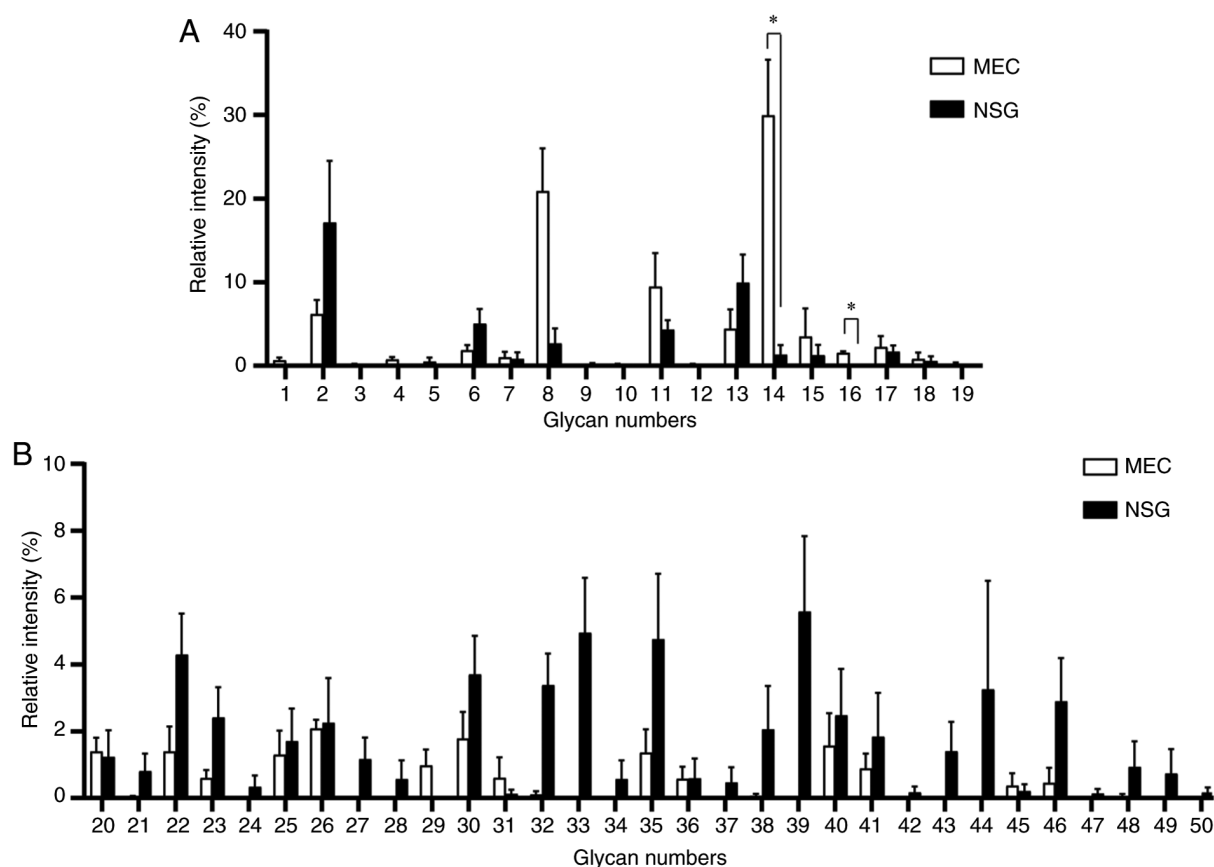

Figure 3. Two-group comparisons of all glycan signals between MEC and NSGs. Bar height represents the mean of the relative intensity of the three bands (i, ii and iii) for MEC (white) and six bands (iv-ix) for NSGs (black). Error bars indicate the SEM. *P<0.001. (A) Sialoglycans and (B) neutral glycans. MEC, mucoepidermoid carcinoma; NSG, normal salivary gland.

