ARTHRITIS & RHEUMATISM
Vol. 52, No. 5, May 2005, pp 1571–1576
DOI 10.1002/art.21023

Autoantibodies to a 140-kd Polypeptide, CADM-140, in Japanese Patients With Clinically Amyopathic Dermatomyositis

Shinji Sato,[1] Michito Hirakata,[1] Masataka Kuwana,[1] Akira Suwa,[1] Shinichi Inada,[2] Tsuneyo Mimori,[3] Takeji Nishikawa,[1] Chester V. Oddis,[4] and Yasuo Ikeda[1]

Objective. **To identify novel autoantibodies specific for dermatomyositis (DM), especially those specific for clinically amyopathic DM (C-ADM).**

Methods. **Autoantibodies were analyzed by immunoprecipitation in 298 serum samples from patients with various connective tissue diseases (CTDs) or idiopathic pulmonary fibrosis (IPF). Antigen specificity of the sera was further examined by immunoblotting and indirect immunofluorescence (IF). The disease specificity and clinical features associated with the antibody of interest were determined.**

Results. **Eight sera recognized a polypeptide of ~140 kd (CADM-140 autoantigen) by immunoprecipitation and immunoblotting. Immunoreactivity was detected in the cytoplasm, and indirect IF revealed a granular or reticular pattern. Anti–CADM-140 antibodies were detected in 8 of 42 patients with DM, but not in patients with other CTDs or IPF. Interestingly, all 8 patients with anti–CADM-140 antibodies had C-ADM. Among 42 patients with DM, those with anti–CADM-140 autoantibodies had significantly more rapidly progressive interstitial lung disease (ILD) when compared with patients without anti–CADM-140 autoantibodies (50% versus 6%; $P = 0.008$).**

Conclusion. **These results indicate that the presence of anti–CADM-140 autoantibodies may be a novel marker for C-ADM. Further attention should be directed to the detection of rapidly progressive ILD in those patients with anti–CADM-140 autoantibodies.**

Polymyositis (PM)/dermatomyositis (DM) is a chronic inflammatory disorder that culminates in injury to the skin and muscle and, sometimes, is associated with interstitial lung disease (ILD) and/or neoplasia (1,2). A number of autoantibodies are associated with myositis, including those specific for aminoacyl–transfer RNA synthetase (anti-ARS) (3), signal recognition particle (anti-SRP) (4), and Mi-2 (5). These autoantibodies have proven to be clinically useful in the diagnosis and classification of these diseases and are predictive of responses to treatment.

It has been known for some time that certain patients may have the typical skin manifestations of DM but no evidence of myositis, a condition known as amyopathic DM. Recently, Sontheimer proposed the existence of a unique subgroup of patients with DM who have the clinical cutaneous features of DM but no evidence of clinical myositis symptoms for at least 2 years after the onset of skin manifestations (referred to as clinically amyopathic DM [C-ADM]) (6). In other words, C-ADM includes patients with amyopathic DM and patients with hypomyopathic DM (patients with subclinical signs of myositis and DM skin manifestations). Some patients with C-ADM, especially those in Japan (7), have been noted to develop rapidly progressive ILD. This condition in many of these patients is resistant to treatment, and fatal outcomes have been observed.

Because of the severity of ILD accompanying C-ADM, a marker autoantibody would be useful for early diagnosis and treatment monitoring. Potential marker autoantibodies have been described by Targoff et al, who, in a preliminary study, described specificity for a 95-kd Se protein, as well as an unidentified 155-kd protein (8). However, a full survey of the autoantibodies

[1]Shinji Sato, MD, Michito Hirakata, MD, Masataka Kuwana, MD, Akira Suwa, MD, Takeji Nishikawa, MD, Yasuo Ikeda, MD: Keio University School of Medicine, Tokyo, Japan; [2]Shinichi Inada, MD: Tokyo Metropolitan Ohtsuka Hospital, Tokyo, Japan; [3]Tsuneyo Mimori, MD: Kyoto University Graduate School of Medicine, Kyoto, Japan; [4]Chester V. Oddis, MD: University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Shinji Sato, MD, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: shins@sc.itc.keio.ac.jp.

Submitted for publication August 11, 2004; accepted in revised form February 3, 2005.

associated with C-ADM has not been performed. In the present study, we examined the sera from 15 Japanese patients with C-ADM to identify additional autoantibodies associated with this disease.

PATIENTS AND METHODS

Patients and sera. Serum samples were obtained from 255 randomly selected Japanese adult patients with connective tissue diseases (CTDs) who were being followed up in clinics at Keio University in Tokyo and collaborating medical centers. These sera were obtained, prior to therapy, from a cohort of 61 patients with PM, 42 with DM (including 15 with C-ADM), 50 with rheumatoid arthritis, 46 with systemic lupus erythematosus, 27 with mixed CTD/overlap syndrome, 22 with systemic sclerosis, and 7 with Sjögren's syndrome. Sera from 43 patients with idiopathic pulmonary fibrosis (IPF) and 16 normal human sera were used as control sera. The diagnosis of C-ADM was based on diagnostic criteria proposed by Sontheimer (6), i.e., DM patients with no clinical muscle symptoms for more than 2 years after the onset of skin manifestations.

The patients were diagnosed as having ILD according to the results of chest radiography, chest computed tomography (CT), and pulmonary function testing, which included the percent predicted values for forced vital capacity and diffusing capacity for carbon monoxide. A subset of patients with rapidly progressive ILD was defined as those presenting with progressive dyspnea and progressive hypoxemia, and a worsening of interstitial change on the chest radiograph within 1 month from the onset of respiratory symptoms.

Immunoprecipitation. The immunoprecipitation assay was performed using extracts of the leukemia cell line, K562, as previously described (9). A total of 10 μl of patient serum was mixed with 2 mg of polypeptide A–Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) in 500 μl of immunoprecipitation buffer (10 m*M* Tris HCl, pH 8.0, 500 m*M* NaCl, 0.1% Nonidet P40) and incubated for 2 hours at 4°C, and then washed 3 times with immunoprecipitation buffer.

For polypeptide studies, antibody-coated Sepharose beads were mixed with 100 μl of ^{35}S-methionine–labeled K562 cell extracts derived from 2×10^5 cells, and rotated at 4°C for 2 hours. After 6 washes, the Sepharose beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer and the polypeptides were fractionated by 6% SDS–polyacrylamide electrophoresis gels. Radiolabeled polypeptide components were analyzed by autoradiography.

For analysis of RNA, the antigen-bound Sepharose beads were incubated with 100 μl of K562 cell extracts (6×10^6 cell equivalents per sample) for 2 hours at 4°C. To extract bound RNA, 30 μl of 3.0*M* sodium acetate, 30 μl of 10% SDS, 2 μl of carrier yeast transfer RNA (10 mg/ml; Sigma, St. Louis, MO), and 300 μl of phenol:chloroform:isoamyl alcohol (50:50:1, containing 0.1% 8-hydroxyquinoline) were added. After ethanol precipitation, the RNA was resolved using a 7*M* urea–10% polyacrylamide gel, which was subsequently silver-stained (Bio-Rad, Hercules, CA).

Immunoblotting. Immunoblotting analysis was performed using K562 cell extracts in a modification of the procedure described by Towbin et al (10).

Immunodepletion. A 10-μl aliquot of the prototype serum of autoantibodies to the 140-kd polypeptide was mixed with 2 mg of Sepharose beads and incubated for 2 hours at 4°C, followed by 3 washes with immunoprecipitation buffer. Another serum that recognized the 140-kd polypeptide was added in a dose-dependent manner (0 μl, 10 μl, 25 μl, and 50 μl) and then incubated. After 3 washes, immunoprecipitation for polypeptide analysis was performed as described above.

Indirect immunofluorescence (IF). Indirect IF was performed using HEp-2 cells and fluorescein-labeled anti-human immunoglobulin (Inova Diagnostics, San Diego, CA).

Clinical studies. The patients whose sera immunoprecipitated a 140-kd polypeptide were examined for their clinical symptoms, clinical course, muscle enzyme levels (creatine kinase [CK] and aldolase), results on chest radiographic and CT scans, and findings of skin pathology. An assessment of muscle weakness was performed using a manual muscle test (11). Some patients were also examined by electromyogram and muscle magnetic resonance imaging (MRI), and by pathologic analysis of the muscle.

Statistical analysis. The 2 groups of DM patients with or without autoantibodies to the 140-kd polypeptide were compared. The results of comparisons between groups were analyzed using the chi-square test, with Yates' correction where appropriate.

RESULTS

Detection of anti–140-kd polypeptide antibodies in patients with C-ADM. We screened 298 patient sera and 16 normal human sera by immunoprecipitation. Sera from 8 (19%) of 42 patients with DM immunoprecipitated a polypeptide of ~140 kd from ^{35}S-methionine–labeled K562 cell extracts (Figure 1A, lanes 1–8). All 8 patients were diagnosed as having C-ADM, a subtype of DM. In the analysis of RNA specificity, these sera did not immunoprecipitate any nucleic acid band, except for 1 patient's serum, which precipitated hYRNA of SSA/Ro components.

The C-ADM sera that immunoprecipitated the 140-kd polypeptide were also used to immunoblot K562 cell extracts. These sera from C-ADM patients displayed a similar reaction on immunoblot, with a polypeptide band of similar molecular weight (results not shown).

