ギラン・バレー症候群における顔面神経麻痺(口頭)	関口　縁, 三澤園子, 澁谷和幹, 三津間さつき, 岩井雄太, 渡辺慶介, 磯瀬　沙希里, 大森茂樹, 桑原　聡.	第25回日本末梢神経学会学術集会	2014年8月29日～30日	京都
POEMS症候群の末梢神経障害：軸索機能検査・超音波検査による検討(口頭)	三津間さつき, 三澤園子, 澁谷和幹, 関口縁, 岩井雄太, 渡辺慶介, 磯瀬沙希里, 大森茂樹, 別府美奈子, 桑原聡.	第25回日本末梢神経学会学術集会	2014年8月29日～30日	京都
日本におけるGuillain-Barré症候群の予後予測スコア（mEGOS）の有用性(口頭)	関口　縁, 三澤園子, 澁谷和幹, 三津間さつき, 岩井雄太, 渡辺慶介, 磯瀬　沙希里, 大森茂樹, 別府美奈子, 桑原　聡.	第26回日本神経免疫学会学術集会	2014年9月4日～6日	金沢
移植後再発のPOEMS症候群の治療	三澤園子, 関口　縁, 三津間さつき, 渡辺慶介, 澁谷和幹, 岩井雄太, 桑原　聡.	第26回日本神経免疫学会学術集会(口頭)	2014年9月4日～6日	金沢
小児発症の慢性炎症性脱髄性多発神経炎の臨床・電気生理学的特徴(ポスター)	別府美奈子, 三澤園子, 藤井克則, 渋谷和幹, 関口縁, 三津間さつき, 岩井雄太, 渡辺慶介, 磯瀬沙希里, 大森茂樹, 網野寛, 桑原聡.	第44回日本臨床神経生理学会学術大会	2014年11月19日～21日	福岡
後天性特発性全身性無汗症（AIGA）の治療反応関連因子に関する解析.(口頭)	朝比奈正人, 藤沼好克, 山中義崇, 荒木信之, Anupama Poudel, 片桐明, 桑原　聡.	第32回日本神経治療学会総会	2014年11月20日～22日	東京

重度の四肢麻痺に 進展した Fisher/咽頭頚部上腕型 Guillain-Barrė重複症候群の一例(口頭)	岩井雄太, 森雅裕, 関口縁, 西村寿貴, 竹井祥子, 金井哲也, 内田智彦, 桑原聡	第211回日本神経学会関東・甲信越地方会	2014年11月29日	東京
『抹消神経障害の臨床』(口頭)	桑原　聡	北海道医師会認定生涯教育講座学術講演会	2014年2月7日	札幌
「CIDP治療の現状」(口頭)	桑原　聡	第8回CIDP医療講演会	2014年3月30日	東京
炎症性ニューロパチーの最新治療の動向.(口頭)	桑原　聡	第55回日本神経学会学術大会	2014年5月21日～24日	福岡
「CIDPの診断・治療 update」(口頭)	桑原　聡	第11回静岡神経免疫フォーラム	2014年7月4日	静岡
『ギラン・バレー/フィッシャー症候群　update』(口頭)	桑原　聡	第五回神経免疫の集い	2014年9月11日	東京
Guillain-Barre症候群の電気生理学(口頭)	桑原　聡	第44回日本臨床神経生理学会学術大会	2014年11月20日	福岡
Cervical spinal nerve 8 and thoracic vertebrae 1 innervation of forearm flexor muscles(ポスター発表).	Chiba T, Imafuku I, Oishi C, Konoeda F, Higashihara M,Hokkoku K, Hatanaka Y, Sonoo M	61st annual scientific meeting of American association of neuromuscular and electrodiagnostic medicine	Savanna	国内
後根神経節炎における脛骨神経刺激体性感覚誘発電位（SEP）の検討	田村暁子, 園生雅弘, 大石知瑞子, 伊藤和博, 蔦田強司, 嶋田裕之, 伊藤義彰	第44回日本臨床神経生理学会学術大会	福岡	国内
CIDPの診断と治療：診療ガイドラインをめぐって(教育講演)	祖父江元	京都(第25回日本末梢神経学会学術集会)	2014年8月30日	国内
新ガイドライン6「CIDP/MMN」(シンポジウム)	祖父江元	福岡(第55回日本神経学会学術大会)	2014年5月21日～24日	国内
IgG4関連ニューロパチーの臨床と病理(シンポジウム)	大山健、小池春樹、祖父江元	福岡(第55回日本神経学会学術大会)	2014年5月21日～24日	国内
ニューロパチーの病態におけるIgG4の意義(口演)	大山健、小池春樹、飯島正博、高橋美江、橋本里奈、川頭祐一、祖父江元	福岡(第55回日本神経学会学術大会)	2014年5月21日～24日	国内