Comparison of O-glycans on mucins between MEC and NSGs. Each band of mucin stained with Alcian blue was excised and subjected to reductive β-elimination, in which the *O*-glycans attached to mucins were released and reduced to the corresponding alditols (Fig. 2A). The obtained glycan alditols were permethylated and analyzed using MALDI-TOF MS. All of the observed glycan signals were categorized into two groups: Sialoglycans and neutral glycans, as summarized in Table II. The relative intensities of the glycan signals observed from each band are depicted in heatmap (Fig. 2B). The

O-glycans from MEC (i-iii) mostly consisted of sialoglycans 8 and 14 (orange cells), while the *O*-glycans from NSGs (iv-ix) contained substantial neutral glycans (navy blue cells). This is a distinctive difference in the glycan profiles of MEC and NSGs. In all of the observed glycan signals, the mean relative intensities in the two groups MEC (i-iii) and NSG (iv-ix) were compared (Fig. 3). Notably, glycan 8 and 14 showed high intensities in MEC compared with NSGs. These glycans were mono-sialylated and di-sialylated of a common core structure $(Hex)_2(HexNAc)_2$, respectively. Glycan 1 is
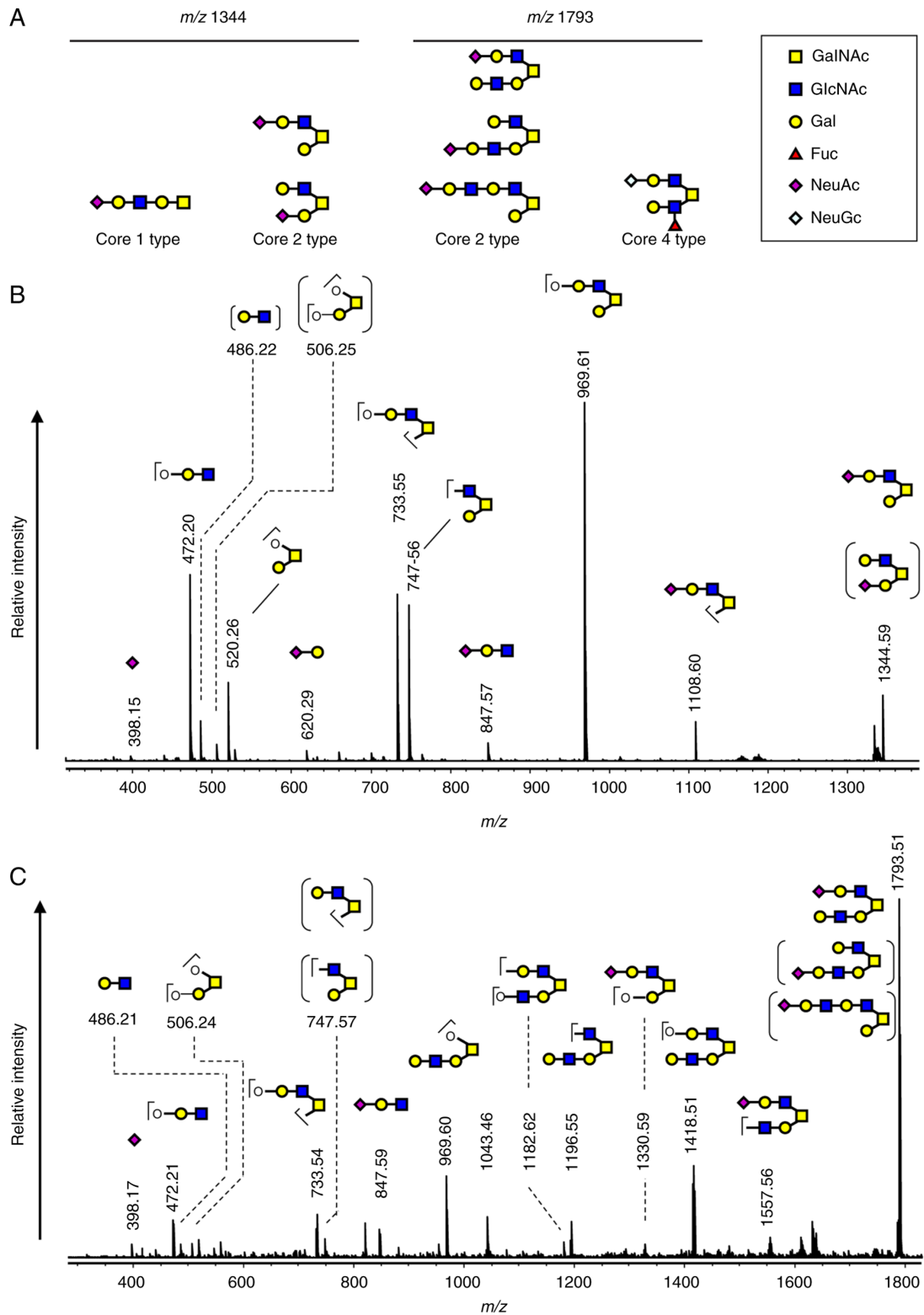
Figure 4. Proposed structures for the two significant glycans, glycan 8 (*m/z*, 1,344) and 16 (*m/z*, 1,793). (A) Possible structures for glycans 8 (*m/z*, 1,344) and 16 (*m/z*, 1,793) based on the biosynthetic pathway. (B) MS/MS spectrum of glycan 8 (*m/z*, 1,344). (C) MS/MS spectrum of glycan 16 (*m/z*, 1,793). The proposed structure and assignment of the fragment ions are indicated in the spectra. The structures in parentheses are considered to be minor components. MS/MS, tandem mass spectrometry.