For identification of novel autoantibodies recognizing the 140-kd molecule, the polypeptide immunoprecipitated by the prototype serum was compared with antigens of similar molecular weight recognized by other known autoantibodies (Figure 1B). The protein recognized by the prototype serum migrated slightly faster than the 140-kd protein recognized by anti-MJ antibody (Figure 1B, lane 2) and faster than that recognized by anti–RNA helicase A antibody (Figure 1B, lane 3), but more slowly than the 120-kd protein precipitated by

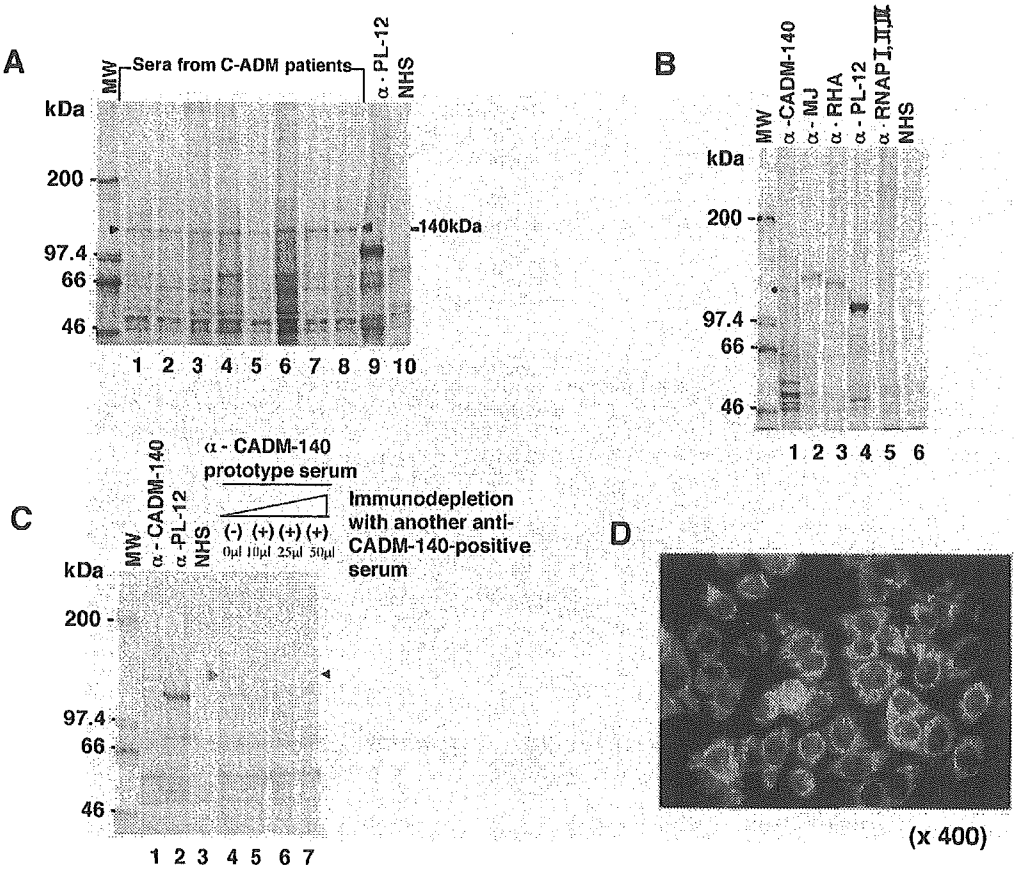

Figure 1. A, Immunoprecipitation of polypeptides with sera from patients with clinically amyopathic dermatomyositis (C-ADM), using ^{35}S-methionine–labeled K562 cell extracts. Lanes 1–8, Sera from C-ADM patients; lane 9, anti–PL-12 serum; lane 10, control normal human serum (NHS). A 140-kd protein was recognized by 8 sera from C-ADM patients (lanes 1–8). **B,** Immunoprecipitation of polypeptides by the prototype serum and by other known autoantibodies. Lane 1, The prototype anti–CADM-140 serum; lane 2, anti-MJ serum; lane 3, anti–RNA helicase A (RHA) serum; lane 4, anti–PL-12 (alanyl–transfer RNA synthetase) serum; lane 5, anti–RNA polymerase I, II, and III (RNAP I, II, and III) serum; lane 6, control NHS. Anti–CADM-140 serum immunoprecipitated an ~140-kd polypeptide that was easily distinguished from that of other known antibodies. **C,** Immunodepletion studies. Sera used for immunoprecipitation were as follows: lane 1, anti–CADM-140; lane 2, anti–PL-12; lane 3, control NHS; lanes 4–7, immunoprecipitation with anti–CADM-140 serum after absorption by another anti–CADM-140–positive serum in a dose-dependent manner. **Arrows** in **A** and **C** denote the 140-kd polypeptide. The sizes of the molecular weight markers are indicated to the left in **A–C. D,** Immunofluorescence pattern of HEp-2 cells stained with anti–CADM-140 serum. A granular or reticular cytoplasmic staining pattern on HEp-2 cells was observed. (Original magnification × 400.)

anti–PL-12 antibody (Figure 1B, lane 4). These results clearly indicate that the 140-kd polypeptide immunoprecipitated by the prototype serum was different from the proteins immunoprecipitated by these other known antibodies. We designated this new autoantibody specificity as anti–CADM-140.

The prototype serum depleted extracts of the 140-kd polypeptide in a dose-dependent manner (Figure 1C, lanes 4–7), and the polypeptide recognized by the prototype serum was no longer immunoprecipitated in these extracts (Figure 1C, lane 7). In contrast, the depletion of radiolabeled K562 cell extracts with the use of autoantibodies of different immunologic specificities did not affect the levels of the anti–CADM-140–specific antigen (results not shown). When sera positive for anti–CADM-140 antibodies were assessed in indirect IF studies, a granular or reticular cytoplasmic staining pattern was observed (Figure 1D).

Table 1. The frequencies of myositis-specific, myositis-associated, and anti–CADM-140 antibodies in patients with connective tissue diseases and IPF*

Autoantibodies	PM (n = 61)	DM (n = 42)		RA (n = 50)	SLE (n = 46)	MCTD/OL (n = 27)	Systemic sclerosis (n = 22)	Sjögren's syndrome (n = 7)	IPF (n = 43)
		Classic DM (n = 27)	C-ADM (n = 15)						
Myositis-specific									
Anti-ARS (anti-Jo-1)	10 (16)	6 (22)	0	0	0	0	0	0	0
Anti-ARS (non–anti-Jo-1)	10 (16)	2 (7)	0	0	0	1 (4)	0	0	4 (9)
Anti-SRP	5 (8)	0	0	0	0	0	0	0	0
Anti–Mi-2	0	2 (7)	0	0	0	0	0	0	0
Myositis-associated									
Anti-SSA/Ro	3 (5)	3 (11)	2 (14)	8 (16)	15 (33)	6 (22)	1 (5)	5 (71)	1 (2)
Anti–U1 RNP	2 (3)	2 (7)	0	1 (2)	18 (39)	23 (85)	2 (9)	0	0
Anti–CADM-140	0	0	8 (53)	0	0	0	0	0	0

* Values are the number (%) of patients. Anti-PM/Scl and other myositis-associated autoantibodies were not detected in any of the sera tested. PM = polymyositis; DM = dermatomyositis; C-ADM = clinically amyopathic dermatomyositis; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; MCTD/OL = mixed connective tissue disease/overlap syndrome; IPF = idiopathic pulmonary fibrosis; anti-ARS = anti–aminoacyl-transfer RNA synthetase; anti-SRP = anti–signal recognition particle.

Disease specificity of the anti–CADM-140 antibodies. The frequencies of myositis-specific antibodies, myositis-associated antibodies, and anti–CADM-140 antibodies are summarized in Table 1. Myositis-specific antibodies are found in most patients with myositis, whereas myositis-associated antibodies are frequently found in patients without myositis (12). Among the patients with CTDs or IPF, myositis-specific antibodies (33 with anti-ARS, 5 with anti-SRP, 2 with anti–Mi-2) and myositis-associated antibodies (44 with anti-SSA/Ro, 48 with anti–U1 RNP, none with anti-PM/Scl or other myositis-associated antibodies) were detected. Anti–CADM-140 autoantibodies were found in 19% of sera from patients with DM (especially in 53% with the C-ADM subtype), but were not detected in patients with other CTDs or IPF.

Clinical features of C-ADM patients with anti–CADM-140. Clinical findings were compared between DM patients (including those with C-ADM) with anti–CADM-140 autoantibodies and those without anti–CADM-140 autoantibodies (Table 2). There were no significant differences in the frequencies of skin symptoms. However, the frequency of rapidly progressive ILD was significantly increased in anti–CADM-140–positive patients compared with that in anti–CADM-140–negative patients (50% versus 6%; $P = 0.008$). No myositis-specific antibodies were found in patients with anti–CADM-140; nevertheless, there was no significant

Table 2. Comparison of clinical features in anti–CADM-140–positive versus anti–CADM-140–negative patients with dermatomyositis

Feature	Anti–CADM-140–positive (n = 8)	Anti–CADM-140–negative (n = 34)	*P*
Age at onset, mean ± SD years	44.5 ± 12.7	46.5 ± 15.7	NS
No. male/no. female	2/6	8/26	NS
Gottron's sign or papules	75	88	NS
Heliotrope rash	50	53	NS
Muscle weakness	0	76	0.02
Elevation of CK	25	74	0.03
Fever	25	50	NS
Raynaud's phenomenon	13	24	NS
Arthritis	50	71	NS
Rapidly progressive ILD	50	6	0.008
Malignancy	0	18	NS
MSAs	0	29	NS
MAAs	13	18	NS

* Except where indicated otherwise, values are the percent of patients. NS = not significant; CK = creatine kinase; ILD = interstitial lung disease; MSAs = myositis-specific autoantibodies; MAAs = myositis-associated autoantibodies.

difference in the frequency of these autoantibodies in comparison with the anti–CADM-140–negative group.