POEMS症候群における表皮内神経線維密度(口演)	大山健、橋本里奈、高橋美江、川頭祐一、飯島正博、小池春樹、祖父江元	京都(第25回日本末梢神経学会学術集会)	2014年8月30日	国内
慢性炎症性脱髄性多発神経炎(CIDP)における筋萎縮の検討(ポスター)	大山健、小池春樹、勝野雅央、高橋美江、川頭祐一、飯島正博、渡辺宏久、祖父江元	金沢(第26回日本神経免疫学会)	2014年9月14日～16日	国内
Muscle Atrophy in Chronic Inflammatory Demyelinating Polyneuropathy: A Computed Tomography Assessment (Poster)	Ken Ohyama, Haruki Koike, Masahisa Katsuno, Mie Takahashi, Rina Hashimoto, Yuichi Kawagashira, Masahiro Iijima, Hiroaki Adachi, and Gen Sobue	Germany(Inflammatory Neuropathy Consortium)	2014年7月13日～16日	国外
高度の神経肥厚を特徴とするCIDPの臨床的検討(口演)	飯島正博、小池春樹、川頭祐一、大山健、高橋 美江、祖父江 元	京都(第25回日本末梢神経学会学術集会)	2014年8月30日	国内
EANによる脱髄に伴う軸索障害機序の解明(口演)	飯島正博、小池春樹、川頭祐一、大山健、高橋 美江、祖父江 元	金沢(第26回日本神経免疫学会)	2014年9月14日～16日	国内
脱髄性ニューロパチーにおけるランビエ絞輪部周辺のチャネル、接着分子の分布異常(口演)	川頭祐一、高橋美江、大山健、橋本里奈、飯島正博、小池春樹、祖父江元	福岡(第55回日本神経学会学術大会)	2014年5月21日～24日	国内
抗MAG抗体陽性ニューロパチーにおけるランビエ絞輪部周辺のイオンチャンネル、接着分子の分布異常(口演)	川頭祐一、高橋美江、大山健、飯島正博、小池春樹、祖父江元	京都(第25回日本末梢神経学会学術集会)	2014年8月30日	国内

アミノ酸系除草剤グリホサートカリウム塩による過敏性血管炎に伴う末梢神経障害の1例(ポスター)	川頭祐一、川畑和也、高橋美江、大山健、橋本里奈、飯島正博、小池春樹、祖父江元	京都(第25回日本末梢神経学会学術集会)	2014年8月30日	国内
MORPHOLOGY OF NONMYELINATING SCHWANN CELLS IN CHRONIC INFLAMMATORY DEMYELINATING POLYNEUROPATHY	Haruki Koike, Mie Takahashi, Ken Ohyama, Yuichi Kawagashira, Masahiro Iijima, and Gen Sobue	Germany(Inflammatory Neuropathy Consortium)	2014年7月13日～16日	国外
TAG-1ノックアウトによる再髄鞘化機転への影響と軸索脆弱性の解析	飯島正博、小池春樹、川頭祐一、橋本里奈、大山健、高橋美江、祖父江元	福岡(第55回日本神経学会学術大会)	2014年5月21日～24日	国内
サルコイドーシスによる末梢神経障害の病理学的特徴	高橋美江、小池春樹、飯島正博、大山健、橋本里奈、川頭祐一、祖父江 元	福岡(第55回日本神経学会学術大会)	2014年5月21日～24日	国内
顕微鏡的多発血管炎に伴うニューロパチーと非全身性血管炎性ニューロパチーの臨床病理学的対比	高橋美江、小池春樹、飯島正博、大山健、橋本里奈、川頭祐一、祖父江 元	京都(第25回日本末梢神経学会学術集会)	2014年8月30日	国内
ガングリオシド複合による抗原抗体反応増強の機序に関する検討	内堀歩、千葉厚郎	第55回 日本神経学会学術大会	2014年5月	国内 (福岡)
Guillain-Barré症候群症例のガングリオシドとの抗原抗体反応におけるセラミド添加の影響	内堀歩、千葉厚郎	第26回 日本神経免疫学会学術集会	2014年9月	国内 (金沢)
Molecular story of Guillain-Barré syndrome	Atsuro Chiba	第26回 日本神経免疫学会学術集会	2014年9月	国内 (金沢)
A study on the mechanism of antigen-antibody reaction increased by ganglioside-complex in Guillain-Barr é syndrome	Ayumi Uchibori, Atsuro Chiba	12th International Congress of Neuroimmunology	2014年11月	国外 (Mainz, Germany))