sialyl Tn antigen, a well-known glycan biomarker for various malignant tumors (27,28), and was detected in two out of three MEC samples, but not in the NSG samples. On the other hand, many highly fucosylated neutral glycans (glycan 33, 35, 39, and 44) were abundantly observed in NSGs.

Structural analysis of the glycans from MEC. The significant glycan 8 and 14 were mono- and di-sialylated structures of the same core structure $(Hex)_2(HexNAc)_2$, respectively. Another significant glycan, glycan 16, is the mono-sialylated structure of $(Hex)_3(HexNAc)_3$. To elucidate the core structures of the

significant glycans in MEC, we acquired the MS/MS spectra of glycan 8 and 16. Based on the biosynthetic pathway of the *O*-glycans, we proposed potential structures for glycans 8 (*m/z* 1,344) and 16 (*m/z* 1,793), respectively (Fig. 4A). The MS/MS spectrum of glycan 8 (*m/z* 1,344) revealed that glycan 8 was a mixture of the positional isomers of sialylation of the same structure that was galactosylated at the GlcNAc of core 2 (Fig. 4B). The MS/MS spectrum of glycan 16 (*m/z* 1,973) was in agreement with the calculated fragments of the *N*-acetyl lactosamine (LacNAc) elongated core 2 (Fig. 4C).