None of the 8 patients with anti–CADM-140–positive sera were treated with steroids or other immunosuppressive medications prior to being assessed for C-ADM. All of these patients had Gottron's sign or papules, or periorbital heliotrope erythema and skin biopsy specimens yielding results compatible with DM. None of these patients had muscle weakness. CK levels were in the normal range in 6 patients (75%) and slightly elevated in the remaining 2 patients. Of the 6 patients assessed for the muscle enzyme aldolase, levels were normal in 2 patients. Of the 2 patients who underwent muscle MRI, neither showed findings compatible with a diagnosis of myopathy. Four patients had a muscle biopsy, and 2 of the muscle specimens exhibited mild infiltration of inflammatory cells, but there was no evidence of necrosis of muscle fibers, variation in fiber size, regeneration, or phagocytosis. Of the 7 patients with ILD (88%), 4 developed rapidly progressive disease.

DISCUSSION

We have identified novel autoantibodies (anti–CADM-140 autoantibodies) to an ~140-kd polypeptide in patients with DM. Anti–CADM-140 antibodies were detected specifically in patients with DM, especially those with C-ADM. In addition, anti–CADM-140 antibodies were associated with rapidly progressive ILD.

It has been reported that amyopathic DM may be accompanied by rapidly progressive ILD, especially in Japanese patients and other Asian patients (7). In contrast, rapidly progressive ILD was shown to be rare in patients with amyopathic DM in a North American population (13). In our series, 5 of 15 patients with C-ADM (33%) (4 of whom had anti–CADM-140 antibodies) had rapidly progressive ILD during their clinical course. Rapidly progressive ILD was more frequent in our series compared with that reported previously in North American populations (13). Although the number of patients that we studied was very limited, it remains possible that racial differences are the reason for this discrepancy, because other clinical studies of Japanese patients also demonstrated findings similar to ours (7).

Furthermore, in a recent preliminary report, using immunoprecipitation and immunoblotting of HeLa cell extracts, Targoff et al documented the presence of antibodies to a 155-kd protein and/or Se protein in patients with C-ADM (8). Thirteen of 18 C-ADM sera possessed an anti–155-kd polypeptide antibody, and 6 also immunoprecipitated a 95-kd polypeptide (anti-Se antibody). In contrast, Oddis et al identified the anti-MJ antibody, which was also found to recognize a 140-kd polypeptide, in patients with juvenile DM (14,15). We have been able to conclude that anti–CADM-140 is distinctively different from anti-MJ, because the molecular weights of the immunoprecipitated polypeptides are different. Moreover, the clinical features of anti-MJ are quite different from those associated with anti–CADM-140. Anti-MJ is detected mainly in juvenile DM, has been observed in the US and Argentina, and is clinically characterized by severe DM with a chronic and polycyclic course, sometimes accompanied by vasculitis (14). In order to elucidate the racial differences in the frequency of these antibodies, the examination of a larger number of patients from several different populations is required.

Our results have thus demonstrated the presence of anti–CADM-140 autoantibodies in patients with C-ADM, and these were found to be associated with rapidly progressive ILD. Further studies of this novel autoantibody specificity may provide insight into the pathogenic mechanisms of C-ADM accompanied by rapidly progressive ILD.

ACKNOWLEDGMENTS

We thank Ms N. Fertig for preparing the prototype anti-MJ serum, and Ms Mutsuko Ishida for assisting in RNA immunoprecipitation assays.

REFERENCES

1. Plotz PH, Rider LG, Targoff IN, Raben N, O'Hanlon TP, Miller FW. Myositis: immunologic contributions to understanding cause, pathogenesis, and therapy. Ann Intern Med 1995;122:715–24.
2. Pearson CM. Polymyositis and dermatomyositis. In: Koopman WJ, editor. Arthritis and allied conditions. Baltimore: Williams & Wilkins; 1997. p. 742–61.
3. Hirakata M, Mimori T, Akizuki M, Craft J, Hardin JA, Homma M. Autoantibodies to small nuclear and cytoplasmic ribonucleoproteins in Japanese patients with inflammatory muscle disease. Arthritis Rheum 1992;35:449–56.
4. Targoff IN, Johnson AE, Miller FW. Antibody to signal recognition particle in polymyositis. Arthritis Rheum 1990;33:1361–70.
5. Targoff IN, Reichlin M. The association between Mi-2 antibodies and dermatomyositis. Arthritis Rheum 1985;28:796–803.
6. Sontheimer RD. Would a new name hasten the acceptance of amyopathic dermatomyositis (dermatomyositis sine myositis) as a distinctive subset within the idiopathic inflammatory dermatomyopathies spectrum of clinical illness? J Am Acad Dermatol 2002;46:626–36.
7. Tokiyama K, Tagawa H, Yokota E, Nagasawa K, Kusaba T, TsudaY, et al. Two cases of amyopathic dermatomyositis with fatal rapidly progressive interstitial pneumonitis. Ryumachi 1990;30: 204–11. In Japanese.
8. Targoff IN, Trieu EP, Sontheimer RD. Autoantibodies to 155 kD

and Se antigens in patients with clinically-amyopathic dermatomyositis [abstract]. Arthritis Rheum 2000;43 Suppl 9:S194.
9. Hirakata M, Suwa A, Nagai S, Kron MA, Trieu EP, Mimori T, et al. Anti-KS: identification of autoantibodies to asparaginyl-transfer RNA synthetase associated with interstitial lung disease. J Immunol 1999;162:2315–20.
10. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci U S A 1979;76: 4350–4.
11. Lane RJ, Emslie-Smith A, Mosquera IE, Hudgson P. Clinical, biochemical and histological responses to treatment in polymyositis: a prospective study. J R Soc Med 1989;82:333–8.
12. Targoff IN. Laboratory testing in the diagnosis and management of idiopathic inflammatory myopathies. Rheum Dis Clin North Am 2002;28:859–90.
13. Euwer RL, Sontheimer RD. Amyopathic dermatomyositis: a review. J Invest Dermatol 1993;100:124S–7S.
14. Oddis CV, Fertig N, Goel A, Espada G, Confalone Gregorian M, Maldonado Cocco JA, et al. Clinical and serological characterization of the anti-MJ antibody in childhood myositis [abstract]. Arthritis Rheum 1997;40 Suppl 9:S139.
15. Espada G, Confalone Gregorian M, Ortiz Z, Fertig N, Londino AV, Oddis CV, et al. Serum autoantibodies in juvenile idiopathic inflammatory myopathies (IIM) in a cohort of Argentine patients [abstract]. Arthritis Rheum 1997;40 Suppl 9:S140.

Int. J. Cancer: **116,** 925–933 (2005)

Immune responses to DNA mismatch repair enzymes hMSH2 and hPMS1 in patients with pancreatic cancer, dermatomyositis and polymyositis

Takaho Okada[1,2], Shinobu Noji[1], Yasufumi Goto[1], Takashi Iwata[1], Tomonobu Fujita[1], Tsutomu Okada[1], Yuriko Matsuzaki[1], Masataka Kuwana[1,3], Michito Hirakata[3], Akira Horii[4], Seiki Matsuno[2], Makoto Sunamura[2] and Yutaka Kawakami[1]*

[1]*Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan*
[2]*Department of Surgery, Division of Gastroenterological Surgery, Tohoku University Graduate School of Medical Science, Sendai, Japan*
[3]*Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan*
[4]*Department of Pathology, Division of Molecular Pathology, Tohoku University Graduate School of Medical Science, Sendai, Japan*

To identify tumor antigens useful for diagnosis and immunotherapy of patients with pancreatic ductal adenocarcinoma, we applied a SEREX approach with a cDNA library made from 5 pancreatic cancer cell lines and sera obtained from 8 patients with pancreatic cancer, and isolated total 32 genes, including 14 previously characterized genes and 18 genes with unknown functions. Among these isolated antigens, serum IgG antibodies for 2 isolated DNA mismatch repair enzymes, Homo sapiens *mutS* homolog 2 (*hMSH2*) and Homo sapiens postmeiotic segregation increased 1 (*hPMS1*), were detected in patients with pancreatic ductal adenocarcinoma and dermatomyositis (DM), and polymyositis (PM), but not in sera from healthy individuals. Immunohistochemical study demonstrated that hMSH2 and hPMS1 were over-expressed in pancreatic ductal adenocarcinoma compared to normal pancreatic ducts. These results suggested that hMSH2 and hPMS1 may be useful as CD4+ helper T cell antigens for immunotherapy of pancreatic cancer patients and that serum IgG antibodies may be useful for diagnosis of patients with pancreatic ductal adenocarcinoma and DM/PM.

Key words: SEREX; tumor antigens; pancreatic cancer; DNA mismatch repair enzyme

Early diagnosis and curative treatment are still difficult for pancreatic ductal adenocarcinoma despite of increased incidence rate.[1] New diagnostic and therapeutic methods, including new chemotherapy, intra-operative irradiation and cytology of pancreatic juice for detection of minimally infiltrated carcinoma *in situ*, are being applied. However, local recurrences or remote metastases, particularly in liver, often result in poor prognosis even in the patients for whom curative resection was performed.[2,3] Immunotherapy may be one of the additional therapeutic modalities for patients with pancreatic cancer since immunotherapies previously conducted on the patients with pancreatic cancer appeared to demonstrate some anti-tumor effects. However, it has not been easy to improve the immunotherapies, since mechanisms for the immunological tumor rejection has not been analyzed well in patients with pancreatic cancer partly due to the limited number of identified pancreatic cancer antigens, including MUC-1, K-ras, Her2/neu and p53.