New Autoantibodies Against Neutral Glycolipids In Encephalomyeloradiculoneuropathy.	Mutoh T, Shima S, Ishikawa T, Ueda A, Asakura K.	The 66th American Academy of Neurology Annual Meeting; Philadelphia	2014年4月29日	国外
CIDP患者における神経叢の可視化および定量の試み	石川等真, 宮下忠行, 引地智加, 福井隆男, 伊藤信二, 朝倉邦彦, 武藤多津郎.	第55回日本神経学会学術大会 福岡	2014年5月21日～24日	国内
Anti-neutral Glycolipid Antibodies In Encephalomyeloradiculoneuropathy.	Shima S, Ishikawa T, Ueda A, Iwabuchi K, Mutoh T.	第55回日本神経学会学術大会 福岡	2014年5月21日	国内
CIDP患者における神経叢の可視化および定量の試み	石川等真, 引地智加, 福井隆男, 島さゆり, 植田晃広, 木澤真努香, 朝倉邦彦, 武藤多津郎.	第25回日本末梢神経学会学術集会	2014年8月29日	国内
抗中性糖脂質抗体陽性の脳脊髄根末梢神経炎(EMRN)の1例	植田晃広, 島さゆり, 石川等真, 福井隆男, 村手健一郎, 廣田政古, 引地智加, 木澤真努香, 伊藤信二, 朝倉邦彦, 武藤多津郎.	第19回日本神経感染症学会学術集会	2014年9月4日～6日	国内
抗中性糖脂質抗体が陽性であったEMRNの一例	水谷泰彰, 村手健一郎, 福井隆男, 廣田政古, 引地智加, 石川等真, 島さゆり, 植田晃広,木澤真努香, 伊藤信二, 朝倉邦彦, 武藤多津郎.	第140回 日本神経学会東海北陸地方会	2014年11月1日	国内

両側顔面神経麻痺で発症したギラン・バレー症候群と考えられる１例	河合宏, 石川等真, 福井隆男, 廣田政古, 引地智加, 島さゆり, 木澤真努香, 朝倉邦彦, 武藤多津郎.	第224回　日本内科学会地方会	2014年11月2日	国内
Profiling of miRNAs in cerebrospinal fluid from patients with amyotrophic lateral sclerosis（口演）	Machida A, Ohkubo T, Matsuo H, Tsunoda A, Kishimoto S, Maehara T, Kosaka N, Ochiya T, Mizusawa H, Yokota T.	The 66th American Academy of Neurology Annual Meeting, Philadelphia	2014年4月30日	国外
髄液miRNA定量による筋萎縮性側索硬化症の新規診断法の開発―多巣性運動ニューロパチーとの比較検討（ポスター）	町田明, 大久保卓哉, 八木洋輔, 関口輝彦, 叶内匡, 水澤英洋, 横田隆徳	第6回日本RNAi研究会	2014年8月1日	国内
Profiling of miRNAs in cerebrospinal fluid from patients with multifocal motor neuropathy and amyotrophic lateral sclerosis（口演）	Ohkubo T, Machida A, Mogushi K, Matsuo H, Tsunoda A, Maehara T, Noto Y, Shimizu T, Kuwabara S, Kanda T, Kosaka N, Ochiya T, Yokota T.	The 37th Annual Meeting of the Japan Neuroscience Society, Yokohama.	2014年9月13日	国内

2．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所 （学会誌・雑誌等名）	発表した時期	国内・外の別
Binding specificity of anti-HNK-1 IgM M-protein in anti-MAG neuropathy: Possible clinical relevance.	Hamada Y, Hirano M, Kuwahara M, Samukawa M, Takada K, Morise J, Yabuno K, Oka S, Kusunoki S.	Neurosci Res 2015; 91: 63-68	2015年2月	国外
A multicenter prospective study of Guillain-Barré syndrome in Japan: a focus on the incidence of subtypes.	Mitsui Y, Kusunoki S, Arimura K, Kaji R, Kanda T, Kuwabara S, Sonoo M, Takada K, the Japanese GBS Study Group.	J Neurol Neurosurg Psychiatry 2015; 86: 110-114	2015年1月	国外
Increased proinflammatory cytokines in sera of patients with multifocal motor neuropathy.	Furukawa T, Matsui N, Fujita K, Miyashiro A, Nodera H, Izumi Y, Shimizu F, Miyamoto K, Takahashi Y, Kanda T, Kusunoki S, Kaji R.	J Neurol Sci 2014; 346: 75-79	2014年11月	国外
A pediatric case of peripheral polyneuropathy with IgM anti-GM1 antibody associated with a group A beta-hemolytic Streptococcus infection.	Ishikawa N, Kobayashi Y, Fujii Y, Samukawa M, Kusunoki S, Kobayashi M.	Pediatr Neurol 2014; 51: 441-443.	2014年9月	国外
Taste impairment in Miller Fisher syndrome.	Yagi Y, Yokote H, Watanabe Y, Amino T, Kamata T, Kusunoki S.	Neurol Sci, on line, DOI: 10.1007/s10072-014-1916-0	2014年8月	国外

