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LETTER TO THE EDITOR

ABNORMAL SUDOMOTOR AXON REFLEX AND ANTIGANGLIOSIDE ANTIBODIES

Antiganglioside antibodies are frequently present in sera from patients with such autoimmune neuropathies as Guillain–Barré syndrome (GBS) and Miller Fisher syndrome (MFS). Autonomic dysfunction sometimes occurs in autoimmune neuropathies,[6] but its relationship to antiganglioside antibodies is unknown. The quantitative sudomotor axon reflex test (QSART), which can evaluate the postganglionic sudomotor axon quantitatively,[4] can provide a measure of autonomic dysfunction. To investigate the relationship between antiganglioside antibodies and autonomic dysfunction in GBS or MFS, we performed QSART in 15 such patients, and compared the severity of QSART with the results of the antiganglioside antibody assay.

We investigated 6 MFS and 9 GBS patients admitted to our hospital between 1992 and 2004. None of the patients had other diseases affecting sweating function, such as diabetes mellitus. QSART was performed in the left distal leg and foot.[4] The severity of QSART abnormality was rated by the age- and gender-matched sudomotor subscore of the Composite Autonomic Scoring Scale[4] (CASS-sud) in which 0 indicates no deficit and 3 represents maximal deficit. IgM and IgG antibodies against GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, GQ1b, GA1, and Gal-C in patients' sera were investigated by enzyme-linked immunosorbent assay (ELISA).[3] Fisher's exact test was performed and $P < 0.05$ was considered statistically significant. The patients consented to participate in this study, which had the approval of our institutional review board.

CASS-sud scores were 2 or 3 in 5 of the 15 patients, and 1 or 0 in the remaining 10 patients. Among those 5 patients with high CASS-sud scores, 4 had anti-GQ1b IgG antibodies and 1 had IgM and IgG antibodies against both GM1 and GD1b (Table 1). However, only 1 of the 10 patients with a low CASS-sud demonstrated anti-GQ1b IgG antibody. Occurrence of IgG anti-GQ1b antibody was more frequent in patients with a CASS-sud of 2 or more ($P < 0.05$). High anti-GQ1b titer (++ or +++) was associated with high (3) CASS-sud score, but larger numbers of patients are necessary for statistical analysis.

Table 1. Relationship between CASS-sud and serum antiganglioside antibodies.

Age (years)	Gender	Diagnosis	Antiganglioside antibodies	CASS-sud
52	M	MFS	GQ1b (IgG)++	3
26	M	MFS	GQ1b (IgG)+++	3
22	F	MFS	GQ1b (IgG)+	2
42	F	MFS	GQ1b (IgG)+	2
20	M	GBS	GM1(IgM)+,(IgG)+, GD1b(IgM)+,(IgG)+	2
67	F	GBS	GD1b(IgG)+++	1
28	F	GBS	GM1(IgM)+,(IgG)+	1
13	M	MFS	GQ1(IgG)+	0
57	M	GBS	GM1(IgM)++,(IgG)+	0
21	M	MFS	–	0
26	M	MFS	–	0
29	M	MFS	–	0
22	M	GBS	–	0
17	F	GBS	–	0

Antibody titers are expressed semiquantitatively according to the OD value of the ELISA. OD <0.1 is negative –; + is 0.1–0.3; ++ is 0.3–0.5; and +++ is >0.5. Occurrence of IgG anti-GQ1b antibody was significantly more frequent in patients with a QSART score ≥2 ($P < 0.05$). GBS, Guillain–Barré syndrome; MFS, Miller Fisher syndrome.

Because other autonomic tests such as head-up tilt or the Valsalva maneuver could not be performed in patients with severe weakness, we focused our attention on the QSART in this study. We have shown an association between postganglionic sudomotor dysfunction and anti-GQ1b antibodies. Birklein et al. showed that local injection of botulinum toxin type A reduced focal hyperhidrosis.[1] Botulinum toxin A–G shows a high binding affinity to GQ1b.[2] Anti-GQ1b antibodies therefore might also decrease the activity of postganglionic sudomotor axons by binding to GQ1b as does botulinum toxin. Plomp et al. reported human anti-GQ1b antibodies bind to mouse neuromuscular junctions to induce acetylecholine release from nerve terminals and eventually to block neuromuscular transmission.[5] IgG antibodies against GQ1b therefore might induce a transmission block at the neuroglandular junction by a similar mechanism as at the neuromuscular junction.

Yoshimasa Kuzumoto, MD, PhD
Mitsuaki Shioyama, MD, PhD
Mikihiro Kihara, MD, PhD
Susumu Kusunoki, MD, PhD

Department of Neurology
Kinki University School of Medicine
Osaka, Japan

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PAPER

Anti-ganglioside complex antibodies in Miller Fisher syndrome

K Kaida, M Kanzaki, D Morita, K Kamakura, K Motoyoshi, M Hirakawa, S Kusunoki

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See end of article for authors' affiliations

Correspondence to: Dr Susumu Kusunoki, Department of Neurology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan; kusunoki-tky@umin.ac.jp

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Background: Some ganglioside complexes (GSCs) are target antigens for serum antibodies in patients with Guillain–Barré syndrome (GBS). Anti-GSC antibodies may be associated with particular clinical features of GBS.
Objective: To investigate antibodies to GSCs in the sera of patients with Miller Fisher syndrome (MFS) characterised by elevation of the IgG anti-GQ1b antibody.
Results: In all, 7 of 12 (58%) consecutive patients with MFS were found to have IgG antibodies to GSCs containing GQ1b, of whom 5 had IgG antibodies to GQ1b-GM1 complex (GQ1b/GM1) and 2 had antibodies to GQ1b/GD1a; 4 of 5 patients without sensory symptoms had anti-GQ1b/GM1 antibodies.
Conclusions: At least three different specificities in MFS-associated antibodies, GQ1b-specific, anti-GQ1b/GM1-positive and anti-GQ1b/GD1a-positive, were observed. In patients with MFS not only GQ1b itself but also clustered epitopes of GSCs, including GQ1b, may be considered to be prime target antigens for serum antibodies. A tendency to escape sensory disturbances is shown by anti-GQ1b/GM1-positive MFS.

We recently reported that some ganglioside complexes (GSCs) are target antigens for serum antibodies in patients with Guillain–Barré syndrome (GBS), an acute immune-mediated polyradiculoneuropathy, and suggested that anti-GSC antibodies may be associated with particular clinical features of GBS.[1] Because glycolipids including gangliosides tend to form clustered complexes with cholesterols in lipid rafts in the plasma membrane,[2] anti-GSC antibodies are likely to cause nerve dysfunction through binding to GSCs in lipid rafts in neuronal membranes.

Miller Fisher syndrome (MFS) is characterised by a clinical triad of ophthalmoplegia, ataxia and areflexia, and is considered to be a variant of GBS.[3] The presence of the IgG anti-GQ1b antibody in serum is an excellent diagnostic marker for MFS.[4] This antibody often cross reacts with GT1a[4 5] and is pathophysiologically associated with ophthalmoplegia or ataxia in MFS and GBS.[5–7] Thus, MFS is a clinically and serologically well-defined syndrome with a pathophysiological mechanism similar to that of GBS, which suggests that patients with MFS may also have anti-GSC antibodies. Here, we examined the serum samples of patients with MFS and found antibodies specific for a mixture of two gangliosides, including GQ1b or GT1a.

METHODS

ELISA for anti-GSC antibodies in serum from patients with MFS

Antibodies to GSC were investigated in acute-phase serum samples collected from consecutive patients with MFS, who were diagnosed at the National Defense Medical College hospital, Saitama-Ken, Japan, between April 1994 and December 2004. The diagnosis of MFS was based on acute self-limited ophthalmoplegia, ataxia and areflexia without marked limb weakness, the involvement of CNS or other neurological diseases. The ELISA was carried out for antibodies to the gangliosides GM1, GM2, GD1a, GD1b, GT1a, GT1b and GQ1b, as described previously.[8 9] When the corrected optical density was >0.1, the serum was considered to be positive. The ELISA for anti-GSC antibodies was carried out as described in our previous report.[1] GSCs used in the ELISA contained two of the above seven ganglioside antigens. Gangliosides were mixed for 30 min before their application to the ELISA. Anti-GSC antibody-positive samples were overlaid for thin-layer chromatography immunostaining, as described previously,[1] and the clinical features of anti-GSC antibody-positive patients with MFS were analysed. The above procedures were carried out at room temperature.

Immunoabsorption of anti-GSC antibody-positive serum samples

Anti-GSC antibodies were absorbed in antigen-coated ELISA wells, as described previously.[5 9] Ganglioside antigens used for the absorption test were GSCs, a mixture of two gangliosides (250 ng each) or 500 ng of each ganglioside. Uncoated wells were used as controls. Anti-GSC antibody-positive serum diluted 1:40 with 1% bovine serum albumin in phosphate-buffered saline was used, and the residual activities of the supernatants on the GSCs were estimated with ELISA. The percentage absorption of anti-GSC antibody activity was calculated as described previously.[9]

RESULTS

Anti-ganglioside antibody assay and representative serum data

Acute-phase serum samples were collected from 12 patients with MFS, 10 (83%) of whom had IgG anti-GQ1b antibodies. The results from the ELISA showed that 7 of the 12 (58%) patients had serum antibodies to GSCs, such as GQ1b/GM1, GQ1b/GD1b, GQ1b/GD1a, GT1a/GM1, GT1a/GD1b, GT1a/GD1a and GQ1b/GT1b (table 1), but not to GSCs without GQ1b or GT1a. Antibodies to GQ1b/GM1, GT1a/GM1 and GT1a/GD1b were frequent. One patient (patient 7) had no

Abbreviations: GBS, Guillain–Barré syndrome; GSC, ganglioside complex; MFS, Miller Fisher syndrome

Table 1 Anti-ganglioside complex antibodies in 12 consecutive patients with Miller Fisher syndrome