Discussion

In this study, MEC was found to abundantly produce MUC1, which was characteristically modified with core 2 type sialoglycans. The secretion of mucins in the oral cavity and the number of sialic acids on the mucins have been reported to decrease with aging (29). MEC samples 1 and 2 and NSG sample 7 were obtained from middle-aged patients (38-41 years old) and showed dark bands in Alcian blue staining during SMME analysis, while other samples from patients over 50 years of age showed pale bands. This is consistent with the previous report described above. MUC1 is involved in cell proliferation and signal transduction (11,30) and has been known to be overexpressed in malignant tumors (31). Immunohistochemistry using the MUC1 antibody showed that higher levels of MUC1 expression were positively correlated with the malignancy and prognosis of MEC (17,18). In our experiments, MUC1 was only detected in MEC. Although MEC sample 3 appeared to be MUC1-negative, all three MEC samples became MUC1-positive by increasing the exposure time of chemiluminescence imaging (Fig. S1A). On the other hand, all NSGs in our experiment were found to be MUC1-negative. The incidence of MEC is high in relatively young patients (average age: 48.8 years old) (32). Notably, MUC1 was not detected even in the NSGs of relatively young patients (6, 7, 8, 9: 41-57 years old). MUC1 is expressed as a membrane-bound mucin in the ducts of all normal salivary gland tissues (33,34). In the present study, only the water-soluble fraction was analyzed, suggesting that the expression of water-soluble MUC1 was closely related to the development of MEC. The MUC1 antibody, MY.1E12, recognizes MUC1 with a sialyl T (Neu5Acα2-3Galβ1-3GalNAc) structure (23). We could not deny that NSGs produce MUC1 without this sialyl T structure. To clarify this point, the SMME membranes were stained using another anti-MUC1 antibody, VU4H5 which recognizes a tandem repeat domain of MUC1. The results supported the finding that MEC but not the NSGs expressed MUC1 (Fig. S1B). The origin spots of the MEC samples were strongly stained, which suggested that MUC1 exists in its insoluble form in the sample solution (Fig. S1A and B). MUC1, one of the membrane-bound mucins, has a cleavage site in the extracellular domain (35,36). The cleaved MUC1 is able to migrate to the upper part during SMME, while the membrane-bound form remains in the origin. On the Alcian blue stained membrane, NSG sample 10 showed a mucin band at almost the same position as MUC1 from the MEC samples. However, the band could not be stained with either of the MUC1 antibodies, MY.1E12 and VU4H5 (Fig. S1).

All of the MEC samples in this study were obtained from minor salivary glands. For NSG, samples 4-8 were obtained from submandibular glands, while samples 9 and 10 were obtained from the sublingual gland and the minor salivary gland, respectively. The difference in the types of salivary glands may affect mucin expression. The staining of the SMME membranes with Alcian blue showed clear differences between the minor salivary glands (1-3, and 10) and submandibular glands (4-8). Moreover, the sublingual gland (9) was different from the other two types. Although the number of cases will need to be increased to confirm differences depending on the type of salivary gland, the fact that MUC1 was only detected in MEC (1-3), and not NSGs (10), obtained from minor salivary glands is a key result. In the present study, only three MEC samples were compared with the NSGs. It would have been better if we could have compared MEC lesions and normal areas in the same patients, but all of the MEC in this study were minor salivary glands, and we could not clearly distinguish between normal areas and lesions. Therefore, we cannot deny that individual differences may have affected the present results. In the future, we plan to conduct similar experiments involving the analyses of MECs in the major salivary glands and other mucus-producing salivary gland tumors, such as acinic cell carcinoma, low-grade salivary duct carcinoma, mammary analog secretory carcinoma, and mucinous variant of myoepithelioma, all of which are MUC1 positive (37-40). We thereby aim to clarify whether the origin and types of salivary gland tumors affect the glycan structure of tumor-associated MUC1.

The glycan profiles of mucin were also clearly different between MEC (i-iii) and NSG (iv-ix) The glycans from the MEC mucins largely consisted of sialoglycans, while those from NSG mucins contained many neutral glycans. Although sialic acids have been reported to decrease with aging (29), NSG mucin (vi) from the submandibular gland of a 41-year-old patient had fewer sialoglycans and NSG mucin (vii) compared to the submandibular gland of a 55-year-old patient, whose glycan profile showed a high ratio of sialoglycans. It was also unclear whether the types of salivary glands had an effect on the glycan profiles of the mucins in our experiment. In the two group comparisons between MEC and NSG, the intensities of glycan 8 and 14 were found to be significantly high in MEC. The two glycans were sialylated core 2 glycans. The mRNA expression of core 2-β-1,6-*N*-acetylglucosaminyltransferase-1 (GCNT1), one of the key enzymes responsible for the core 2 branched structure, is associated with the progression of several cancers, including colorectal cancer, pulmonary adenocarcinoma, and prostate cancer (41-43). It has also been suggested that MUC1 carrying core 2 glycans contribute to the ability of cancer cells to evade attack by natural killer cells (44). Thus, MUC 1 carrying core 2 glycans expressed in MEC may play an important role in malignant cellular behavior. The sialyl Tn (STn) antigen has been widely reported as a tumor-associated glycan antigen. In the present study, STn (glycan 1) was only detected in MEC samples, but with a very small intensity. The STn antigen in MUC1 has potential as a specific biomarker for MEC, however, the sialylated core 2 glycans described above are more