Prevalence of anti-ganglioside antibodies and their clinical correlates with Guillain-Barré syndrome in Korea: a nationwide multicenter study.	Kim JK, Bae JS, Kim DS, Kusunoki S, Kim JE, Kim JS, Park YE, Park KJ, Song HS, Kim SY, Lim JG, Kim NH, Suh BC, Nam TS, Park MS, Choi YC, Sohn EH, Na SJ, Huh SY, Kwon O, Lee SY, Lee SH, Oh SY, Jeong SH, Lee TK, Kim DU.	J Clin Neurol 2014; 10: 94-100	2014年4月	国外
慢性炎症性脱髄性多発根ニューロパチー(CIDP)非典型的CIDP	福島和広、池田修一	Clinical Neuroscience	2014年3月	
Peripheral sympathetic nerve dysfunction in adolescent Japanese girls following immunization with the human papillomavirus vaccine	Kinoshita T, Abe RT, Hineno A, Tsunekawa K, Nakane S, Ikeda S	Intern Med	2014年4月	
Motor branch biopsy of the pronator teres muscle in a patient with painful forearm neuropathy	Kinoshita T, Fukushima K, Abe RT, Ogawa Y, Nakagawa M, Katoh N, Yoshida T, Kato H, Ikeda S	Case Rep Neurol	2014年7月	
Guillain-Barré syndromeの予後改善のための治療戦略～現状と展望	海田賢一	Peripheral Nerve 末梢神経 2014; 25(2): 229-232.	2014; 25(2): 229-232. 平成26年12月1日発行	国内
免疫介在性ニューロパチーの自己抗体と免疫グロブリン静注療法	海田賢一	Peripheral Nerve 末梢神経 2014; 25(2): 284-289.	2014; 25(2): 284-289. 平成26年12月1日発行	国内
「ギラン・バレー症候群：IGOS」：病態解明・新規治療を目指した神経疾患の患者レジストリシステム、第4回。	海田賢一	BRAIN and NERVE 2014; 66(12): 1496-1502.	平成26年12月1日発行	国内

多巣性運動ニューロパチー	松井尚子	今日の治療指針　2014年度版 885-886 医学書院	2014	国内
多巣性運動ニューロパチー（MMN）　病態と検査所見	松井尚子、古川貴大、梶龍兒	GBS と CIDP－診療 New Standards Clinical Neuroscience 2014 年 Vol.32(3) 324-326 中外医学社	2014	国内
V　脱髄性疾患、遺伝性ニューロパチー　多巣性運動ニューロパチー（MMN）	古川貴大、松井尚子、梶龍兒			
Are multifocal motor neuropathy patients underdiagnosed? An epidemiological survey in Japan.	Miyashiro A, Matsui N, Shimatani Y, et al.	Muscle Nerve 2014; 49(3): 357-61.	2014	国内
Increased proinflammatory cytokines in sera of patients with multifocal motor neuropathy.	Furukawa T, Matsui N, Fujita K, et al.	J Neurol Sci 2014; 346(1-2):75-79.	2014	国外
Sera from multifocal motor neuropathy patients disrupt the blood-nerve barrier.	Shimizu F, Omoto M, Sano Y, Tasaki A, Matsui N, Miyashiro A, Koga M, Kaji R, Kanda T	J Neurol Neurosurg Psychiatry	2014	国外
Severity and patterns of blood-nerve barrier breakdown in patients with chronic inflammatory demyelinating polyradiculoneuropathy: correlations with clinical subtypes.	Shimizu F, Sawai S, Sano Y, Beppu M, Misawa S, Nishihara H, Koga M, Kuwabara S, Kanda T	PLoS One	2014	国外
Increased proinflammatory cytokines in sera of patients with multifocal motor neuropathy.	Furukawa T, Matsui N, Fujita K, Miyashiro A, Nodera H, Izumi Y, Shimizu F, Miyamoto K, Takahashi Y, Kanda T, Kusunoki S, Kaji R	J Neurol Sci	2014	国外