Patient number	Age (year)	Sex	Involved cranial nerves*	Sensory signs†	Corrected OD: Anti-GQ1b	Anti-GT1a	Anti-GQ1b/GM1	Anti-GQ1b/GD1b	Anti-GT1a/GM1	Anti-GT1a/GD1b	Anti-GQ1b/GD1a	Anti-GT1a/GD1a	Anti-GQ1b/GT1b	Other anti-glycolipid antibodies
1	32	M		+	0.13	0.72	(−)	(−)	(−)	(−)	(−)	(−)	(−)	
2	65	F	7	+	0.12	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	
3	38	M		+	0.86	0.55	(−)	(−)	(−)	(−)	(−)	(−)	(−)	
4	68	F		++‡	0.85	0.74	(−)	(−)	(−)	(−)	(−)	(−)	(−)	
5	27	M		+	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	
6	56	F		+	0.55	(−)	0.79	(−)	0.28	0.10	(−)	(−)	(−)	
7	12	F		−	(−)	(−)	0.11	(−)	0.55	(−)	(−)	(−)	(−)	
8	64	M	7	−	0.42	(−)	0.67	(−)	0.70	0.39	(−)	(−)	(−)	
9	69	M		−	0.77	(−)	1.08	(−)	1.02	0.56	(−)	(−)	(−)	
10	38	F		−	0.11	(−)	0.70	0.63	0.84	0.60	(−)	(−)	(−)	
11	35	M		++‡	0.20	(−)	(−)	(−)	(−)	(−)	0.40	0.22	0.42	
12	52	F	9, 10	+	1.28	1.76	(−)	(−)	(−)	(−)	1.56	(−)	1.68	IgM: GM1

OD, optical density; (−), antibody negative.
ELISAs were repeated twice, and the mean OD of the two experiments was calculated.
*Affected cranial nerves other than 3, 4, and 6. Because of the lack of anti-GT1a and GD1b in patient 6 and anti-GQ1b and GM1 in patient 7, anti-GT1a/GD1b and anti-GQ1b/GM1 in these patients were considered to be positive.
†Criteria for sensory signs: −, no sensory signs or symptoms; +, only paraesthesia or dysaesthesia; and ++, sensory deficits.
‡Deep sensory disturbance.

anti-GQ1b or anti-GT1a antibodies, but had antibodies to GQ1b/GM1 and GT1a/GM1. In contrast with anti-GSC antibodies in GBS, no antibodies to the GSCs consisting of two of the four major gangliosides, GM1, GD1a, GD1b and GT1b, were found in patients with MFS.

The specificity of the anti-GSC antibodies was investigated with a representative serum sample from an anti-GSC antibody-positive patient with MFS (patient 10). An ELISA showed that the serum had IgG antibody activities for GQ1b/GM1, GQ1b/GD1b, GT1a/GM1 and GT1a/GD1b, but little activity against GQ1b and GT1a (fig 1A–C). Thin-layer chromatography studies also showed specific immunoreactivity against the overlapping portion of two gangliosides, including GT1a/GM1, GQ1b/GM1 and GT1a/GD1b (fig 1D,E).

An immunoabsorption study with serum from patient 10 showed that the serum antibodies were specifically immunoreactive with GQ1b/GM1 and GT1a/GM1, but not with GM1 or GT1a, although the antibodies appeared to cross react with GQ1b. The percentage absorption of anti-GQ1b/GM1 antibody activity was 1.8% by the GM1 antigen, 43% by GQ1b, 8.8% by GT1a, 96% by GQ1b/GM1 and 83% by GT1a/GM1. The percentage absorption of anti-GT1a/GM1 antibody activity was 7.6% by GM1, 47% by GQ1b, 8.5% by GT1a, 94% by GQ1b/GM1 and 70% by GT1a/GM1.

Fine specificity of anti-GSC antibodies and clinical features of anti-GSC antibody-positive patients with MFS

The ELISA showed that the fine specificity of serum antibodies in patients with MFS was heterogeneous (table 1). Some serum antibodies had stronger activity with GQ1b/GM1 or GT1a/GM1 than with either GQ1b or GT1a alone, whereas others had little or no activity against GQ1b/GM1 or GT1a/GM1, despite showing intense activity with GQ1b or GT1a. On the basis of the presence of anti-GSC antibodies, the 12 patients with MFS could be subdivided into the three groups: anti-GSC negative (patients 1–5), anti-GQ1b/GM1 positive (patients 6–10) and anti-GQ1b/GD1a positive (patients 11 and 12; table 1).

Sensory signs were infrequent in patients with MFS with antibodies to GQ1b/GM1 and GT1a/GM1 (table 1), but otherwise there were no remarkable differences in clinical features between anti-GSC antibody-positive and antibody-negative patients with MFS. All the patients had antecedent respiratory infections: *Haemophilus influenzae* was identified from a throat swab of patient 4 and influenza B virus was serologically proved to be a pathogen in the antecedent infection of patient 7.

DISCUSSION

This study confirmed that the anti-GQ1b antibody is a useful marker for MFS, but the fine specificity of anti-ganglioside antibodies in MFS was more diverse than expected. Antibodies to GSCs containing GQ1b or GT1a, and anti-GQ1b and anti-GT1a antibodies, may be crucial for the development of MFS. Antecedent respiratory infection in patients with MFS may be associated with production of antibodies to GSCs containing GQ1b or GT1a.

According to the results of the ELISA, serum antibodies in five patients (6–10) most strongly bound to GQ1b/GM1 and GT1a/GM1. Hence, a combination of [Galβ1–3GalNAc] and [NeuAcα2–8NeuAcα2–3Galβ1–3GalNAc] in the terminal residues of gangliotetraose structures may be important as an antigenic epitope for those anti-GSC antibodies (fig 1F). Serum antibodies in patients 11 and 12 bound most strongly to GQ1b/GD1a and GQ1b/GT1b, and a combination of [NeuAcα2–3Galβ1–3GalNAc] and [NeuAcα2–8NeuAcα2–3Galβ1–3GalNAc] in the terminal residues may be a target antigen in those patients. On the other hand, sera from patients 1–4 had antibodies to GQ1b or GT1a but not to GSCs, and showed attenuation of these activities on addition of another ganglioside antigen, suggesting that the serum antibodies had monospecific activities to GQ1b or GT1a. Thus, there are at least three different specificities in MFS-associated antibodies. Such differences in antibody specificity seem to have some influence on clinical features in MFS, because sensory signs were infrequent in patients with MFS with antibodies specific to GQ1b/GM1 and GT1a/GM1, but were commonly seen in patients with other antibodies. The clinical relevance of such anti-GSC antibodies, however, needs to be investigated in a larger number of patients with MFS before firm conclusions can be drawn.

Much evidence has indicated a pathophysiological role for the IgG anti-GQ1b antibody in the development of MFS. Immunohistochemical, ex vivo or in vitro studies with monoclonal anti-GQ1b antibody have shown not only specific localisation of GQ1b in the human peripheral nerves but also the neuroparalytic action of the anti-GQ1b