Human tumor antigens recognized by T cells have recently been identified using various methods, including cDNA expression cloning with tumor reactive T cells[4–6] or patients' serum IgG antibodies (Ab) and various systematic genetic analysis along with currently well established gene databases. Since generation of tumor reactive T cells are relatively difficult in many cancers in the exception of melanoma, we have applied cDNA expression cloning with sera from cancer patients, called SEREX (serological identification of tumor antigens by cDNA expression cloning), for the identification of tumor antigens in various cancers including melanoma,[7] glioma,[8] colon cancer[9] and bladder cancer.[10]

In our study, we isolated tumor antigens using SEREX with sera from patients with pancreatic ductal adenocarcinoma. Among the identified antigens, 2 DNA mismatch repair enzymes, hMSH2 and hPMS1, were found to over-express in pancreatic ductal adenocarcinoma by immunohistochemical analysis, and their antibodies were detected in sera from patients with various cancers, particularly with pancreatic ductal adenocarcinoma, and in sera from patients with dermatomyositis/polymyositis (DM/PM), but not sera from healthy individuals. Therefore, hMSH2 and hPMS1 are immunogenic antigens in multiple patients with pancreatic ductal adenocarcinoma, indicating that they may be useful for immunotherapy of pancreatic cancer. Furthermore, serum Ab may be useful for diagnosis of pancreatic cancer and DM/PM.

Material and methods

Cell lines and tissues

Pancreatic ductal adenocarcinoma cell lines, PK1, PK8, PK9, PK45P and PK59, were established in Tohoku University, cultured in RPMI1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 6 μg/l epidermal growth factor, 150 units/l insulin, 0.5 mg/l hydrocortisone, 10 mg/l transferrin, 100 IU/ml penicillin and 100 μg/ml streptomycin. Pancreatic ductal adenocarcinoma cell lines, Panc-1, MIAPaCa-2, AsPC-1 and BxPC-3, and colon cancer cell lines, Colo201, DLD-1 and Nota, were cultured in 10%FBS RPMI1640. Colon carcinoma cell lines, Colo320, SW837 and LoVo, were cultured in 10%FBS DMEM. Tumor specimens were obtained from patients who had undergone surgical resection at Tohoku university hospital or Keio university hospital with informed consent according to institution guidelines. These specimens were stored in −80°C.

Profiles of patients with pancreatic ductal adenocarcinoma used for immunoscreening of the cDNA library and of patients with DM/PM

Sera from 8 patients with advanced pancreatic ductal adenocarcinoma patients (Table I) admitted in Tohoku University hospital or Keio University hospital were used for immunoscreening. Sera from patients with 119 DM/PM, 10 rheumatoid arthritis, 10 systemic lupus erythematodes (SLE), 10 sclerosis, 10 tuberculosis and 2 brain abscesses in Keio University hospital were used for serum screening.

Grant sponsor: Ministry of Education, Science, Sport and Culture of Japan; Grant numbers: 10470264, 12557109, 14104013, 14571226; Grant sponsor: Ministry of Health and Welfare, Japan; Grant sponsor: Keio University; Grant sponsor: Keio Gijuku Academic Development Funds.
***Correspondence to:** Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: 81-3-5362-9259. E-mail: yutakawa@sc.itc.keio.ac.jp
Received 15 September 2004; Accepted after revision 14 February 2005
DOI 10.1002/ijc.21118
Published online 26 April 2005 in Wiley InterScience (www.interscience.wiley.com).

Publication of the International Union Against Cancer

TABLE I - PROFILES OF THE PATIENTS AND NUMBERS OF THE ISOLATED CLONES BY SEREX

Patient	Age	Sex	Clinical stage	Pathological feature	Number of screened clones	Positive clones	Number of total antigens	Known antigens	Uncharacterized antigens
PA1	70	F	IVb	Moderately differentiated adenocarcinoma					
PA2	66	F	IVb	Poorly differentiated adenocarcinoma	7.0×10^5	18	11	10	1
PA3	61	M	IVb	N.D.					
PA4	54	F	III	Adenosquamous carcinoma					
PA5	64	F	IVb	Moderately differentiated adenocarcinoma					
PA6	49	M	III	Intraductal-papillary mucinous carcinoma	6.4×10^5	12	7	4	3
PA7	63	M	III	Moderately differentiated adenocarcinoma					
PA8	83	M	IVb	N.D.					
Total					1.3×10^6	30	18	14	4

Construction of the cDNA library from human pancreatic ductal adenocarcinoma cell lines and immunoscreening with patients' sera (SEREX)

Total RNA was extracted from human pancreatic ductal adenocarcinoma cell line PK1, PK8, PK9, PK45P and PK59, by cesium gradient ultracentrifugation. Poly(A)+ RNA was purified twice from a mixture of 5 pancreatic cancer total RNA with latex beads coated with oligo-dT (Oligotex-dT 30 super mRNA Purification kit; Takara, Kyoto, Japan). A lambda phage cDNA library was constructed using lambda ZAP II (Stratagene, La Jolla, CA) as previously described.[7] The primary size of the library was 3.1×10^6 pfu. For immunoscreening, 2 mixtures of sera from 4 patients with pancreatic ductal adenocarcinoma were diluted 1:15 in 5% skim milk supplemented Tris-buffered saline (TBS) with 0.01% sodium azide. To remove anti-*E.coli* and anti-lambda phage antibodies, they were first absorbed with *E. coli* lysates overnight and then the 1:100 diluted solution in 5% skim milk TBS was absorbed with no insert lambda phages on nitrocellulose membrane. The cDNA library was screened with the serum mixtures as previously described.[11] Briefly, cDNA library was plated at the 1.5×10^4/dish and transferred to nitrocellulose membranes with IPTG. The membranes were incubated with the serum mixtures and then clones recognized by serum IgG Ab were detected by enzymatic dye reaction with alkaline phosphatase conjugated anti-human IgG-Fc antibody. Positive clones were further purified by additional immunoscreening. The isolated cDNAs were amplified by PCR with T3 sense and T7 antisense primers and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit and ABI Prism 310 or 3100 automated sequencer (Foster City, CA). The isolated cDNAs were analyzed by comparing genetic databases.

Real-time RT-PCR and Northern blot analysis

Total RNA was extracted from various cell lines and pancreatic ductal adenocarcinoma tissues by cesium gradient ultracentrifugation. Total RNAs of various normal tissues were purchased from CLONTEC (Palo Alto, CA), BioChain Institute (Hayward, CA), Cell Applications (SanDiego, CA), CHEMICON International (Temecula, CA) and Stratagene. cDNAs were synthesized from total RNA by reverse transcriptase (M-MLV Reverse Transcriptase, Promega Corp., Madison, WI). The mRNAs for hMSH2 and hPMS1 were quantitatively measured by TaqMan real-time RT-PCR with the specific probes (Hs00179857 and Hs00153333) and the TaqMan Universal PCR Master Mix Kit (Applied Biosystems) and the ABI Prism 7900 Sequence Detection System. GAPDH was used as an internal control and amplified in a same tube to normalize variance of the input RNA. The level of target mRNA in various samples was estimated by the 2[-Delta Delta C(T)] relative quantification method. A ratio of the mRNA in various samples to that of colon tissue was determined.

Northern blot analysis was performed using 10 μg of total RNAs as previously described.[7] Briefly, total RNA was electrophoresed in a 1.0% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-XL; Amersham Biosciences, Piscataway, NJ). The membranes were incubated in QuickHyb Hybridization Solution (Stratagene) with P-32 labeled cDNA probes prepared using the High Prime DNA Labeling kit (Boehringer Mannheim), washed in 0.1% SDS 0.1×SSC solution and radioactivity was detected with BAS-2500 or 5000 (Fujifilm, Tokyo, Japan).

Immunohistochemistry

Three micrometer thick slices were prepared from formalin-fixed and paraffin-embedded tissues, deparaffinized in xylene and soaked in 100% ethanol. The slides were processed in 0.3% hydrogen peroxide aqua/99% methanol for 30 min to block the endogenous peroxidase and dipped into 10 mM citrate buffer, and heat-treated at 120°C for 10 min for antigen retrieval. Blocking was performed by incubation with 10% mouse serum or rabbit serum for 30 min. The tissues were then treated with 200-fold diluted anti-human MSH2 monoclonal antibody G219-1129 (PharMingen, San Diego, CA) or 1,000-fold diluted anti-human PMS1 polyclonal antibody sc-615 (Santa Cruz, Santa Cruz) overnight at 4°C. The tissues were reacted with the secondary antibody, either Histofine Simple Stain PO (M) or (R) (Nichirei, Tokyo, Japan), at room temperature for 30 min. Detection was performed by dipping into 150 ml of 10 mM DAB (WAKO, Osaka, Japan). Sections were counterstained with methylgreen.

In vitro *transcription/translation of hPMS1 and immunoprecipitation assay*

In vitro transcription/translation was performed using Single Tube Protein System 3, T7 (Takara Shuzo). Briefly, isolated *hPMS1* cDNA clone was amplified by 30 cycles of PCR using the Pyrobest kit (Takara Shuzo) with the sense primer (5′-AATGAAA-CAATTGCCTGCGGC-3′) and the antisense primer (5′-TGCTGT-TTTATGACAGAACCA-3′). It was mixed with the PCR products and incubated with End Conversion mix at 22°C for 5 min and then incubated with 4 U of T4 DNA ligase, pT7 Blue-2 Blunt Vector at 22°C for 1 hr. This cDNA template was amplified by 30 cycles of PCR using Ex Taq kit (Takara Shuzo) with the R20mer (5′-CAGC-TATGACCATGATTACG-3′) and the *hPMS1*-antisense primer. Amplified cDNA template was used for *in vitro* transcription and translation reaction with the S-35-labeled methionine. Translated proteins were separated and identified in 10% SDS-PAGE. Radioisotope was detected in BAS 2500 or 5000 (Fujifilm).