A multi-center prospective study of Guillain-Barré syndrome in Japan: a focus on the incidence of subtypes.	Mitsui Y, Kusunoki S, Arimura K, Kaji R, Kanda T, Kuwabara S, Sonoo M, Takada K, the Japanese GBS Study Group	J Neurol Neurosurg Psychiatry	2015	国外
Combined immunomodulatory therapies resulted in stepwise recovery in autoimmune autonomic ganglionopathy.	Nishihara H, Koga M, Higuchi O, Tasaki A, Ogasawara JI, Kawai M, Nakane S, Kanda T	Clin Exp Neuroimmunol	2015	国内
A nationwide survey of combined central and peripheral demyelination in Japan.	Ogata H, Matsuse D, Yamasaki R, Kawamura N, Matsushita T, Yonekawa T, Hirotani M, Murai H, Kira JI	J Neurol Neurosurg Psychiatry.	2015年2月11日	国外
Acquired and genetic channelopathies: in vivo assessment of axonal excitability.	Kuwabara S, Misawa S.	Experimental neurology 2015;263:368-371	2015年1月	国外
Involvement of group Ia afferents in Fisher syndrome: Neuronopathy or axonopathy?	Kuwabara S, Sekiguchi Y, Misawa S.	Clinical Neurophysiology 2014;125(1):213-4	2014年1月	国外
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Serum cytokine and chemokine profiles in patients with chronic inflammatory demyelinating polyneuropathy	Beppu M, Sawai S, Misawa S, Sogawa K, Mori M, Ishige T, Satoh M, Nomura F, Kuwabara S.	Journal of Neuroimmunology 2015;279:7-10	2015年2月	国外

Duration of the distal compound muscle action potential for diagnosis of chronic inflammatory demyelinating polyneuropathy: effects of low-cut filters.	Isose S, Misawa S, Sonoo M, Shimuzu T, Oishi C, Shibuya K, Nasu S, Sekiguchi Y, Mitsuma S, Beppu M, Omori S, Komori T, Kokubun N, Inaba A, Hirashima F, Kuwabara S; Tokyo Metropolitan Neuromuscular Electrodiagnosis Study Group	J Clin Neurophysiol. 2014;31(5):441-3	2014年1月	国外
Different electrophysiological profiles and treatment response in 'typical' and 'atypical' chronic inflammatory demyelinating polyneuropathy.	Kuwabara S, Isose S, Mori M, Mitsuma S, Sawai S, Beppu M, Sekiguchi Y, Misawa S.	J Neurol Neurosurg Psychiatry. Inpress	inpress	国外
A multicentre prospective study of Guillain-Barre Syndrome in Japan: a focus on the incidence of subtypes.	Mitsui Y, Kusunoki S, Arimura K, Kaji R, Kanda T, Kuwabara S, Sonoo M, Takada K; and the Japanese GBS Study Group.	J Neurol Neurosurg Psychiatry 2015;86(1):110-4	2015年1月	国外

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Ⅳ. 研究成果の刊行物・別刷

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Binding specificity of anti-HNK-1 IgM M-protein in anti-MAG neuropathy: Possible clinical relevance

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ABSTRACT

Anti-myelin-associated-glycoprotein (MAG) neuropathy is an intractable autoimmune polyneuropathy. The antigenic region of MAG is the human natural killer-1 (HNK-1) carbohydrate. We and others previously suggested that the extension of antibody reactivities to HNK-1-bearing proteins other than MAG was associated with treatment resistance, without statistical analyses. In this study, we established an ELISA method with recombinant proteins to test binding specificities of currently available monoclonal antibodies to MAG and another HNK-1-bearing protein, phosphacan. Using this system, we found the distinct binding specificities of anti-MAG antibody in 19 patients with anti-MAG neuropathy. Their clinical relevance was then determined retrospectively with the adjusted 10-points INCAT disability score (0 = normal and 10 = highly disable). The results showed that strong reactivities of anti-MAG antibodies to phosphacan were significantly associated with treatment resistance or progressive clinical courses, indicating a possible clinical relevance of the binding specificities.

1. Introduction

Accumulating evidence suggests the causal relationship between IgM monoclonal gammopathy and peripheral neuropathy (Steck et al., 2006). A review article described that 1.5% of patients with neuropathy were associated with IgM monoclonal gammopathy (Nemni et al., 1994). Among such patients, 50–60% of patients have antibody activity to myelin-associated glycoprotein (MAG) (Nemni et al., 1994). IgM anti-MAG-antibody-associated neuropathy (anti-MAG neuropathy) is a chronic demyelinating neuropathy that predominantly affects sensory nerves initially but later involves the motor system, and thus referred also to distal acquired demyelinating sensory (DADS) polyneuropathy (Steck et al., 2006). No specific therapeutic option has been established by large-scale studies, however, intravenous immunoglobulin infusion (IVIg), plasma exchange (PE) and rituximab therapy have been effective in some patients (Nobile-Orazio et al., 2000). This fact may suggest that such treatment-responsiveness or prognosis may not be attributed to the certain treatment itself, but may depend on other unknown factors.