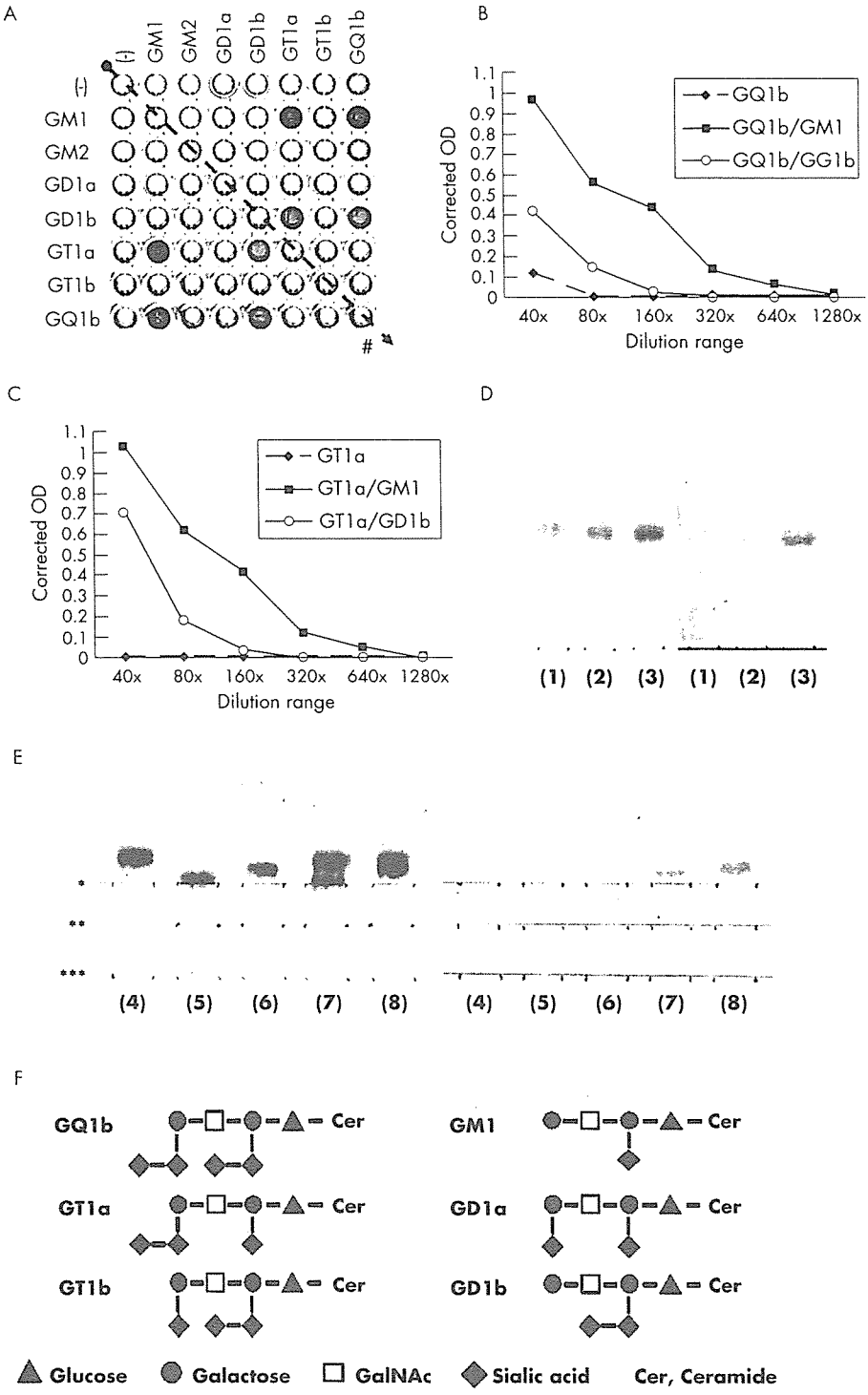

Figure 1 ELISA and thin-layer chromatogram (TLC) immunostaining of the serum of patient 10. (A) In the ELISA, reactions in wells containing GQ1b/GM1, GQ1b/GD1b, GT1a/GM1 and GT1a/GD1b (w/w = 0.1/0.1) were much stronger than those in wells containing only GQ1b. The number of control wells (#) on the plate is indicated by oblique dotted arrows. (B,C) Serum in the ELISA was diluted serially from 1:40 to 1:1280 and the antibody titres were as follows: IgG anti-GQ1b/GM1, 1:320; anti-GQ1b/GD1b, 1:80, anti-GQ1b, 1:40; anti-GT1a/GM1, 1:320; anti-GT1a/GD1b, 1:80; and anti-GT1a, negative results. (D,E) In TLC immunostaining, the developing solvent consisted of chloroform, methanol and 0.2% $CaCl_2 \cdot 2H_2O$ (50:45:10, v/v). The following ganglioside antigens were developed in each lane: (1) GT1a (2 μg), (2) GD1b (2 μg), (3) GT1a and GD1b (2 μg each), (4) GM1 (2 μg), (5) GT1a (2 μg), (6) GQ1b (2 μg), (7) GT1a and GM1 (2 μg each) and (8) GQ1b and GM1 (2 μg each). The start line for each antigen is indicated by asterisks: (*) for GQ1b, (**) for GT1a and (***) for GM1. Serum was diluted 1:100. Peroxidase-conjugated goat anti-human IgG Fc antibody (ICN Biomedicals, Aurora, Ohio, USA; diluted 1:200) was used as the secondary antibody. Left panel: TLC results visualised by orcinol reagent; right panel: TLC immunostaining. Positive immunostaining in lane (3) on the right panel indicates an antibody reaction to the GT1a–GD1b complex (GT1a/GD1b) (D). Positive staining in lanes (7) and (8) on the right panel indicates antibody reactions to GT1a/GM1 and GQ1b/GM1, respectively (E). Antibody reactions to GT1a, GD1b, GM1, GT1a and GQ1b were negative. (F) Carbohydrate structures of GQ1b, GT1a, GD1b, GM1, GT1b and GD1a are shown. OD, optical density.

antibodies, such as conduction block at motor nerve terminals.[5 6 10 13] Similar studies with antibodies monospecific to GSCs comprising GQ1b and GT1a may be useful for elucidation of the pathogenetic mechanisms of MFS.

The characteristic formation of clusters of gangliosides in the plasma membrane may result in anti-GSC antibodies causing nerve dysfunction more efficiently than monospecific anti-GQ1b antibody. Whether anti-GSC and anti-GQ1b antibodies bind to identical sites in neuronal membranes remains unclear, and future investigations on the localisation and possible roles of GSCs in the plasma membrane are required to deal with this issue.

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Authors' affiliations

K Kaida, M Kanzaki, K Kamakura, K Motoyoshi, Third Department of Internal Medicine, National Defense Medical College, Tokorozawa, Saitama-Ken, Japan

D Morita, M Hirakawa, S Kusunoki, Department of Neurology, Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan

Competing interests: None.

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CSF tau protein: A new prognostic marker for Guillain-Barré syndrome

Abstract—We measured the CSF tau protein levels in 26 patients with Guillain-Barré syndrome. The levels of the poor outcome group (Hughes grade at 6 months was between II and VI, n = 6) were higher than those of the good outcome group (0 or I, n = 20) ($p < 0.0005$). The higher levels of CSF tau may reflect axonal degeneration and could predict a poor clinical outcome in Guillain-Barré syndrome.

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K. Jin, MD; A. Takeda, MD, PhD; Y. Shiga, MD, PhD; S. Sato, MD, PhD; A. Ohnuma, MD, PhD; H. Nomura, MD, PhD; H. Arai, MD, PhD; S. Kusunoki, MD, PhD; M. Ikeda, PhD; and Y. Itoyama, MD, PhD

Since the introduction of plasma exchange or IV immunoglobulins as standard therapies for Guillain-Barré syndrome (GBS), the general prognosis has dramatically improved. Although most patients with GBS treated show good recovery from neurologic impairments, 11% of GBS cases still die and 16% have long-term disability.[1] A poor outcome in GBS is correlated with some clinical findings, such as older age at onset, longer time to nadir, necessity for ventilatory support, presence of antecedent diarrhea, and electrophysiologic signs of axonal degeneration.[1] However, it is generally difficult to predict the clinical outcome in the early phase of GBS.

Tau protein (tau) is a microtubule-associated protein primarily localized in neuronal cells. In damaged brain, tau is released into the CSF from the neuronal cytoplasm and the CSF tau levels are known to be a good diagnostic marker for several CNS disorders.[2-4] Although tau also exists in the PNS,[5] there have been few studies concerning the CSF tau levels in PNS disorders.[4] In this study, we measured the CSF tau levels in patients with GBS to clarify whether it may reflect the degree of axonal damage and could predict the clinical outcome.

Methods. *Patients.* We reviewed medical records of consecutive patients who were admitted to our hospitals between April 1998 and March 2004. Twenty-six patients (17 men, mean age 51.4 years) fulfilled the criteria for GBS,[6] all of whom were recruited to the present study. They were evaluated at the nadir and 6 months after the onset according to the Hughes functional grading scale,[7] and were divided into two groups based on the Hughes grade at 6 months. When the Hughes grade at 6 months was 0 or I, the cases were classified into the good outcome group (n = 20). The other cases whose Hughes grades at 6 months were between II and VI were classified into the poor outcome group (n = 6). Antecedent episodes in the 4 weeks before the onset of symptoms were determined. Electrophysiologic studies were conducted within 14 days from the onset. Pretreatment serum and CSF samples obtained within 24 days from the onset were frozen and stored at −80 °C for subsequent analyses.

Electrophysiologic studies. Nerve conduction studies were performed using standard procedures with a Neuropack electromyographic machine (Nihon Kohden, Tokyo, Japan). According to the electrodiagnostic criteria,[8] patients were classified into five categories; normal, demyelinating, axonal, inexcitable, and equivocal.

Laboratory analyses. The presence of antibodies for glycolipid antigens (GalNAc-GD1a and GM1) was determined by an enzyme linked immunosorbent assay (ELISA) because they were reported to be a good marker for axonal degeneration in GBS.[9] The serum was considered to be seropositive for each of the antigens when the corrected OD was more than 0.1. The CSF tau levels were measured using an ELISA kit, Fino Scholar hTAU (Innogenetics, Ghent, Belgium).[2]

Statistical analyses. The Mann–Whitney *U* test was used for the comparison of age at onset, time to nadir, Hughes grade, and CSF findings. The Fisher's exact probability test was used for the comparison of some other clinical features. Both univariate and multivariate models were developed for analysis of the outcome using ordinal logistic regression. The outcome variable was defined in the three ordered categories (0, the Hughes grade at 6 months was 0; 1, it was I; 2, it was between II and VI). Independent variables for this model included age at onset, time to nadir, need for ventilatory support, axonal pattern, CSF tau levels, and timing of when the CSF specimens were acquired. Results were classed as significant if $p < 0.05$.

Results. The age at onset, sex, time to nadir, Hughes grade at nadir, choice of initial treatments, and CSF protein levels were not significantly different between the good and poor outcome groups. On the other hand, the incidence of diarrhea and axonal pattern on the electrodiagnostic criteria showed a higher tendency in the poor outcome group, but the difference was not significant. The incidence of necessity for ventilatory support and the CSF tau levels were higher in the poor outcome group (table 1, figure). Anti-GalNAc-GD1a or GM1 antibodies were positive in five of six patients with high CSF tau levels.

The univariate model of ordinal logistic regression analysis showed that the poor outcome in GBS was associated with necessity for ventilatory support ($p < 0.05$), axonal pattern ($p < 0.05$), and higher CSF tau levels ($p < 0.01$), but not with older age at onset and longer time to nadir. The multivariate model revealed that the poor outcome in GBS was only associated with higher CSF tau levels (table 2).

Discussion. We showed here that the CSF tau levels were significantly higher in GBS cases with poor outcomes and could be used as an independent prog-

From the Department of Neurology (K.J., S.S., A.O., H.N.), Kohnan Hospital; Department of Neurology (K.J.), Iwate National Hospital; Departments of Neurology (A.T., Y.S., Y.I.) and Geriatric and Complementary Medicine (H.A.), Center for Asian Traditional Medicine Research, Tohoku University Graduate School of Medicine; Department of Neurology (S.K.), Kinki University School of Medicine; and Institute of Industrial Ecological Sciences (M.I.), University of Occupational and Environmental Health, Japan.

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Address correspondence and reprint requests to Dr. Takeda, Department of Neurology, Tohoku University Graduate School of Medicine, 1-1, Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan; e-mail: atakeda@em.neurol.med.tohoku.ac.jp

Table 1 *Comparison of clinical and laboratory features between the good and poor outcome groups of patients with Guillain-Barré syndrome*

	Good outcome, n = 20	Poor outcome, n = 6	*p* Value
Age at onset, y	47.6 ± 19.9	64.2 ± 19.2	0.078
M/F	15:5	2:4	0.084*
Diarrhea, %	20.0	66.7	0.051
Time to nadir, d	7.6 ± 5.0	5.8 ± 1.7	0.46
Need for ventilatory support, %	5.0	50.0	0.028
Hughes grade at nadir	3.5 ± 0.8	4.2 ± 0.8†	0.11
Initial treatment, %			
IV immunoglobulins	85.0	83.3	0.68
Plasmapheresis	10.0	16.7	0.88
Axonal pattern‡	20.0	66.7	0.051
Protein values in CSF, mg/mL	85.3 ± 79.1	46.7 ± 17.3	0.24*
Tau protein values in CSF, ng/mL	159.6 ± 67.4	341.7 ± 44.5	0.00026
Timing of when CSF specimens were acquired, d	7.2 ± 6.2	7.5 ± 8.0	0.85

Values are means ± SD where applicable.

* Though not significant, a male predominance and higher CSF protein levels in the good outcome group were observed, possibly due to the small scale of this study. It was well-established that sex and CSF protein levels do not influence the outcome of patients with GBS.[1]

† One patient was excluded because she died 7 days after the onset (n = 5).