Immunoprecipitation was performed with the *in vitro* translated S-35-labeled hPMS1 protein. Ten microliters of sera was mixed with 2 mg of Protein A- Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ) in TBS buffer (10 mM Tris-HCl, pH 7.4, and 140 mM NaCl) and incubated overnight at 4°C. Sepharose absorbed in IgG was washed in immunoprecipitation (IPP) buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl and 0.1%Nonidet P-40) 3 times. Absorbed Sepharose was mixed with 5 μl of the *in vitro* translated protein and incubated at 4°C for 2 hr. The Sepharose was washed by IPP buffer 5 times, dissolved in 1×SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and heat-treated for 100°C

TABLE II – ISOLATED ANTIGENS BY SEREX WITH SERA FROM PATIENTS WITH PANCREATIC CANCER

Clone name	Number of isolated clone	Length of isolated clone (kbp)	Identity	Accession Number	Chromosomal locarization
f1	4	3.2	hPMS1	BF210947	Ch.2
f2	2	2.2	hMSH2	AU131598	Ch.2
f3	2	2.5	SOX13	AI014340	Ch.1
f4	2	1.4	HRY	AL552433	Ch.3
f5	2	5	KIAA0580	AU140056	Ch.4
f6	1	2.1	Translocase of inner mitochondrial membrane (TIM) 44	AU121643	Ch.19
f7	1	1.5	Galectin 9	BF347161	Ch.17
f8	1	2.5	CTCL tumor antigen se2-2	AF176816	Ch.12
f9	1	1.3	Mitochondrial ribosomal protein (MRP) L12	BG104344	Ch.17
f10	1	1.2	HS1 binding protein (HAX1)	BF212858	Ch.1
f11	1	1.6	ZNF 207	BE617467	Ch.17
s1	5	1.5	hnRNP methyltransferase (HMT)1	AU131177	Ch.19
s2	2	2.3	IMAGE: 3480396 3	BF059745	Ch.22
s3	1	2	Kinectin 1	BE816123	Ch.14
s4	1	2.3	Chromosome22 clone RP-43L2	AI814302	Ch.22
s5	1	3.2	Hypothetical protein DKFZp761 D1823	AI089375	Ch.9
s6	1	2.3	RUNX2	BF059745	Ch.6
s7	1	2.5	p53	BF342477	Ch.17

TABLE III – FREQUENCY OF SERUM IgG Ab SPECIFIC FOR THE SEREX IDENTIFIED ANTIGENS

	Healty individuals (n = 34)	Pancreatic cancer (n = 37)	Renal cell cancer (n = 8)	Esophageal cancer (n = 10)	Melanoma (n = 10)	Prostate cancer (n = 10)	Bladder cancer (n = 10)	Colon cancer		Endometrial cancer (n = 6)	Pancreatitis (n = 7)
								MSI (+) (n = 10)	MSI (−) (n = 7)		
hMSH2	0	5	1	0	0	1	0	0	0	1	0
hPMS1	0	3	1	0	0	1	0	0	0	0	0
HRY	0	3	0	1	0	1	0	0	0	0	0
SOX13	0	2	0	0	0	0	0	0	0	1	0
MRPL12	0	2	0	0	1	1	0	0	0	0	0
HMT1	0	2	0	0	0	2	0	0	0	0	0
HAX1	0	1	0	1	0	0	0	0	0	0	0
ZNF207	0	1	0	0	0	1	0	0	0	0	0
RP-43L2	0	1	0	0	1	0	0	0	0	1	0
KTN1	4	8	0	0	0	5	2	0	2	1	3
IMAGE:3480396 3′	4	5	1	1	0	1	0	2	0	0	0
KIAA0580	2	4	0	2	1	1	0	1	0	1	0
RUNX2	4	4	3	1	0	2	0	1	0	0	0
TIM44	1	2	0	0	0	1	1	1	0	1	0
p53	1	2	0	2	0	0	0	0	0	0	0

for 5 min. The products were separated by 10% SDS-PAGE, sensitized in 0.5 M sodium salicylate solution for 10 min and dried with a gel dryer. The radioisotope was detected in BAS 2500 or 5000 (Fujifilm).

Preparation of recombinant hMSH2 protein and Western blot analysis

The *hMSH2* cDNA was amplified by PCR with primers containing *Bam*H1(5′) and *Not*1(3′) sites (5′-GGATCCATGGCGGTGCAGCCGAAGGA-3′ and 5′-GCGGCCGCTCACGTAGTAACTTTTATTC-3′) from the lambda phage isolated by SEREX. The PCR products were digested with *Bam*H1 and *Not*1 (TAKARA), and ligated into the expression plasmid, pET16b. The recombinant protein was expressed in *E.coli AD494(DE3)pLys S* (Novagen) with IPTG induction, and purified by Ni^{2+} affinity chromatography, Hi Trap Chelating (Amersham Biosciences).

Western blot analysis was performed as previously described.[9] Briefly, 2.5 μg of recombinant hMSH2 protein was electrophoresed on 8.5% polyacrylamide-SDS gel and transferred onto nitrocellulose membrane. The membrane was blocked by 5% nonfat milk and incubated with sera at 1:100 dilution or mouse anti-His antibody (Amersham Biosciences) at 1:3,000 dilution and then reacted with alkalinephosphatase-conjugated goat anti-human IgG antibodies or goat anti-mouse IgG antibodies at 1:3,000 dilution. The Ab reacted bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tertazorium.

Statistical analysis

Fisher's exact test was used to evaluate correlation between complication of cancer and serum anti-hPMS1 or hMSH2 antibody in DM/PM patients.

Results

Isolation of tumor antigens by cDNA expression cloning with IgG Ab in sera from 8 patients with pancreatic ductal adenocarcinoma

A total of 1.3×10^6 clones of the cDNA library made from 5 pancreatic ductal adenocarcinoma cell lines, PK1, PK8, PK9, PK45P and PK59, were screened with 2 mixtures of sera from 4 patients (total 8 patients) with pancreatic ductal adenocarcinoma. A total of 30 cDNA clones representing 18 genes (f1–f11 with the first serum mixture and s1–s7 with the second serum mixture) was isolated (Table I). These represented 14 previously characterized and 4 uncharacterized genes. Some antigens were isolated more than 2 times. Five arginine methyltransferase *HMT1*, 4 DNA mismatch repair enzyme *hPMS1* and 2 DNA mismatch repair enzyme *hMSH2* were isolated (Table II). These isolated antigens include molecules possibly related to formation of malignant phenotypes, including

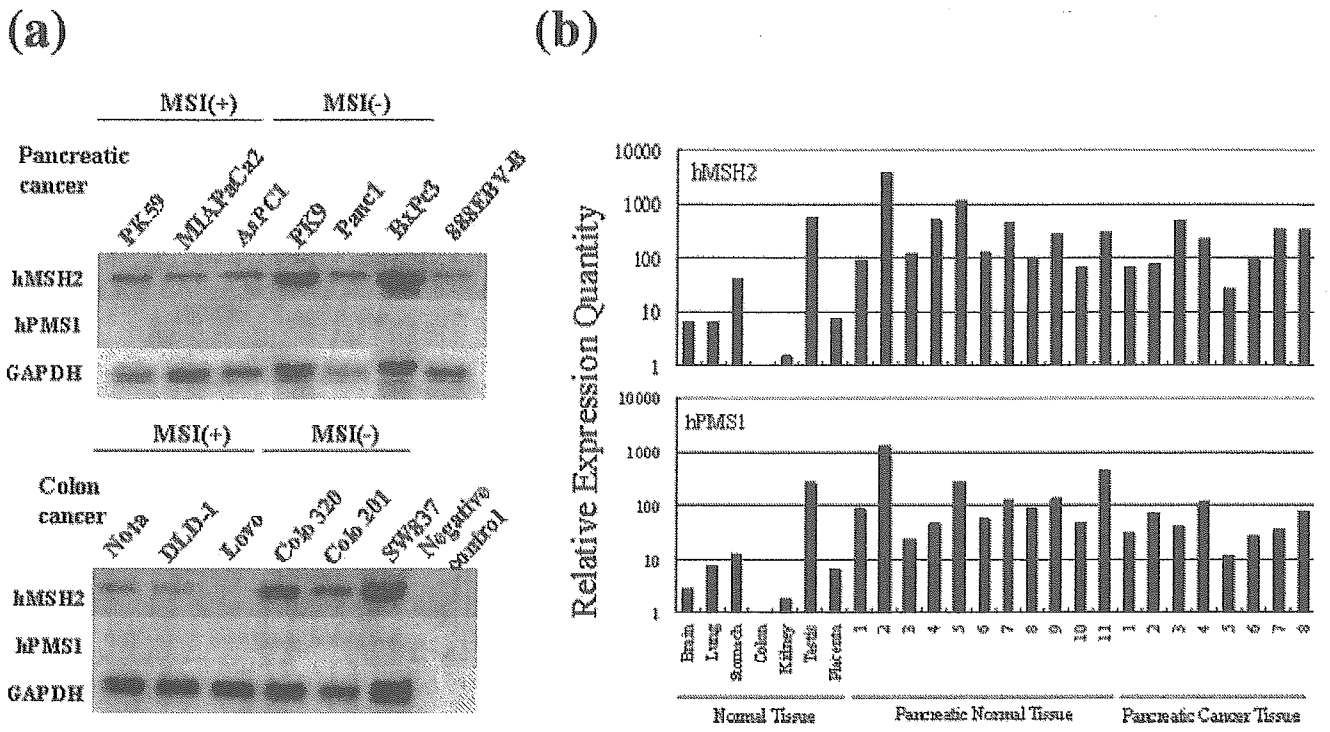

FIGURE 1 – mRNA expression of hMSH2 and hPMS1. (*a*) High expression of hMSH2 and hPMS1 in MSI negative cancer cell lines. Northern blot analysis was performed for hMSH2 and hPMS1 in MSI positive and negative cancer cell lines. Both hMSH2 and hPMS1 showed lower expression in MSI positive pancreatic ductal adenocarcinoma cell lines (PK59, MIAPaCa2 and AsPC1) and colon cancer cell lines (Nota and DLD1) than MSI negative pancreatic ductal adenocarcinoma cell lines (PK9, Panc1 and BxPC3) and colon cancer cell lines (Colo320, Colo201 and SW837). Colon cancer cell line LoVo with homozygous hMSH2 gene deletion only expressed hPMS1. (*b*) The expression of hMSH2 and hPMS1 in various normal tissues and pancreatic cancer tissues. The mRNAs for hPMS1 and hMSH2 were quantitatively measured in various normal tissues, including brain, kidney, lung, colon, stomach, testis, placenta and 11 normal pancreas tissues, and 8 pancreatic cancer tissues using TaqMan real-time RT-PCR. GAPDH was used as an internal control. The mRNAs in various samples were estimated by the 2[-Delta Delta C(T)] relative quantification method. A ratio of the mRNA of various samples to that of colon was shown.

transcription factor *SOX13*, oncogene *p53*, methyltransferase *HMT1* and DNA mismatch repair enzymes *hMSH2* and *hPMS1*.