likely to be used as biomarkers because of their prominent intensities. It has also been reported that the sialic acid levels are increased in malignant tumors (45), which is in good agreement with our results. The abnormal sialylation of cancer cells is a characteristic alteration associated with malignant properties, including invasiveness and metastatic potential (46). Although it has been reported that high MUC1 expression is positively correlated with malignancy, it is still unclear whether the glycan structures of the tumor-associated MUC1 are correlated to the MEC grade because our experiments used only low-grade MEC samples. Using immunohistochemistry, a previous study revealed that the positivity rate of Tn and Lewis a is decreased in high-grade MEC (28). We intend to obtain experimental data on the correlation between the grades and glycan structures of tumor-associated MUC1 in the water-soluble fractions of MEC samples in the future.

In this study, we analyzed the soluble fractions obtained from the homogenates of salivary glands and identified a characteristic mucin, namely MUC1 with sialylated core 2 glycans. Unlike MUC7, which is secreted by goblet cells via exocytosis from the salivary glands into the saliva, MUC1 is usually not secreted, even in the case of soluble MUC1, which is cleaved at the extracellular domain. Soluble MUC1 may enter the blood circulation from lesion areas and has potential for use as a serum biomarker for several tumors that produce MUC1 with characteristic glycans, such as STn and sialyl Lewis a (47). A previous study reported that the serum levels of CA153, a MUC1 antigen recognized by the two anti-MUC1 antibodies DF3 and 115D8, are elevated in some diseases, including various cancers, type 2 diabetes, and coronary heart disease (48). The authors proposed that pathological leakage of CA153 from the epithelium, in addition to the decreased CA153 clearance rate may cause the MUC1 antigen to appear in the blood of patients with the aforementioned diseases. When MUC1 with sialylated core 2 glycans can be used as a serum biomarker for MEC, CA153 in the serum of patients with these diseases may be a confounding variable for tumor-associated MUC1 from MEC. To avoid such confusion, it may be important to distinguish the differences in the glycans of MUC1. KL6 for lung disease and WFA-positive MUC1 for intrahepatic cholangiocarcinoma are known examples in which the glycans of MUC1 differ among primary tumor tissues (49,50). Regrettably, there is no information on the glycans of MUC1 in salivary glands. To clarify these issues in the future, it is necessary to analyze the expression of MUC1, including its glycans, in other oral tumors, and investigate its leakage into the bloodstream.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the MassIVE repository (https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=441d55f0338f4e09a75d00be9f0acbc0; accession no. MSV000087578).

Authors' contributions

EI, TN, KK, UA and AK designed the study. KH performed pathological evaluation of the salivary glands. EI, TS and AK performed SMME and glycan analysis. EI, TS and AK confirmed the authenticity of all the raw data. EI and AK interpreted the acquired data and prepared the manuscript. TN, KK and UA contributed to critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the Tokyo Dental College Ichikawa General Hospital Ethics Review Committee (approval no. I16-74; Ichikawa, Japan) and the AIST Committee on Bioethics of Experiments Involving Human-derived Materials (approval no. h-213; Tokyo, Japan). Written informed consent was obtained for experimentation with human subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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