The antigenic region of MAG is the human natural killer-1 (HNK-1) carbohydrate, comprising three sugar residues with sulfated modification. HNK-1 is also present in sulfated glucuronyl paragloboside (SGPG), P0, PMP-22, and phosphacan. Thus, such glycoconjugates other than MAG might be recognized or targeted by anti-MAG antibodies. Experimentally-raised HNK-1-binding monoclonal antibodies, such as HNK-1 and Cat-315, recognized multiple HNK-1 bearing proteins, however, fine specificities of these antibodies differed considerably (Dino et al., 2006). Consistent with this experimental finding, we and others previously suggested that the extension of antibody reactivities to various HNK-1-bearing proteins other than MAG might be associated with treatment resistance (Shiina et al., 2001; Weiss et al., 1999). For example, antibody reactivities to P0 and PMP22 other than MAG tended to be associated with poor responsiveness to immunotherapy. However, both studies used the human brain and peripheral nerves, and lacked statistical analyses, indicating the difficulties in application to a large-scale analysis and possibly in reproducibility.

In this study, we established an ELISA method with recombinant proteins to test binding specificities of three available monoclonal antibodies to HNK-1-bearing proteins, such as MAG and phosphacan. Using essentially the same assay method, we found the distinct binding specificities of anti-MAG antibody in 19 patients with

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anti-MAG neuropathy. Their clinical relevance was then determined retrospectively with the adjusted 10-points INCAT disability score (*0 = normal and 10 = highly disable*) (*Hughes et al., 2001*). Effectiveness of treatment or clinical progression was evaluated with the ΔINCAT score (the increase in INCAT score) and with the INCAT score progression index (ΔINCAT score per month).

2. Methods

2.1. Monoclonal antibodies

The monoclonal antibody HNK-1 was purchased from the American Type Culture Collection. Cat-315 was purchased from Millipore (MAB1581). 6B4 was kindly provided from Dr. N. Maeda (Tokyo Metropolitan Institute of Medical Science). 6B4 and Cat-315 had been thought to recognize phosphacan and aggrecan, respectively. But later these antibodies were found to react the HNK-1 carbohydrate and thus other HNK-1-bearing proteins (Morise et al., 2014).

2.2. Antigens

The expression plasmid pIRES-GlcAT-P/HNK-1ST (pIRES-P/ST), which expresses rate-limiting enzymes for production of the HNK-1 epitope, has been described previously (Kizuka et al., 2006). Human IgG Fc-tagged MAG (MAG-Fc) and phosphacan (phosphacan-Fc) were prepared by subcloning of extracellular domains of mouse MAG and full length rat phosphacan cDNA amplified by PCR into pEF-Fc to give pEF-MAG-Fc and pEF-phosphacan-Fc, respectively. COS-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C until 50–70% confluent. After culture medium was replaced with Opti-MEM I (GIBCO), cells were transfected with a combination of pEF-MAG-Fc or pEF-phosphacan-Fc in the presence or absence of pIRES-P/ST, using *Lipofectamine 2000* (*Invitrogen*). After transfection for 6 h, Opti-MEM I was replaced with ASF Medium 104 (Ajinomoto). Cells were incubated for another 4 days to obtain culture medium containing secreted proteins. MAG-Fc and phosphacan-Fc with or without HNK-1 carbohydrate epitope were purified with a protein G-Sepharose TM4 Fast Flow column (GE Healthcare), which exhibits high affinity for the human IgG Fc region. The presence of the HNK-1 epitope on the prepared antigens was confirmed by the monoclonal antibodies (HNK-1, Cat-315, and 6B4). By contrast, the antigens without the HNK-1 epitope were not recognized by any of those monoclonal antibodies. Each antigen was aliquoted and kept frozen until use.

2.3. ELISA

For examinations of monoclonal antibodies, wells of an ELISA plate were coated with 50 ng antigen (phosphacan or MAG). Phosphacan and MAG with no expression of the HNK-1 epitope were used as controls. Nonspecific binding sites on the wells were saturated with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 60 min at room temperature. 1st antibodies diluted 1:2000 in 3% BSA/PBS was added to the antigen-coated, after which the plates were incubated for 120 min at room temperature. The wells were washed three times with 0.1% TritonX-100 in PBS and incubated for 90 min at room temperature with peroxidase-conjugated rabbit anti-mouse IgM antibody (Zymed Laboratories, South San Francisco, CA, USA) diluted 1:50,000 in 3% BSA in PBS and washed three times with 0.1% TritonX-100 in PBS. Then the plates were incubated with SureBlue™ TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA). The reaction was stopped by addition of 0.5 N HCl. The color reaction was read at 450 nm with Infinite M200 (Tecan, San Jose, CA, USA). The wash buffer containing 0.1% Tween 20 instead of 0.1% TritonX-100 was also used in our experiment, the result of which was essentially the same.