Screening of IgG Ab reacting to the SEREX identified antigens for evaluation of their immunogenicity in sera from patients with various cancers and healthy individuals

These 18 antigens were screened for evaluation of their immunogenicity using sera from patients with various cancers and healthy individuals. To identify target antigens involved in antitumor immune responses, we selected antigens for which immune responses was raised preferentially in cancer patients. By screening each isolated antigen with serum IgG Ab from 34 healthy individuals, we first selected 15 antigens which reacted with less than 5 healthy donor sera and further evaluated their immunogenicity with sera from patients with various cancers, including 37 pancreatic ductal adenocarcinomas, 8 renal cell cancers, 10 esophageal cancers, 10 melanomas, 10 prostate cancers, 10 bladder cancers, 17 colorectal cancers (10 with MSI+ and 7 with MSI- cancer) and 6 endometrial cancers. Sera from 7 patients with acute pancreatitis were also screened to exclude possible immune response to normal pancreas (Table III).

IgG Ab specific for hMSH2, hPMS1, HRY (hairy Drosophila-homolog), SOX13 (sex determining region Y-box 13), MRPL12 (mitochondrial ribosomal protein L12), HMT1, HAX1 (HS1 binding protein), ZNF207 (zinc finger protein 207) and RP-43L2 were detected in sera from the patients with pancreatic ductal adenocarcinoma and other cancers but not detected in any sera from 34 healthy individuals or 7 patients with pancreatitis. Among these 9 antigens reacted with only sera from cancer patients, serum IgG Abs specific for hMSH2 or hPMS1 were detected in 5 of 37 (13.5%) or 3 of 37 (8.1%) patients with pancreatic ductal adenocarcinoma, respectively, and in 3 of 71 or 2 of 71 patients with other cancers, respectively.

Expression of the SEREX identified antigens in various normal tissues and cancers evaluated by RT-PCR and Northern blot analysis

Tissue specific expression of these 9 antigens for which IgG Ab were detected in sera from patients with various cancers but not in sera from healthy individuals were first evaluated by RT-PCR analysis on various normal tissues, primary cultured normal cells, cancer cell lines and pancreatic ductal adenocarcinoma tissues, and these antigens were found to express ubiquitously in various normal tissues and cancers (data not shown). However, by Northern blot analysis, *hMSH2* and *hPMS1* were found to express higher in most cancer cell lines tested, including PK9 pancreatic cancer, 526mel melanoma, KIS lung cancer, TE8 esophageal cancer, KU7 bladder cancer, MDA231 breast cancer and RCC6 renal cancer cell lines, than in various normal tissues, suggesting over-expression of *hMSH2* and *hPMS1* in various cancer cells (data not shown). Interestingly, when compared expression of these genes between MSI positive and MSI negative cancer cell lines in pancreatic ductal adenocarcinoma and colon cancer, *hMSH2* and *hPMS1* appeared to express higher in the MSI negative cancer cell lines than in MSI positive cancer cell lines, although the expression of *hPMS1* was lower than *hMSH2* (Fig. 1*a*). The colon cancer cell line LoVo with homozygous deletion of *hMSH2* expressed only *hPMS1*. Although these results may suggest possible involvement of these DNA mismatch repair enzymes in MSI status, and possible higher immune response to these enzymes in patients with MSI negative cancers, we could not detect the specific serum Ab in any colon cancer

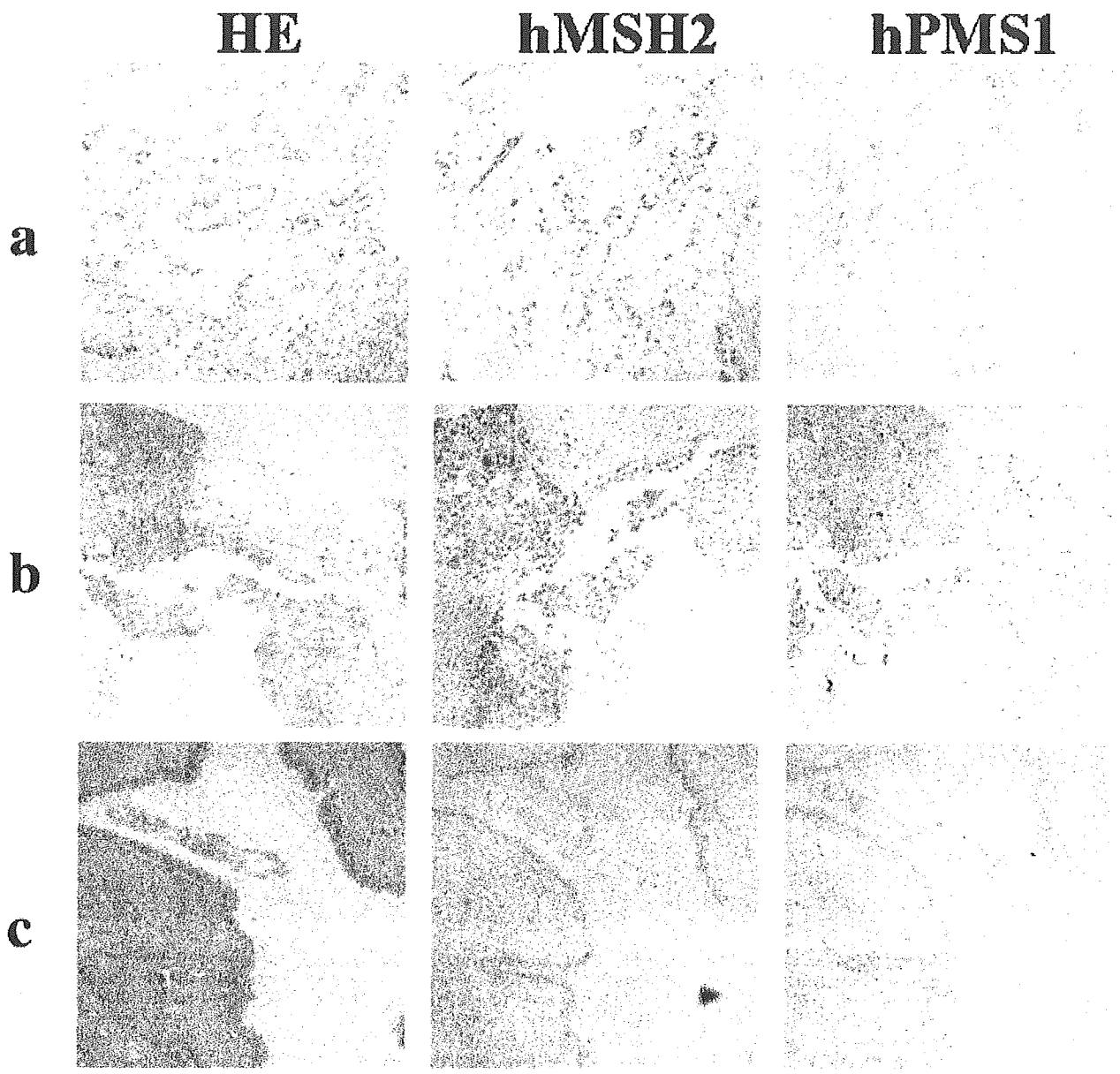

FIGURE 2 – Overexpression of the hMSH2 and hPMS1 proteins in pancreatic cancer. Immunohistochemical study was performed for hMSH2 and hPMS1 on pancreatic ductal adenocarcinoma and normal pancreas tissues. One representative pancreatic ductal adenocarcinoma (*a*) obtained from a patient with positive serum IgG Ab for both hMSH2 and hPMS1, and 1 representative pancreatic ductal adenocarcinoma (*b*) from a patient without the specific Ab was shown. One representative normal pancreas tissue (*c*) from a patient without the specific Ab was shown. The nucleus and cytoplasm of the pancreatic ductal adenocarcinoma cells were strongly stained for hMSH2 and hPMS1. No significant difference of the intensity was found between tumor cells from patients with or without serum anti-hMSH2 and hPMS1 Ab. Epithelial cells of the normal pancreatic ducts were weakly stained.

patients tested, and MSI status was unknown in the pancreatic cancer patients whose serum Ab were evaluated for their reactivity.

We then evaluated the expression of *hMSH2* and *hPMS1* in 8 pancreas cancer tissues and various normal tissues including 11 pancreas tissues using quantitative real time PCR. Among normal tissues, relatively high expression was observed in testis and pancreas. Although pancreatic cancer tissues expressed these 2 genes at relatively high amounts, no difference was observed between pancreatic cancer and normal pancreas (Fig. 1*b*). We also evaluated expression of these 2 genes using publicly available gene data bases. In the Unigene database, *hMSH2* and *hPMS1* were found in cDNA libraries from various normal tissues, including brain, lung, heart, stomach, colon, liver, kidney, testis, skin, muscle, blood, bone marrow and so on, but expression is low (227 *hMSH2* sequences and 243 *hPMS1* sequences of total 5×10^6 registered sequences). In the SAGE databases, the expression of *hMSH2* and *hPMS1* was also low in various normal tissues including pancreas. No significant difference in the expression of these 2 genes was observed between normal pancreas and pancreatic cancer, although upregulation of both genes was found in breast cancer. These results indicated that mRNA for *hMSH2* and *hPMS1* may not be overexpressed in pancreatic cancer.