‡ Axonal pattern included the primary axonal and inexcitable groups of Hadden's electrodiagnostic criteria.[8]

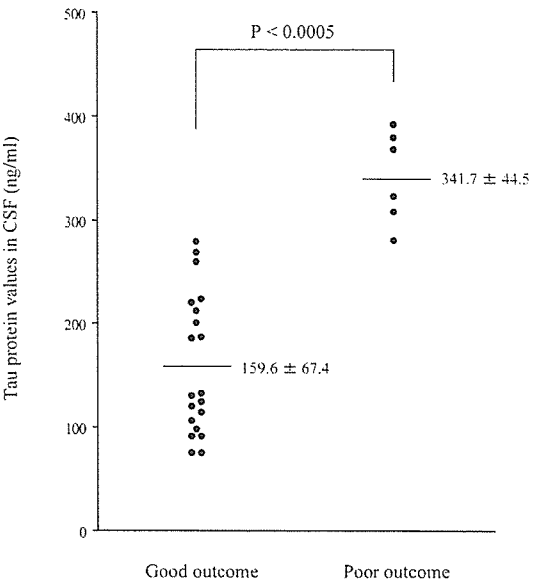

Figure. Comparison of tau protein (tau) levels in CSF between the good outcome group (n = 20) and the poor outcome group (n = 6) in patients with Guillain-Barré syndrome. The functional grading was evaluated at 6 months after the onset according to the Hughes scale as follows: 0, healthy; I, minor symptoms or signs, able to run; II, ambulates independently; III, able to walk 5 meters with assistance; IV, bed bound; V, requiring assisted ventilation; VI, dead.[7] When the Hughes grade at 6 months was 0 or I, the cases were classified into the good outcome group (n = 20). Other cases whose Hughes grades at 6 months were between II and VI were classified into the poor outcome group (n = 6). The mean values of tau are indicated with horizontal lines. The poor outcome group showed higher tau levels than those in the good outcome group (p < 0.0005). All of the six poor outcome cases showed high levels of CSF tau (≥280 pg/mL). In contrast, none of the good outcome cases showed high levels of CSF tau.

nostic marker for GBS. In contrast with previous reports,[1] neither age nor time to nadir were associated with poor clinical courses in our study, which could be due to the smaller size of the present study. Although the univariate model of logistic regression analysis showed that the necessity for ventilatory support and axonal pattern were associated with poor outcomes in GBS, the multivariate model analyses failed to show significance (table 2). Thus, the high CSF tau levels were much more closely associated with a poor prognosis of GBS. Because the CSF tau level is known to increase gradually with age,[3] the higher levels of CSF tau in the poor outcome group might have been influenced by the older age. However, the higher CSF tau levels were independently associated with poor outcomes, even after adjustment for age (table 2). Thus, CSF tau appears to be a useful and reliable prognostic marker for GBS. Concerning the CSF tau levels in patients with PNS disorders, only one article showed that the tau levels were low in five patients with GBS,[4] but this was a small study and the clinical features of each case were not fully described.

Tau is a phosphorylated microtubule-associated protein primarily localized in neuronal axons and it is present not only in the CNS but also in the PNS.[5] It has been established that the concentration of CSF tau can reflect the degree of neuronal damage in CNS disorders such as AD, Creutzfeldt-Jakob disease, encephalitis, stroke, and active MS.[2-4] The present results suggest that CSF tau is associated with the degree of PNS damage. Because some GBS cases were reported to be complicated with CNS dysfunctions as shown in Miller Fisher syndrome, the CSF tau might be derived from the putative CNS damage in GBS. Moreover, incidental complications of subclinical AD might account for the elevated CSF tau in poor outcome groups. However, it is plausible that the CSF tau in GBS was derived from PNS lesions, because no cases in the poor outcome group showed either clinical or neuroimaging signs of CNS

Table 2 *Ordinal logistic regression analysis of factors associated with poor outcome in patients with Guillain-Barré syndrome (n = 26)*

Variable	Coefficient	Standard error	Odds ratio (95% CI)	*p* Value
Age at onset, y	0.080	0.054	1.08 (0.97–1.20)	0.14
Time to nadir, d	−0.12	0.20	0.89 (0.59–1.32)	0.55
Need for ventilatory support	−5.01	2.60	0.01 (0.000041–1.09)	0.054
Axonal pattern	−1.90	2.06	0.15 (0.0026–8.50)	0.36
Tau protein values in CSF, ng/mL	−0.039	0.016	0.96 (0.93–0.99)	0.015
Timing of when CSF specimens were acquired, d	−0.076	0.18	0.93 (0.65–1.32)	0.67

involvement during the at least 1 year of observation. On the other hand, 66.7% and 83.3% of the patients with higher CSF tau levels showed an axonal pattern (table 1) and positive anti-GalNAc-GD1a or GM1 antibodies, respectively. These results suggest that the CSF tau levels in GBS are closely associated with axonal injury. Thus, the measurement of CSF tau appeared to be useful for estimating the presence of axonal degeneration, even in the early phase of GBS. Most of the tau detected in CSF is a proteolytic product of the N-terminal peptide,[10] and therefore we cannot determine which form of tau is mainly detected using this method. Further studies are needed to elucidate the mechanisms responsible for the elevated CSF tau in GBS.

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Genetic mechanisms for the synthesis of fucosyl GM1 in small cell lung cancer cell lines

Noriyo Tokuda[2], Qing Zhang[2], Shoko Yoshida[2,3], Susumu Kusunoki[4], Takeshi Urano[2], Keiko Furukawa[2], and Koichi Furukawa[1,2]

[2]Department of Biochemistry II, Nagoya University School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan; [3]Department of Internal Medicine II, Nagoya City University School of Medicine, 1 Kawasumi, Mizuho-ku, Nagoya 467-8602, Japan; and [4]Department of Neurology, Kinki University School of Medicine, 377-2 Ohno-higashi, Sayama, Osaka 589-8511, Japan

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Fucosyl GM1 has been reported to be specifically expressed in small cell lung cancer (SCLC) cells. However, the genetic basis for the synthesis of fucosyl GM1 has not been investigated. We analyzed the glycosyltransferases responsible for the synthesis of fucosyl GM1 in SCLC cell lines. In four SCLC cell lines expressing fucosyl GM1, both *FUT1* and *FUT2* mRNAs were detected, indicating that either one or both of α1,2-fucosyltransferases may be involved in the expression of fucosyl GM1. However, three of these four lines contained function-loss mutations in the *FUT2* coding region, suggesting that FUT1 is mainly involved in the α1,2-fucosylation of GM1. The expression levels of the *GM1 synthase* gene showed no correlation with those of fucosyl GM1, whereas the co-transfection of *GM1 synthase* cDNA with *FUT1* or *FUT2* into SK-LC-17 clearly enhanced the neo-expression of fucosyl GM1, indicating its essential role. In contrast, the co-transfection of *GD3 synthase* cDNA reduced the expression levels of fucosyl GM1 with *FUT1* or *FUT2*. Consequently, FUT1 seems to mainly contribute to the expression of fucosyl GM1, although both FUT1 and FUT2 are capable of generating the antigen. These results should promote the functional analysis of fucosyl GM1 leading to the development of novel therapies for SCLC.

Key words: fucosyl GM1/fucosyltransferase/H enzyme/ small cell lung cancer

Introduction

The nervous tissues of vertebrates are enriched with gangliosides, sialic acid-containing glycosphingolipids, that play crucial roles in the maintenance of the tissue integrity and normal function (Wiegandt, 1985). Some of them are also expressed in neuroectoderm-derived cancer cells, such as malignant melanomas (Portoukalian *et al.*, 1976; Carubia, *et al.*, 1984), neuroblastomas (Cheung *et al.*, 1985), and gliomas (Fredman *et al.*, 1986). Therefore, gangliosides such as GD3 and GD2 have been considered to be tumor markers and have been utilized as targets for immunotherapy (Houghton *et al.*, 1985). Recent studies using cDNAs for the synthetic enzymes of gangliosides have revealed that those antigens are involved in the malignant properties of cancer cells, such as the prominent proliferation and invasiveness (Yoshida *et al.*, 2001; Hamamura *et al.*, 2005).

Fucosyl GM1 is a member of the gangliosides with a unique structure consisting of a part of GM1 and α1,2-fucosylated galactose at the nonreducing end (Klenk, 1942). This structure was detected in particular animal cells under developmental regulation (Suchy *et al.*, 1988; Kusunoki *et al.*, 1992) and in restricted sites of mammalian nervous tissues (Kusunoki and Inoue, 1991; Kusunoki *et al.*, 1992) and has sometimes become one of the target antigens in autoimmune neuronal diseases (Yoshino *et al.*, 1993; Yuki and Ariga, 1997). Fucosyl GM1 is one of major gangliosides in the rat pheochromocytoma cell line, PC12 (Ariga *et al.*, 1988; Nishio *et al.*, 2004), and its expression was further enhanced by the transfection of *GM1 synthase* cDNA in our study (Ferrari *et al.*, 1995). However, no rigorous study on the function of fucosyl GM1 in nervous systems has ever been performed, in contrast to GM1 (Ferrari *et al.*, 1995; Mutoh *et al.*, 1995; Nishio *et al.*, 2004) or b-series gangliosides (Fukumoto *et al.*, 2000).

More than 10 years ago, Lindholm and others reported that fucosyl GM1 was expressed in human small cell lung cancer (SCLC) cells (Nilsson *et al.*, 1986). Livingston and others extensively analyzed the expression pattern of fucosyl GM1 in human normal tissues and various cancer tissues (Brezicka *et al.*, 1989; Zhang *et al.*, 1997), showing that fucosyl GM1 was expressed in very few normal tissues and also in SCLC tissues. Consequently, fucosyl GM1 has been considered to be a candidate for a tumor marker for SCLC (Vangsted *et al.*, 1994). Furthermore, Livingston and others have tried to develop a vaccine with fucosyl GM1 for the treatment of patients with SCLC and have reported significant responses of anti-fucosyl GM1 antibodies in the patients who underwent vaccination with fucosyl GM1 conjugated with KLH (Dickler *et al.*, 1999). These results indicate that fucosyl GM1 can be a target antigen in antibody immunotherapy for SCLC. However, no studies have ever been performed on the mechanisms for the synthesis and expression of fucosyl GM1.