For examinations of patients' sera, wells of an ELISA plate were coated with 25 pg antigen (phosphacan or MAG). Phosphacan or MAG with no expression of the HNK-1 epitope was also coated in different wells. Uncoated wells were used as controls. Nonspecific binding sites on the wells were saturated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature. A 50 μl serum sample diluted 1:400 in 1% BSA in PBS was added to the antigen-coated and uncoated wells, after which the plates were incubated for 120 min at room temperature. The wells were washed three times with 0.1% BSA in PBS and incubated for 90 min at room temperature with 50 μl of peroxidase-conjugated goat anti-human IgM antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:10,000 in 1% BSA in PBS and washed three times with 0.1% BSA in PBS. Then the plates were incubated with 40 mg/dl o-phenylenediamine dihydrochloride and 0.006% H_2O_2 in phosphate-citrate buffer, pH 5.0, for 2 min. The reaction was stopped by addition of 50 μl of 8 N H_2SO_4. The color reaction was read at 492 nm with an ELISA reader (BioRad, Hercules, CA, USA). The optical density at 492 nm (OD 492) of the uncoated well was subtracted from that of the antigen-coated well. The difference in OD was considered to indicate the antibody activity against MAG or phosphacan. The ratios of anti-phosphacan to anti-MAG antibody activities (P/M ratio) were calculated.

2.4. Patients

We examined sera from patients diagnosed with neuropathy associated with IgM monoclonal gammopathy from January 2005 to December 2011, using methods described previously (Shiina et al., 2001). Anti-SGPG and anti-MAG IgM antibodies were found in 36 patients. The clinical features and details of medical treatment were obtained for 24 of these patients (20 men and 4 women) from attending physicians. Among those 24 patients, the observation period for one patient was only one month, 3 patients were not treated, and one patient also had IgG antibodies against multiple gangliosides such as GM1, GM2, GM3, GD1a, GT1b, and GQ1b. These 5 patients were excluded, leaving data for 19 patients (16 men and 3 women) for analysis.

2.5. Clinical features

Clinical features including initial symptoms, cerebrospinal fluid (CSF) protein levels, serum IgM levels, treatment periods, the adjusted 10-points INCAT scores before treatment, and latest INCAT scores were obtained retrospectively. Effectiveness of treatment or clinical progression was evaluated with ΔINCAT score and with the INCAT score progression index.

2.6. Statistical analysis

Differences in percentages were tested by Chi-square test or Fisher exact test. Differences in medians were examined by Mann–Whitney *U* test. Two-tailed *p*-values <0.05 were considered significant. Correlation was tested by Spearman rank correlation analysis. All calculations were performed using SPSS v.20 (IBM Japan).

3. Results

3.1. Reactivities of monoclonal antibodies

For examination of the preferential reactivity of the monoclonal antibodies HNK-1, 6B4, and Cat-315, we prepared MAG or phosphacan as a carrier protein with a different type of the HNK-1

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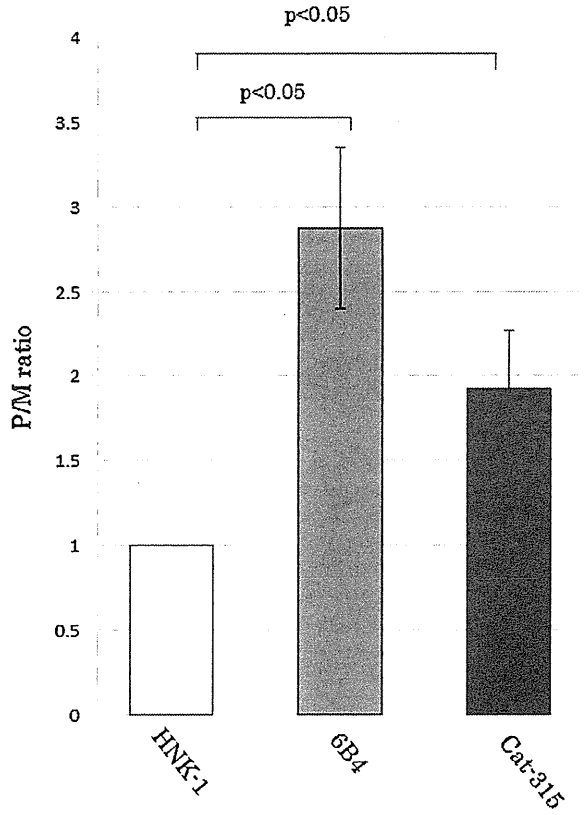

Fig. 1. Preferential immunoreactivity of the monoclonal antibodies HNK-1, 6B4, and Cat-315 against the HNK-1 carbohydrate epitope expressed on phosphacan and MAG. Wells of an ELISA plate were coated with phosphacan and MAG. The immunoreactivity of HNK-1, 6B4, and Cat-315 were measured as described in Section 2. The relative reactivity of each monoclonal antibody against phosphacan to MAG (P/M ratio) was calculated. Bar graphs showed the relative reactivity of 6B4 (gray bar) and Cat-315 (black bar) normalized by that of HNK-1 (white bar). Data show relative ratio ± SD.

carbohydrate antigen. Both 6B4 and Cat-315 preferentially recognized the HNK-1 carbohydrate epitope on phosphacan than that on MAG (Fig. 1).