Over-expression of hMSH2 and hPMS1 proteins in pancreatic ductal adenocarcinoma by immunohistochemical analysis

Since mRNAs evaluated by real time PCR analysis were obtained from bulk pancreatic cancer tissues that contained lots of noncancerous stromal cells, we then performed immunohistochemical study to

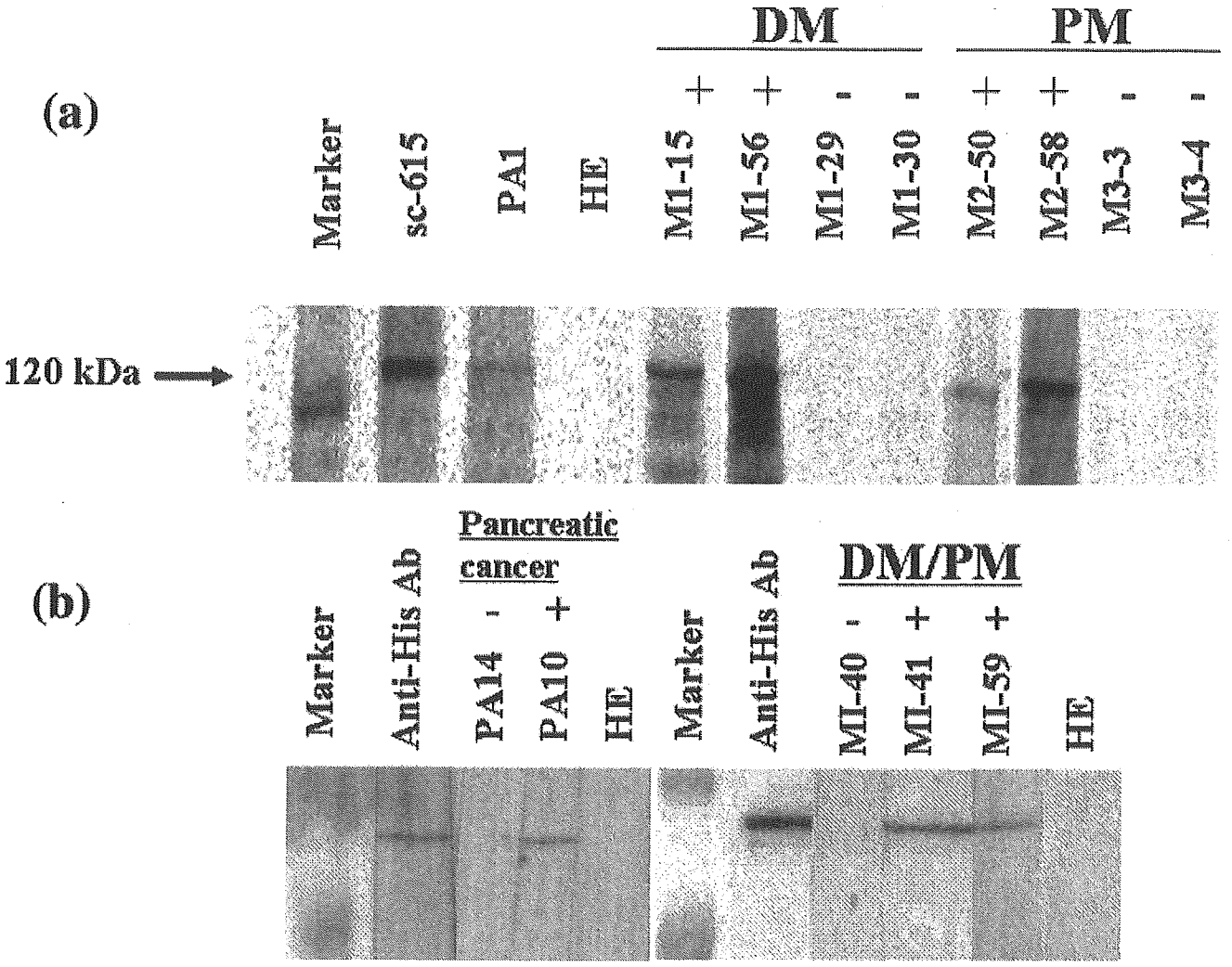

FIGURE 3 – Presence of anti-hMSH2 or hPMS1 antibody in sera from patients with DM/PM. (*a*) Anti-hPMS1 Ab was detected by immunoprecipitation in some of the patients with pancreatic cancer or DM/PM. The hPMS1 protein produced by *in vitro* transcription and translation was immunoprecipitated with anti-hPMS1 rabbit polyclonal Ab sc-615, and sera from a healthy individual (HE), a pancreatic cancer patient (PA1) and DM/PM patients (M1-15, M1-29, M1-30, M1-56, M2-50, M2-58, M3-3 and M3-4). This figure shows 1 of the representative results. The DM patient M1-15 had breast cancer. +, positive bands, −, negative bands. (*b*) Anti-hMSH2 Ab was detected by Western blot analysis in some of the patients with pancreatic cancer or DM/PM. The Western blot analysis was performed with the bacterial recombinant hMSH2 protein, anti-His tag Ab and sera from a healthy individual (HE), patients with pancreatic cancer (PA10 and PA14) and DM/PM (M1-40, M1-41 and M1-59). This figure shows 2 representative experiments. +, positive bands, −, negative bands.

TABLE IV – NO CORRELATION BETWEEN SERUM ANTI-hPMS1 AND hMSH2 IgG Ab AND CANCER COMPLICATION IN DM/PM PATIENTS

	Myositis antibody		Cancer +	Cancer −
hPMS1	PM	+	0	5
		−	0	63
	DM	+	1	2
		−	2	46
hMSH2	PM	+	0	3
		−	0	59
	DM	+	0	2
		−	2	36

evaluate the hMSH2 and hPMS1 protein in cancer cells at a single cell level. Five pancreatic ductal adenocarcinoma specimens obtained from patients who had anti-hMSH2 serum Ab, 3 specimens from patients with anti-hPMS1 serum Ab and 6 specimens from patients without either Ab were stained with anti-hMSH2 mouse monoclonal Ab or anti-hPMS1 rabbit polyclonal Ab. Although anti-hMSH2 and anti-hPMS1 Ab stained nucleus of normal pancreatic duct cells, normal pancreas acinar cells, Langerhans cells and stromal cells, the intensity of the nuclear and cytoplasmic staining was stronger in pancreatic ductal adenocarcinoma cells, indicating over-expression of the hMSH2 and hPMS1 proteins in pancreatic ductal adenocarcinoma cells [Fig. 2, 1 representative example of pancreatic cancer tissue (*a*) from a patient who had serum IgG Ab for both hMSH2 and hPMS1, and 1 representative example of pancreatic cancer tissue from a patient without the specific serum Ab (*b*) and normal pancreas tissue (*c*)]. No different intensity of the staining of pancreatic ductal adenocarcinoma was observed between the specimens from patients with or without the anti-hMSH2 and anti- hPMS1 Abs.

Detection of anti-hPMS1 and anti-hMSH2 Ab in sera from patients with dermatomyositis and polymyositis

Anti-hPMS1 Ab was previously reported as a specific autoantibody detected in approximately 7.5% (4/53) of patients with

dermatomyositis and polymyositis (DM/PM), and it was not detected among patients with systemic lupus erythematodes or sclerderma.[12] Therefore, we evaluated anti-hPMS1 and anti-hMSH2 Ab in sera from DM/PM patients. More than 100 sera from the DM/PM patients were screened for anti-hPMS1 serum Ab by a convenient rapid assay using immunoprecipitation with the S-35 methionine-labeled hPMS1 protein produced by *in vitro* transcription and translation (Fig. 3*a*). Anti-hMSH2 serum antibody was screened using Western blot analysis with the bacterial recombinant hMSH2 protein (Fig. 3*b*) because the hMSH2 protein was not well produced by *in vitro* translation. Anti-hPMS1 antibody was detected in sera from 8 of 119 (6.7%) DM/PM patients with similar frequency to the previous report.[12] Similarly, anti-hMSH2 Ab was detected in sera from 5 of 102 (4.9%) DM/PM patients. The anti-hMSH2 Ab was not detected in sera from patients with other collagen diseases, including 10 rheumatoid arthritis, 10 systemic lupus erythematodes, 10 sclerosis or patients with infectious diseases, including 10 tuberculosis and 2 brain abscesses (data not shown), indicating that anti-hMSH2 Ab may also be a specific autoantibody for DM/PM patients. Since PM/DM patients, particularly DM patients, are known to have complications of various cancers including pancreatic cancer, we have examined possible correlation between cancer complication and serum Ab specific for hPMS1/hMSH2. Although 1 DM patient with positive anti-hPMS1 serum Ab complicated with breast cancer, no significant correlation was found between cancer complication and anti-hPMS1 or anti-hMSH2 serum Ab in our analysis with more than 100 DM/PM patients (Table IV). We also examined other myositis specific autoantibodies, including Ab against aminoacyl tRNA synthetases and signal recognition particle (SRP) in all cancer patients with positive serum anti-hPMS1 Ab, but none of them were positive in the cancer patients (data not shown). Therefore, anti-hPMS1 and anti- hMSH2 Ab appeared to be raised in the DM/PM patients independent of cancer development. These results suggest that serum Ab specific for hMSH2 and hPMS1 may be useful for diagnosis of pancreatic cancer and DM/PM.