In the present study, glycosyltransferases responsible for the synthesis of fucosyl GM1 in SCLC cell lines were analyzed. Simultaneously, other factors that may affect the efficiency of the synthesis and expression of fucosyl GM1 were also investigated. These results should promote the functional analysis of fucosyl GM1 in human cancer cells and contribute to the development of novel therapies for the refractory disease.

[1]To whom correspondence should be addressed; e-mail: koichi@med.nagoya-u.ac.jp

Results

Specific expression of fucosyl GM1 in SCLC cell lines

In a previous study, we reported that GM1 was present in almost all SCLC and non-SCLC cell lines (Figure 1A) (Yoshida *et al.*, 2001). Furthermore, we analyzed the expression of fucosyl GM1 in 22 SCLC cell lines and 22 non-SCLC cell lines with flow cytometry (Figure 1A). It was demonstrated that four SCLC cell lines significantly presented this ganglioside on the cell surface (Figure 1B). Conversely, no non-SCLC cell lines had fucosyl GM1 on the cell surface. Furthermore, to determine whether this glycolipid exists in the cytosol, we attempted to detect fucosyl GM1 using immunocytochemistry. This ganglioside was only detected in the same four SCLC cell lines showing fucosyl GM1 expression on the cell surface (data not shown). These results were in accordance with the report that the expression of this antigen was specifically associated with SCLC cell lines (Nilsson *et al.*, 1986; Brezicka *et al.*, 1989; Zhang *et al.*, 1997).

Expression levels of genes relevant to biosynthesis of fucosyl GM1

Fucosyl GM1 is generated by adding α1,2-linked fucose to the galactose of GM1 at the last step of biosynthetic pathway. In humans, FUT1 and FUT2 enzymes are known as functional α1,2-fucosyltransferases. To analyze the correlation between the expression of fucosyl GM1 and each of the α1,2-fucosyltransferases, we investigated the expression levels of *FUT1* and *FUT2* genes in the SCLC cell lines with northern blotting (Figure 2). *FUT1* mRNA was detected in many cell lines, and *FUT2* mRNA was weakly detected in only two SCLC cell lines. It is showed that all cell lines expressing fucosyl GM1 strongly expressed the *FUT1* gene, and two of them, that is, SBC1 and NCI-H69, also expressed *FUT2* gene. *FUT1* was also expressed in some of the fucosyl GM1-negative cell lines.

Furthermore, the expression levels of the *FUT1* and *FUT2* genes were determined using a quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) analyzer. In this study, we used 11 SCLC cell lines, including the *FUT1* gene-positive and *FUT1* gene-negative lines indicated by northern blotting analysis. As shown in Figure 3, the expression levels of the *FUT1* and *FUT2* genes in fucosyl GM1-positive cell lines were relatively high. The statistical analysis of FUT gene expression levels between fucosyl GM1-positive and fucosyl GM1-negative groups revealed that *p*-value for *FUT2* was 0.03 and that for *FUT1* was 0.06. However, *p*-value for *FUT1* was <0.001, when the value of ACC-LC-5 expressing extraordinarily high GD3 synthase was eliminated.

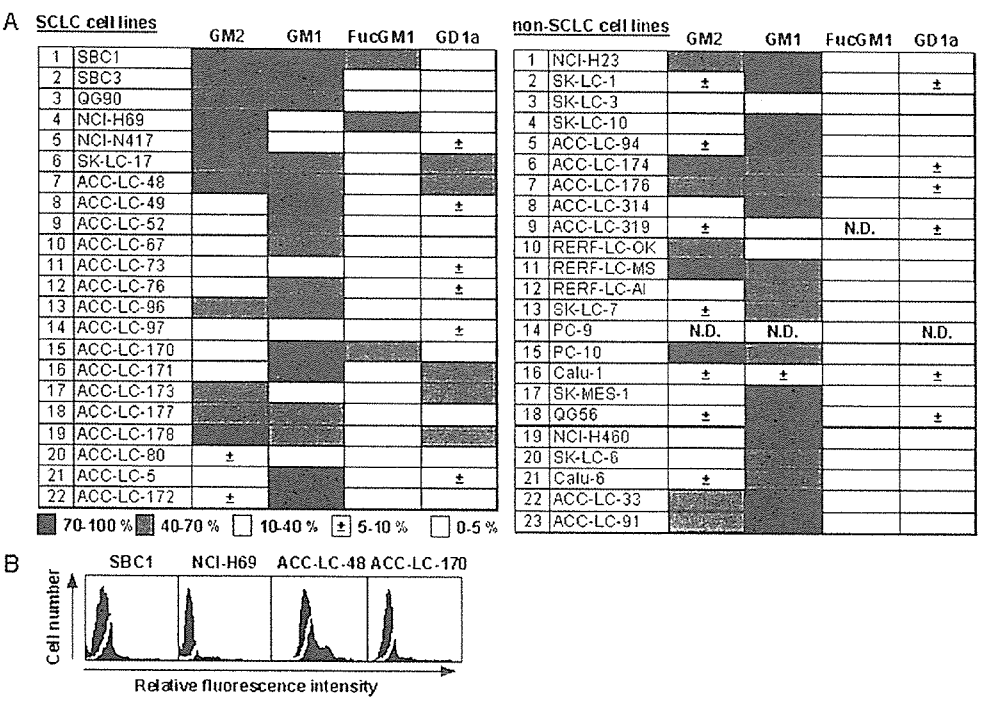

Fig. 1. Expression pattern of gangliosides on lung cancer cell lines. (A) Cell surface expression of gangliosides on 44 lung cancer cell lines (left panel: 1–22, small cell lung carcinoma; right panel: 1–14, adenocarcinoma; 15–18, squamous cell carcinoma; 19–23, large carcinoma) was analyzed by flow cytometry, as described in *Materials and methods*. The expression levels of gangliosides were classified into five groups based on the percentages of positive cells. ND, data not determined. (B) Cell surface expression of fucosyl GM1 on SBC1, NCI-H69, ACC-LC-48, and ACC-LC-170 as analyzed with flow cytometry. Gray lines indicate histograms with anti-fucosyl GM1 antibody, and filled histograms indicate the controls treated by second antibody alone.

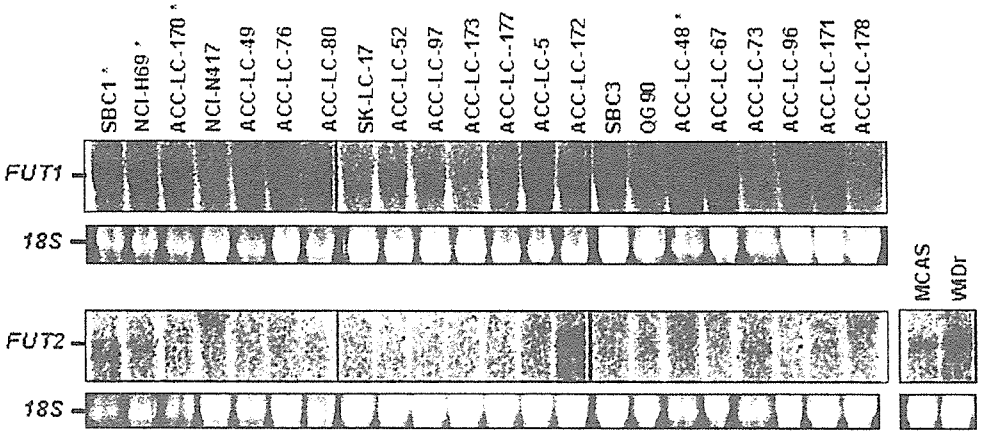

Fig. 2. Expression of the *FUT1* and *FUT2* gene in lung cancer cell lines by northern blotting. Analysis of the *FUT1* (upper column) and *FUT2* (bottom column) gene expression in small cell lung cancer (SCLC) cell lines. Total RNA (15 μg) was separated in 1.25 % agarose gel and blotted onto a nylon membrane, followed by the membrane hybridization with [^{32}P]dCTP-labeled cDNAs. *, fucosyl GM1-positive cell lines. MCAS and WiDr were positive controls of the *FUT2* gene. Control 18S is shown to present the amount of RNA in each lane.

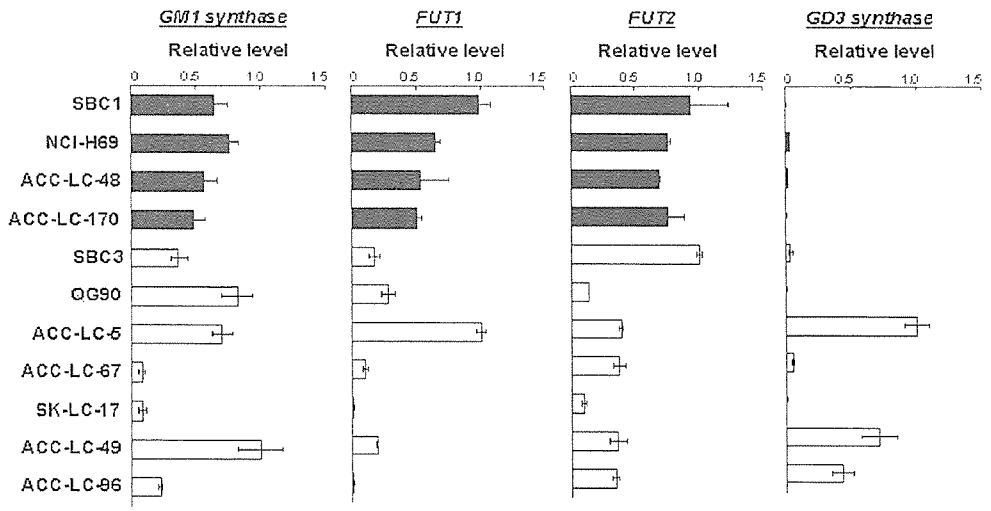

Fig. 3. Correlation between the expression of fucosyl GM1 and the expression levels of glycosyltransferase genes in the small cell lung cancer (SCLC) cell lines. Expression levels of *GM1 synthase*, *FUT1*, *FUT2*, and *GD3 synthase* genes were analyzed quantitatively in FucGM1-positive cell lines (solid bar) and fucosyl GM1-negative cell lines (open bar). The values indicate mean ± SD as ratios to the highest value in the individual genes. The *p*-value of *FUT2* between fucosyl GM1-positive and fucosyl GM1-negative groups was 0.03 and that of *FUT1* between the two groups was 0.06.