3.2. Reactivities of sera from patients with anti-MAG neuropathy

The clinical features of the 19 patients and antibody activities against MAG and phosphacan are shown in Table 1. Reactivities of patients' sera to MAG and phosphacan with the HNK-1 epitope were detectable with considerable variation, while those to MAG or phosphacan without the HNK-1 epitope were undetectable. The mean antibody activities against MAG and phosphacan were 0.35 ± 0.20 and 0.30 ± 0.20, respectively, and the mean ratio of anti-phosphacan to anti-MAG antibody activities (P/M ratio) was 0.85 ± 0.40.

3.3. Clinical information and relevance of the antibody specificities

The initial symptom was leg dysesthesia in 16 patients, muscle weakness in 2, and gait disturbance in one patient. Seventeen patients were treated with IVIg, 9 with prednisolone, 4 with rituximab, and 3 with plasma exchange (Table 2). The INCAT scores were 3.26 ± 1.19 (mean ± SD) at the time of serum sampling and 3.57 ± 2.29 at the latest examination, giving a mean change in INCAT score during the interval of 0.31 ± 2.08 (Table 2). There was no association between the anti-MAG or anti-phosphacan antibody titers and the clinical features of the patients. However, the P/M ratio of the 19 cases showed a positive correlation with the ΔINCAT score and the INCAT progression index (Fig. 2).

The patients were divided into two groups with a P/M ratio ≥1 ($n = 6$) and <1 ($n = 13$). The ΔINCAT score and the INCAT progression index were significantly higher in P/M ratio ≥1 than in <1 ($p < 0.05$) (Fig. 3).

4. Discussion

We established the ELISA method with recombinant proteins. In addition to MAG, we used phosphacan as a representative antigen for HNK-1-bearing glycoproteins, because this protein was soluble enough to be useful for our ELISA system. By contrast, some of HNK-1-bearing glycoproteins associated with neuropathy, such as PMP22, are highly insoluble. Our ELISA system revealed that currently available mouse monoclonal antibodies reactive with the HNK-1 epitope showed differential binding activities to MAG and phosphacan. Using this system, the serum IgMs from patients with

Table 1
Clinical information and ELISA results of patients with anti-MAG neuropathy.

Patients	Sex	Age	Neuropathy duration (years)	Initial symptom	Serum IgM (mg/dl)	CSF protein (mg/dl)	Phosphacan	MAG	P/M ratio
1	M	70	16	Muscle weakness	1340	47	0.12	0.19	0.63
2	F	79	10	Gait disturbance	855	106	0.14	0.10	1.35
3	M	83	5	Leg dysesthesia	688	ND	0.35	0.48	0.73
4	M	67	11	Leg dysesthesia	747	107	0.42	0.53	0.80
5	M	60	6	Leg dysesthesia	1172	71	0.08	0.18	0.46
6	M	66	5	Leg dysesthesia	878	507	0.44	0.71	0.61
7	M	70	4	Leg dysesthesia	ND	92	0.72	0.44	1.64
8	M	83	5	Leg dysesthesia	2079	103	0.35	0.21	1.71
9	M	61	6	Leg dysesthesia	1862	239	0.24	0.36	0.67
10	M	58	4	Leg dysesthesia	486	48	0.31	0.27	1.18
11	F	63	3	Leg dysesthesia	518	92	0.27	0.36	0.76
12	M	65	2	Leg dysesthesia	714	184	0.05	0.15	0.30
13	M	76	5	Leg dysesthesia	446	74	0.02	0.14	0.17
14	M	71	1	Leg dysesthesia	ND	69	0.05	0.05	1.00
15	F	75	2	Leg dysesthesia	514	ND	0.36	0.37	0.98
16	M	75	1	Leg dysesthesia	294	138	0.72	0.81	0.89
17	M	58	2	Leg dysesthesia	207	36	0.28	0.45	0.62
18	M	80	3	Muscle weakness	585	94	0.41	0.40	1.05
19	M	59	1	Leg dysesthesia	507	59	0.36	0.53	0.67

Phosphacan, titers of anti-phosphacan antibody; MAG, titers of anti-MAG antibody.

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