Ganglioside GM1 is a precursor of fucosyl GM1, and the biosynthesis of GM1 is an essential step for the expression of fucosyl GM1. In addition, GD3 metabolism is also a crucial step for the biosynthetic pathway of fucosyl GM1, because GD3 synthase competes with GM1 synthase for the common precursor GM3 via GM2 (Figure 4). To clarify whether the activities of GM1 synthase and GD3 synthase affect the expression of fucosyl GM1 on the cell surface, we measured the expression levels of the *GM1 synthase* and *GD3 synthase* genes (Figure 3). There was no correlation between the expression levels of the *GM1 synthase* gene and those of fucosyl GM1 in the SCLC cell lines. As for *GD3 synthase* gene, it was hard to find statistical differences between the expression levels of the *GD3 synthase* gene and those of fucosyl GM1. This is because there were no cell lines that expressed significant levels of *GD3 synthase* in the fucosyl GM1-positive samples, and *GD3 synthase* levels were widely dispersed from zero to very high in the fucosyl GM1-negative samples. Thus, it was concluded that the expression levels of *GD3 synthase* gene largely affect the expression of fucosyl GM1, because fairly high levels of *GD3 synthase* expression resulted in no expression

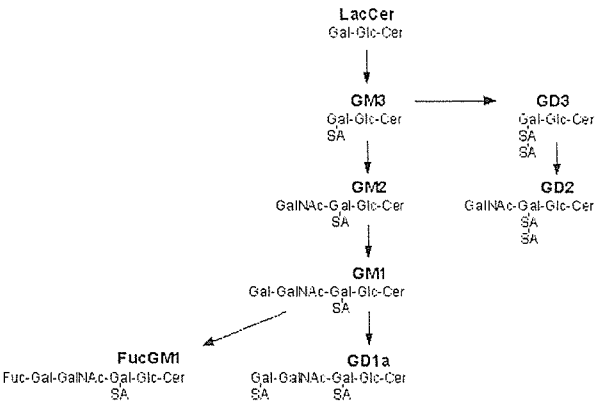

Fig. 4. Biosynthetic pathway of fucosyl GM1. Cer, ceramide; Fuc, fucose; FucGM1, fucosyl GM1; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; LacCer, lactosylceramide; SA, sialic acid.

of fucosyl GM1, even if they showed high levels of α1,2-fucosyltransferases.

Expression of blood group antigens containing α1,2-linked fucose

Similar to fucosyl GM1, some of the histo-blood group antigens contain an α1,2-linked fucose, and they are also synthesized from the precursors by the FUT1 or FUT2 enzyme. To determine whether the high expression of the *FUT1* and *FUT2* genes affects the expression of those antigens, we analyzed the expression levels of H (type 1 and type 2), Lewis b, and Lewis y antigens on the cell surface with flow cytometry. As shown in Figure 5, the cell lines that showed relatively high levels of α1,2-fucosyltransferases expressed these antigens prominently. H Antigen was not detected on the NCI-H69 cell line, presumably as a result of complete metabolic conversion to Lewis b and/or Lewis y antigens. These results suggested that high levels of the α1,2-fucosyltransferase gene expression lead to the synthesis of histo-blood group antigens as well as fucosyl GM1 in SCLC cell lines.

Both FUT1 *and* FUT2 *were capable to synthesize fucosyl GM1 in the transfectant cells*

Although it was suggested that both *FUT1* and *FUT2* genes are involved in the synthesis of fucosyl GM1, it has not been clear whether FUT1 and FUT2 really have the ability to transfer fucose to GM1 in the SCLC cells. We examined this issue with the SK-LC-17 cell line, which does not express fucosyl GM1 and weakly expresses GM1. *FUT1/FUT2* and *GM1 synthase* cDNAs were transiently introduced into SK-LC-17. Although fucosyl GM1 was not clearly expressed by the transfection with either *FUT1* or *FUT2* cDNA alone, and cells transfected with only *GM1 synthase* cDNA did not express fucosyl GM1 (Figure 6A), the co-transfection of *FUT1* or *FUT2* cDNA with *GM1 synthase* cDNA resulted in the definite expression of fucosyl GM1 on the cell surface (Figure 6B). Fucosyl GM1 expression was strongly inhibited by the co-transfection of *GD3 synthase* cDNA (Figure 6C). These results indicated that both FUT1 and FUT2 act onto GM1 to generate fucosyl GM1 in the presence of a sufficient amount of GM1, and GD3 synthase acts as a competitor in the biosynthesis of fucosyl GM1, even when GM1 synthase and α1,2-fucosyltransferase were sufficiently expressed.

Frequent mutations in the FUT2 *gene in fucosyl GM1-positive cell lines*

The inactivating mutations of the *FUT1* and *FUT2* genes have been reported. *FUT1* inactivating mutations are very rare and responsible for the Bombay phenotype characterized by the lack of ABH antigen on erythrocytes and vascular endothelium (Kaneko *et al.*, 1997). In contrast, *FUT2* inactivating mutations are more frequent and responsible for the nonsecretor phenotype (Table I). Individuals with these *FUT2* inactivated forms have been typed "se." It is found in ~20% of the European and North American populations. A low-activity mutation, A385T, named sej, retains ~3% of the FUT2 activity (Kudo *et al.*, 1996), and this

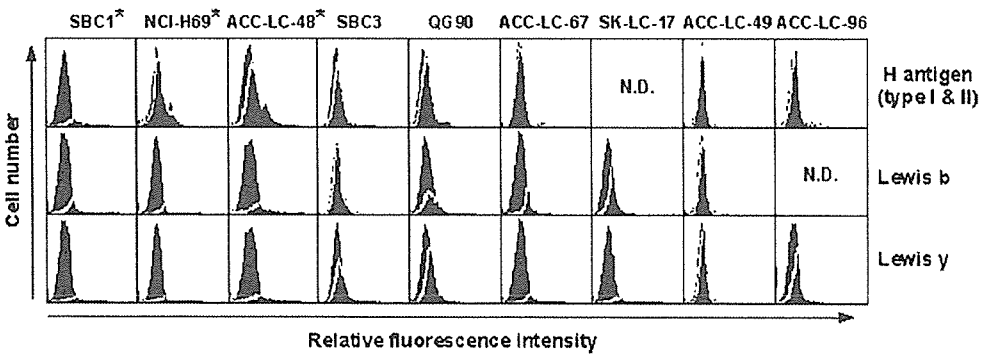

Fig. 5. Expression patterns of H antigen, Lewis b, and Lewis y on the small cell lung cancer (SCLC) cell lines. Expression of H antigen (type I and II), Lewis b, and Lewis y on the cell surface as detected with flow cytometry. Gray lines indicate histograms with antibodies, and filled histograms indicate the controls treated by a second antibody alone. *, fucosyl GM1-positive cell lines.

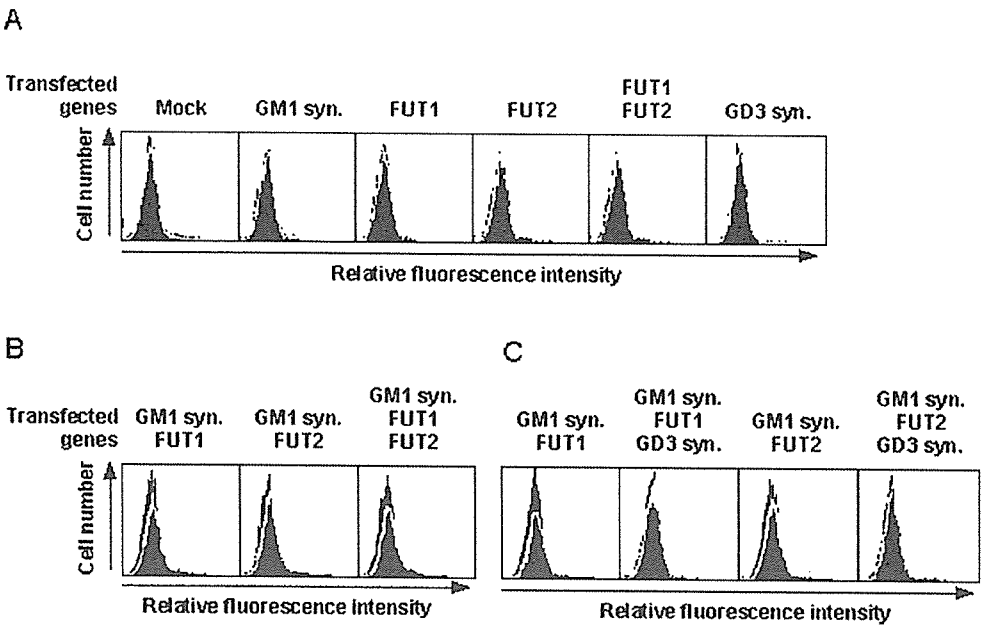

Fig. 6. Expression patterns of fucosyl GM1 on SK-LC-17 cells transiently transfected with glycosyltransferases. (**A**) The cells were transfected with *GM1 synthase*, *FUT1*, *FUT2*, or *GD3 synthase* alone, and *FUT1* and *FUT2* co-transfected as a control of (**B**) and (**C**). Fucosyl GM1 was scarcely expressed under these conditions. (**B**) The cells were transfected with *FUT1* and/or *FUT2* together with *GM1 synthase* cDNAs. (**C**) Effect of co-transfection of *GD3 synthase* cDNA on the expression of fucosyl GM1 under the condition as in (**B**). Syn., synthase.

Table I. Inactivating and partially inactivating mutations found in the *FUT2* gene

Mutation	Effect on enzyme	Population	Reference
211–272 (fusion)	Inactive	Japanese	Koda *et al.* (1996)
A385T (Ile129Phe)	Unstable enzyme	Asian	Kudo *et al.* (1996)
		Indonesian	Henry *et al.* (1996a)
		Polynesian	Henry *et al.* (1996b)
G428A (Try143Stop)	Inactive	Caucasian	Kelly *et al.* (1995)
C571T (Arg191Stop)	Inactive	Polynesian	Henry *et al.* (1996c)
		Chinese	Yu *et al.* (1996)
		Japanese	Koda *et al.* (1996)
C628T (Arg210Stop)	Inactive	Japanese	Koda *et al.* (1996)
C658T (Arg220Stop)	Inactive	Chinese	Liu *et al.* (1999)
685delTGG (Val230 excluded)	Inactive	Taiwanese	Yu *et al.* (1999)

mutation is found in ~16% of the Japanese population (Narimatsu *et al.*, 1998). Then, we analyzed *FUT2* gene mutations with direct sequencing of genome DNA from SCLC cell lines. In 21 cell lines, nine cell lines did not have any mutations (Type Se/Se), as summarized in Table II, and 11 lines had homozygous mutations (Type se/se or sej/sej). Just one cell line had a heterozygous mutation (Type Se/sej). Among the fucosyl GM1-positive cell lines, two cell lines had low-functional *FUT2* with A385T, and one cell line had inactivated *FUT2* with G428A. Therefore, it was indicated that the expression of fucosyl GM1 does not depend on the activity of FUT2 enzyme in these cell lines. Thus, it is suggested that FUT1 enzyme is primarily involved in the expression of fucosyl GM1 on the cell surface in SCLC cell lines.

The FUT2 *mutation, se/se and sej/sej, lead to the loss of fucosyl GM1 synthetic activity*

To clarify whether the mutations, *se/se* and *sej/sej*, lead to the complete loss of fucosyl GM1 synthetic activity, we analyzed the expression patterns of fucosyl GM1 in the cells transfected with these mutant genes. Although the co-transfection

Table II. Genetic type of FUT2 enzyme in small cell lung cancer (SCLC) cell lines

Cell line		FucGM1[a]	*FUT2* mutation point[b]							Type
			Fusion	385A/T	428G/A	571C/T	628C/T	658C/T	685del	
SBC1	SCLC	+	–	A	G	C	C	C	–	Se/Se
SBC3	SCLC	–	–	A	G	C	C	C	–	Se/Se
QG90	SCLC	–	–	A	G	C	C	C	–	Se/Se
NCI-H69	SCLC	+	–	A	A	C	C	C	–	se/se
NCI-N417	SCLC	–	–	A	G	C	C	C	–	Se/Se
SK-LC-17	SCLC	–	–	A	A	C	C	C	–	se/se
ACC-LC-48	SCLC	+	–	T	G	C	C	C	–	sej/sej
ACC-LC-49	SCLC	–	–	T	G	C	C	C	–	sej/sej
ACC-LC-52	SCLC	–	–	T	G	C	C	C	–	sej/sej
ACC-LC-67	SCLC	–	–	T	G	C	C	C	–	sej/sej
ACC-LC-73	SCLC	–	–	T	G	C	C	C	–	sej/sej
ACC-LC-76	SCLC	–	–	A	G	C	C	C	–	Se/Se
ACC-LC-96	SCLC	–	–	A	G	C	C	C	–	Se/Se
ACC-LC-97	SCLC	–	–	A	G	C	C	C	–	Se/Se
ACC-LC-170	SCLC	+	–	T	G	C	C	C	–	sej/sej
ACC-LC-171	SCLC	–	–	ND	ND	ND	ND	ND	–	–
ACC-LC-173	SCLC	–	–	T	G	C	C	C	–	sej/sej
ACC-LC-177	SCLC	–	–	A	G	C	C	C	–	Se/Se
ACC-LC-178	SCLC	–	–	A	G	C	C	C	–	Se/Se
ACC-LC-80	SCLC	–	–	A/T	G	C	C	C	–	Se/sej
ACC-LC-5	SCLC	–	–	T	G	C	C	C	–	sej/sej
ACC-LC-172	SCLC	–	–	T	G	C	C	C	–	sej/sej

–, normal sequence at the position; FucGM1, fucosyl GM1; ND, not determined; se, inactivated type; Se, normal type; sej, low-activated type that has ~3% of normal enzyme activity.
[a]The expression of fucosyl GM1 was analyzed with flow cytometry as shown in Figure 1.
[b]See Table I for detailed information.

of FUT2 wild-type *Se/Se* derived from SBC1 cell line and GM1 synthase cDNA resulted in the definite expression of fucosyl GM1 on the cell surface, the co-transfection of *se/se* derived from NCI-H69 cell line and GM1 synthase cDNA did not induce the expression of fucosyl GM1 on the cell surface at all (Figure 7). In the case of co-transfection of *sej/sej* derived from ACC-LC-48 cell line and GM1 synthase cDNA, the expression of fucosyl GM1 was observed, although the expression level was much lower than that by Se/Se. The transfection of *sej/sej* mutant cDNA derived from ACC-LC-170 cell line also resulted in a low expression level of fucosyl GM1 just as *sej/sej* from ACC-LC-48 cell line (data not shown).

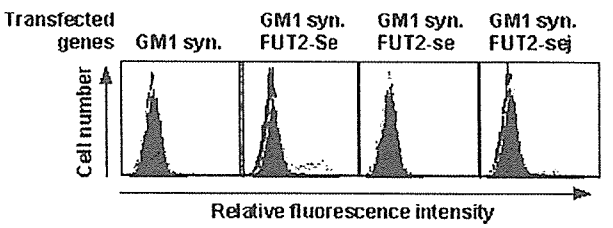

Fig. 7. Expression of fucosyl GM1 on SK-LC-17 cells transiently transfected with *FUT2* cDNAs containing mutations. The cells were transfected with *FUT2* (*Se*), *FUT2* (*se*), or *FUT2* (*sej*) together with the *GM1 synthase* cDNA. *Se*, *se*, and *sej* genes were derived from SBC1, NCI-H69, and ACC-LC-48, respectively, and inserted into pcDNA3.1 expression vector. The fucosyl GM1 expression was analyzed with flow cytometry. Gray lines indicate histograms with antibodies, and filled histograms indicate the controls treated by a second antibody alone. Syn., synthase.

Discussion

Fucosyl GM1 has been thought to be specifically expressed in SCLC cells in both cell lines and tumor tissues (Nilsson *et al.*, 1986; Brezicka *et al.*, 1989; Zhang *et al.*, 1997). Our study results with cultured cell lines also indicated that fucosyl GM1 is specifically expressed in SCLC cells. Among glycosyltransferases involved in the synthesis of fucosyl GM1, the expression levels of the *GM1 synthase* gene did not show any correlation with the expression pattern of fucosyl GM1 in lung cancer cell lines. This might be because the *GM1 synthase* gene was broadly expressed in the majority of cell lines although at low levels, and it might be sufficient to provide some level of the precursor for the synthesis of fucosyl GM1. In contrast, the expression of *FUT1* and/or *FUT2* was considered to be essential for the synthesis of

fucosyl GM1, and the expression levels of the *GD3 synthase* gene oppositely affected fucosyl GM1 expression. In some cell lines in which the *GM1 synthase* level is relatively low, the co-transfection of *GM1 synthase* cDNA was sufficient for FUT1/FUT2 action to make cells express fucosyl GM1. In fact, GM1 synthase and either or both of FUT1/FUT2 effectively bring about fucosyl GM1 in SK-LC-17 cells, indicating its essential involvement in the synthesis of fucosyl GM1.

Both FUT1 and FUT2 are defined as α1,2-fucosyltransferases involved in the synthesis of blood group H antigen, that is, Fucα1,2Galβ1,3/4GlcNAc—structures on glycoproteins and glycolipids (Koda *et al.*, 2001). They are differentially expressed among various tissues and utilize different acceptor structures. FUT1 is expressed in human erythrocytes and vascular endothelial cells and synthesizes type 2 H antigens using type 2 precursor structures. Very rare cases with function-loss mutations in the *FUT1* gene have been called the Bombay or para-Bombay type, with the co-existence or noncoexistence of the *FUT2* mutation, respectively (Koda *et al.*, 2001). FUT2 is mainly expressed in secretions, the gastrointestinal tracts, bronchial tracts, and genital organs and catalyzes the synthesis of type 1 H antigens on mucins. This reaction is, therefore, essential for the synthesis of blood group-carrying mucins such as A, B, and H. Thus, FUT2 has been called Sec enzyme (secretion), because its activity determines the presence of blood group antigens in the saliva.

Preferential usages of type 2 and type 1 chains with FUT1 and FUT2, respectively, were also observed in the transfectant cells with cDNA expression plasmids of individual glycosyltransferases (Lopez-Ferrer and de Bolos, 2002; Mathieu *et al.*, 2004). For example, the expression of FUT1 induced type 2 H and Lewis y or Lewis b antigens and the down-regulation of sialyl Lewis x but did not affect the expression of sialyl Lewis a. As for the synthesis of other type H antigens, such as type 3/4 chains, no definite findings about human fucosyltransferases have been reported, although some animal (rat and bovine) α1,2-fucosyltransferases have been known to prefer Galβ1,3GalNAc acceptor substrates (Barreaud *et al.*, 2000; Sherwood *et al.*, 2001). Bovine fut1 and fut2 synthesize fucosyl GM1 almost equally (Barreaud *et al.*, 2000), whereas the human Se enzyme (FUT2) was reported to act on type 1 and type 3 chain acceptors (Clausen and Hakomori, 1989; Sarnesto *et al.*, 1990). In fact, the preferential production of type 3 H chain with FUT2 has been reported (Lofling *et al.*, 2002). However, little is known about the α1,2-fucosyltransferase responsible for the synthesis of fucosyl GM1 in human tissues.

Thus, whether both FUT1 and FUT2 or either one of them is involved in the synthesis of fucosyl GM1 in SCLC is difficult to be clearly determined based on the obtained results and on the past findings. In fact, these two genes were up-regulated in fucosyl GM1-positive SCLC cell lines, and they behaved in a similar manner in the sublines generated from ACC-LC-170 (data not shown). Furthermore, both of them could induce the expression of fucosyl GM1, when co-transfected into a fucosyl GM1-negative cell line. This was more obvious when co-transfected with *GM1 synthase* cDNA. The fact that three of four fucosyl GM1-positive SCLC cell lines contained mutated *FUT2* gene indicated that FUT1 might be responsible for the synthesis of fucosyl GM1 *in vivo*. Although many reports indicating that similar glycosyltransferase-like molecules function as a molecular chaperone of a real enzyme have recently appeared (Ju and Cummings, 2002), FUT1 seems capable of acting independently in SCLC cells. However, the possibility that FUT1 and FUT2 perform their function more efficiently when present together than when being isolated cannot be excluded, although no additive effects were observed when co-transfected.

As for affinities of the two enzymes toward GM1, there were already reports on the kinetics of FUT1 and FUT2 toward GM1. In bovine, it was shown that K_m values of fut1 and fut2 for GM1 were 1.29 and 1.42 (mM), respectively (Barreaud *et al.*, 2000). In mice, K_m values of Fut1 and Fut2 toward GM1 were 909.1 and 500.0 (μM), respectively (Iwamori and Domino, 2004). All these data suggested that these two enzymes might not have so different affinities toward GM1.

The implications of the expression of α1,2-FUTs in malignant tumor cells have been studied using cloned animal and human cDNAs. In a rat colon carcinoma cell line, the reduction of α1,2-fucosyltransferases FTA or FTB (human FUT1 and FUT2 homologs) with antisense cDNA resulted in an increased tumorigenicity or reduced tumorigenicity, respectively (Hallouin *et al.*, 1999), suggesting differential protein fucosylation among two fucosyltransferases leading to opposite effects. They also affected the sensitivity to apoptosis in the transfectant cells (Goupille *et al.*, 2000). In human pancreatic cancers, α1,2-fucosyltransferase activities were generally down-regulated (Mas *et al.*, 1998), and the restoration of the activity with *FUT1* cDNA decreased the adhesive and metastatic properties (Aubert *et al.*, 2000). Thus, the expression of FUTs appears to affect the expression of ligands of selectins (Mathieu *et al.*, 2004), thermosensitivity (Okamura *et al.*, 2002), and metastatic properties (Aubert *et al.*, 2000). However, precise α1,2-fucosylated structures should be various and have not been clarified in individual experiments.

Whether fucosyl GM1 plays key roles in malignant tumors has not been known at this moment. Because the generation of high expressants of fucosyl GM1 became possible based on the results obtained in this study, the implications of this antigen in the cell proliferation and invasion in SCLC cells will be easily investigated. Although targeted deletions of the *FUT1* and *FUT2* did not show any serious defects in mice (Domino *et al.*, 2001), fucosyl GM1 has been considered to be involved in the signal control (Kosugi *et al.*, 1987). Fucosyl GM1 has also been identified as a target molecule in neurological disorders caused by autoantibodies (Yoshino *et al.*, 1993; Yuki and Ariga, 1997). These facts might suggest that fucosyl GM1 is an important functional molecule expressed on the cell surface of some neuronal cell groups, and it may also play critical roles in the malignant properties of human cancer cells.

The expression of fucosyl GM1 in human tissues should be very restricted compared with that in mice (Iwamori and Domino, 2004) according to the results of immunohistological analyses (Zhang *et al.*, 1997). In mice, the K_m values of

Fut1/Fut2 for GM1 are 5–10 times more than those for nLc4 and Lc4 (Iwamori and Domino, 2004). These findings suggest that antibody therapy might be effective against SCLC particularly because of its specific expression, as fucosyl GM1 can be expressed only when nLc4/Lc4 structures are less abundant and the GD3 synthase activity is not strong.

The discrepancy in the incidence of fucosyl GM1 expression between cell lines and tumor tissue, as analyzed in this study and reported by Brezicka *et al.* (1989) and Zhang *et al.* (1997), respectively, is probably because of the changes in the cell phenotypes during adaptation to *in vitro* culture system after resection. Alternatively, fucosyl GM1 may be a cryptic antigen or may be present in the cytoplasm. Our restricted study of immunocytostaining after fixation suggested that the former interpretation is more likely. Therefore, the possibility that fucosyl GM1 can be used as a target in the immunotherapy of SCLC, such as monoclonal antibodies (Brezicka *et al.*, 1991) and vaccination (Krug *et al.*, 2004), should be convincing, although this issue remains to be carefully investigated. Combined antibody therapy toward multiple tumor antigens might be more effective, as suggested in recent reports (Brezicka *et al.*, 2000; Livingston *et al.*, 2005).

Materials and methods

Cell lines and culture

All human lung cancer cell lines were kindly provided by Dr. T. Takahashi (Nagoya University, Nagoya). Among SCLC cell lines, all ACC-series cell lines (almost Japanese), NCI-N417, and SBC1 and SBC3 were derived from Asian patients. NCI-H69 was derived from a Caucasian patient. They were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 U/mL of benzylpenicillin, and 100 μg/mL of streptomycin sulfate, at 37°C in a humidified 5% CO_2 atmosphere.

Flow cytometry

The cell surface expression of glycolipids was analyzed with FACScan (Becton Dickinson, Mountain View, CA). The cells were incubated with anti-fucosyl GM1 monoclonal antibody CDR73–6 (Kusunoki *et al.*, 1994), anti-Lewis b monoclonal antibody (Seikagaku, Tokyo, Japan), or anti-Lewis y monoclonal antibody (Seikagaku) for 45 min on ice and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibody (Zymed, San Francisco, CA) or IgG antibody (Cappel, Durham, NC). To analyze the expression of GM1, we incubated cells with the cholera toxin (CT) B subunit–biotin conjugates (List Biological Laboratories, Campbell, CA) for 45 min on ice and then incubated with FITC-conjugated avidin (EY Laboratories, San Mateo, CA). Control samples were prepared using the second antibody alone. The intensity of staining was measured and presented in arbitrary units as the log of the fluorescence intensity.

Northern blotting

Total RNA was isolated from lung cancer cell lines with Trizol reagent (Invitrogen, Rockvile, MD) according to the manufacturer's instructions. Fifteen micrograms each of formamide-denatured total RNA was separated by an agarose gel and transferred onto a nylon membrane, GeneScreen Plus (Du Pont New England Nuclear, MA). After baking, the membrane was prehybridized for 3 h and hybridized with [^{32}P]dCTP-labeled cDNA fragment of *FUT1* or *FUT2* as previously described (Yamashiro *et al.*, 1993). The membrane was washed twice with 2× SSC at room temperature, 2× SSC and 1% SDS at 60°C twice, and finally once with 0.1× SSC at room temperature and then exposed to an imaging plate (Fujifilm, Kanagawa, Japan) for 15 h at room temperature. The hybridized bands were detected by BAS-2000 (Fujifilm). MCAS and WiDr were positive controls of the *FUT2* gene, as reported (Koda *et al.*, 1997).

Quantitative real-time RT–PCR

The RNA from each cell line was reverse-transcribed into first-strand cDNA with an oligo dT primer. The quantitative RT–PCR analysis was performed using a DNA Engine Opticon 2 System (Bio-Rad Laboratories, Hercules, CA) with a DyNAmo SYBR Green qPCR Kit (Finnzymes Oy, Espoo, Finland) according to the manufacturer's protocol. The amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured as an internal standard. The PCR was performed using primers specific to each gene through 40 cycles of 95°C for 20 s, 60°C for 10 s, and 72°C for 10 s after preincubation at 95°C for 10 min. The specific primers used for amplification had the following sequences: FUT1-F (5′-CCTGCCAGACTCTGAGTTCC-3′), FUT1-R (5′-AGGCTTAGCCAATGTCCAGA-3′), FUT2-F1 (5′-CTGGTCGTTCAGATGCCTTT-3′), FUT2-R1 (5′-ACTCCCACATGGCTTGAATC-3′), GM1 synthase-F (5′-GCCG TGACCCAGGCCCGG GG-3′), GM1 synthase-R (5′-TC TGGTTCAGGTTCTCCGGA-3′), GM2 synthase-F (5′-AGAGGGTCAGGCAGATCTCA-3′), GM2 synthase-R (5′-CGGACTGTGTCTGCTGTGTT-3′), GM3 synthase-F (5′-AACCCAGAACACCTTTGCAC-3′), GM3 synthase-R (5′-TCACCACTCCCTCTTTGACC-3′), GD3 synthase-F (5′-CAGCATAATTCGGCAAAGGT-3′), GD3 synthase-R (5′-ATTGGCACCAACATCTGACA-3′), GAPDH-F (5′-GTCAGTGGTGGACCTGACCT-3′), and GAPDH-R (5′-TGCTGTAGCCAAAGGCGTTG-3′).

Polymorphism of the FUT2 *gene*

A part of coding region of the *FUT2* gene (185–941) was amplified with PCR using genomic DNA isolated from lung cancer cell lines. PCR was performed with the following primers: FUT2-F2 (5′-GGAACCAGATGGGCGAGTAC-3′) and FUT2-R2 (5′-GCTGCCTCTGGCTTAAAGATT-3′). Then, the product was separated using an agarose gel, followed by extraction from the gel using a QIAEX II Gel Extraction Kit (Qiagen GmbH, Hilden). The purified products were sequenced with the dideoxy termination method using an ABI PRISM 3100 Gene Analyzer (Applied Biosystems, Foster City, CA).

Construction of the expression vectors

pCDM7-FUT1 and *pcDNA1-FUT2* were kindly provided by Dr. J.B. Lowe (Larsen *et al.*, 1990; Kelly *et al.*, 1995). The expression vector *pcDNA3.1-FUT1* was prepared by

inserting an XhoI–XbaI fragment from *pCDM7-FUT1* into the XhoI–XbaI site of a *pcDNA3.1* vector (Invitrogen). *pMIKneo-M1T-9* vector was prepared by inserting an XhoI–XbaI fragment from rat *GM1 synthase* cDNA clone *pM1T-9* (Miyazaki *et al.*, 1997) into the XhoI–XbaI sites of a *pMIKneo* vector (presented by Dr. Maruyama at Tokyo Medical and Dental University).

Gene transfection

SK-LC-17 cells in a 60-mm plastic tissue-culture plate (Becton Dickinson) at a density of 3×10^5 cells/4 mL/plate were transfected transiently with *pMIKneo-M1T-9*, *pcDNA3.1-FUT1*, and/or *pcDNAI-FUT2* using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. After the cells were cultured for 60 h, the glycolipid antigens expressed on the cell surface were detected with flow cytometry analysis.

ACC-LC-170 subcloning

Subclones of the ACC-LC-170 parent cell line were established by the limited dilution method. The ACC-LC-170.10 subline with a low expression of fucosyl GM1 on the cell surface and the ACC-LC-170.31 subline with a high expression of this glycolipid based on the flow cytometry with monoclonal antibody CDR73–6 were established.

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Conflict of interest statement

None declared.

Abbreviations

FUT (or fut), fucosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcription–polymerase chain reaction; SCLC, small cell lung cancer.

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