Discussion

Since pancreatic cancer is one of the problematic cancers for early diagnosis and treatment with conventional therapeutics, new diagnostic and therapeutic methods need to be developed. Clinical trials of immunotherapies attempted on pancreatic cancers demonstrated some anti-tumor effects. Intradermal immunization with the mutated K-ras peptide with GM-CSF resulted in the induction of memory CD4+ T cell response and prolonged survival in the T cell responders compared to the nonresponders.[13,14] Vaccination with GM-CSF transduced allogeneic pancreatic cancer cell lines with adjuvant radiation and chemotherapy following surgical excision demonstrated possible benefit in disease free survival, which appeared to be associated with the increase of postvaccination DTH responses against autologous tumor cells.[15] Therefore, immunotherapy may be one of the additional therapeutic modalities for patients with pancreatic cancer. However, the mechanism for immunological tumor regression has not been well evaluated and improvement of the immunotherapy has been difficult partly because only a limited number of tumor antigens have so far been identified for pancreatic cancer.[16,17] Furthermore, additional tumor markers are required for better diagnosis of pancreatic cancer. Thus, identification of additional antigens is important for development of immunotherapy and diagnostic methods for patients with pancreatic cancer.

In our study, we have isolated tumor antigens by SEREX using the cDNA library made from 5 pancreatic ductal adenocarcinoma cell lines and 8 allogeneic sera from patients with pancreatic ductal adenocarcinoma. These identified antigens included interesting molecules possibly related to cancer phenotypes, such as transcription factor *SOX13*, oncogene *p53*, methyltransferase *HMT1* and DNA mismatch repair enzyme *hMSH2* and *hPMS1*. *SOX13* was recognized by sera from patients with 2 of 37 pancreatic cancers and 1 of 6 endometrial cancers but not recognized by any sera from 34 healthy individuals. *SOX13* is a member of the *SOX* D group that also includes *SOX5* and *SOX6*. We have previously reported that SOX5 and SOX6 were specifically over-expressed in primary brain tumors, and the HMG box regions in SOX 5 and SOX6 were frequently recognized by sera from brain tumor patients.[8] Thus, SOX13 may be expressed in some of the pancreatic cancers and recognized by IgG Ab. Alternatively, antibody raised against SOX5 or SOX6 might cross-react SOX13 because the HMG box has high homology among these *SOX* D group protein.[18] p53 is previously reported to be recognized by T cells from patients with pancreatic cancer,[19] and frequently recognized by serum IgG Ab in patients with various cancers including colon cancers.[20–22] However, the particular p53 clone isolated in our study was recognized by sera from patients with a limited cancer including pancreatic cancer and was not recognized by sera from colon cancer patients. Downregulation of *HMT1* which may upregulate antiproliferating effects of IFNs through interaction with the intracytoplasmic domain of the Type I interferon (IFN) receptor was reported in breast cancer.[23,24] HMT1 was recognized by sera from patients with 2 of 37 pancreatic cancers, 2 of 10 prostate cancers but not recognized by any sera from 34 healthy individuals. Since DNA mismatch repair enzyme hMSH2 and hPMS1 were recognized by sera from 5 of 37 and 3 of 37 patients with pancreatic cancers, respectively, but not recognized by any sera from 34 healthy individuals, they were further investigated for their expression and immunogenicity in various cancers.

Although mRNAs for *hMSH2* and *hPMS1* were ubiquitously detected by RT-PCR analysis, the immunohistochemical study revealed over-expression of the hMSH2 and hPMS1 proteins in pancreatic ductal adenocarcinoma compared to normal pancreatic tissues. Over-expression of hMSH2 in ovarian cancer cells compared to normal ovarian tissues was previously reported by immunohistochemical analysis.[25] Although the expression of hMSH2 in rapidly proliferating normal cells such as intestinal epithelial cells was reported,[26] the mechanism for over-expression in ovarian cancer was not simply explained by higher proliferative ability of cancer cells because no correlation was observed between expression of hMSH2 and Ki-67 antigen. Over-expressed antigens such as galectin9[27] and HER2[28] were previously reported to induce immune response in cancer patients and frequently detected as tumor antigens by SEREX. A heat shock protein, hsp105 over-expressed in various cancers including pancreatic cancer and colon cancers, was previously identified by SEREX with serum from a pancreatic cancer patient.[29] Thus, over-expression of proteins in tumor cells might induce immune responses to hMSH2 and hPMS1 in patients with pancreatic cancer.

Some of the pancreatic cancers are known to have microsatellite instability due to inactivation of DNA mismatch repair genes. Forty percent of MSI positive pancreatic cancer was reported to be caused by silencing of MLH1 through promoter hypermethylation.[30] Genetic alterations of *hMSH2* have not been found.[31] Patients with MSI positive pancreatic cancer was reported to have better prognosis after treatment.[30] In our study, higher expression of hMSH2 and hPMS1 in the MSI negative cancers than the MSI positive cancers was observed in the pancreas and colon cancer cell lines. High proliferative ability of MSI negative cancer with high hMSH2 and hPMS1 expression may be associated with poor prognosis of MSI negative cancers. To investigate relationship among MSI status, hMSH2/hPMS1 expression and their specific Ab, further study on fresh cancer samples along with MSI status is necessary.

Although both $CD4^+$ and $CD8^+$ T cell responses to hMSH2 and hPMS1 remain to be investigated, these antigens may be useful as at least $CD4^+$ helper T cell antigens for immunotherapy, particularly in patients with positive serum Ab. The recognition by IgG Ab suggests that the same antigen activated $CD4^+$ helper T cells (Th) in the patients, meaning that theses antigens are

immunogenic in cancer patients. In addition, many SEREX defined antigens were shown to induce CD8+ cytotoxic T cells (CTL).[32–35] Positive correlation was observed between positive serum IgG antibody and induction of CD8+ CTL against a cancer-testis antigen NY-ESO-1.[32,36] In pancreatic cancer, SEREX defined antigen, coactosin-like protein (CLP), was reported to induce HLA-A2 restricted tumor reactive CTL from PBMC.[37] In the case of over-expressed antigens in tumor cells with relatively lower expression in normal cells, specificity of effecter T cells to cancer cells is defined by density of MHC/peptide complex on tumor cell surface. Thus, relatively low level expression of proteins in normal cells dose not exclude the use of T cells against these over-expressed antigens as previously reported in several tumor antigens including SART-1.[38]

In addition to be target antigens for immunotherapy, anti-hMSH2 and hPMS1 Ab may be useful for diagnosis of pancreatic cancer, since we have previously observed disappearance of serum antibody specific for the SEREX defined antigens in the patients who had good prognosis after treatment.[7,9] Anti-p53 antibody was reported to be utilized even for early diagnosis of esophageal cancers.[39] In our study, we also examined anti-hPMS1 and anti-hMSH2 Ab in sera from DM/PM patients, since anit-hPMS1 antibody was previously reported to be an autoantibody specifically detected in 7.5% of PM/DM patients.[12] The anti-hPMS1 Ab was detected 8 of 119 (6.7%) DM/PM patients in a similar frequency as previously reported. In addition, we found that anti-hMSH2 Ab was also detected in 5 of 102 (4.9%) DM/PM patients at a similar rate but not detected in other collagen diseases or infectious diseases, suggesting that anit-hMSH2 Ab may also be an autoantibody specific for DM/PM and useful for diagnosis of DM/PM patients. Antibodies to other DNA mismatch repair enzymes, including hMSH3, hMSH6, hPMS2 and hMLH1, remain to be investigated. The preferential induction of these Ab in patients with DM/PM and pancreatic cancer indicated that simple tissue destruction is not the reason for these Ab responses.

DM was known to be strongly associated with various cancers, although complication rate was about 10%, and PM was moderately associated with increase of cancer.[40–42] The risks of pancreatic cancer, lung carcinoma, ovarian cancer, gastric cancer, colorectal cancer and non-Hodgkin lymphoma were increased in DM patients and risks of non-Hodgkin lymphoma, lung cancer and bladder cancer were increased in PM patients.[43] Some autoantibodies are known to correlate with specific clinicopathological features in DM/PM patients, including anti-Jo-1 antibody for multiple arthritis/interstitial pneumonia and anti-SRP antibody for treatment-resistant severe myositis. Therefore, we examined whether positive anti-hPMS1/hMSH2 Ab had any correlation with cancer complication in the DM/PM patients. Although 1 patient complicated with breast cancer had serum anti-hPMS1 antibody, no significant correlation was observed among more than 100 patients, suggesting that these antibodies may be raised independently of cancer development with yet unknown mechanism. Alternatively, numbers of patients evaluated in our study are still too small to draw any conclusions, we thus would like to follow up the patients with positive serum autoantibody.

In summary, using SEREX, we identified 2 DNA mismatch repair enzymes, hMSH2 and hPMS1, which are over-expressed in pancreatic cancer cells, and whose antibodies were detected in patients with various cancers, particularly with pancreatic ductal adenocarcinoma, indicating that hMSH2 and hPMS1 are immunogenic antigens in various cancer patients and possibly useful as T cell antigens for immunotherapy. In addition, their antibodies may be useful for diagnosis of patients with both pancreatic cancer and DM/PM patients.

Acknowledgements

We thank M. Mukai for kindly providing some cancer tissues for immunohistochemistry. We also appreciate the expert technical assistance of K. Dan.

References

1. Kedra B, Popiela T, Sierzega M, Precht A. Prognostic factors of long-term survival after resective procedures for pancreatic cancer. Hepatogastroenterology 2001;48:1762–6.
2. Afsari A, Zhandoug Z, Young S, Ferguson L, Silapaswan S, Mittal V. Outcome analysis of pancreaticoduodenectomy at a community hospital. Am Surg 2002;68:281–4; discussion 84–5.
3. Janes RH, Jr., Niederhuber JE, Chmiel JS, Winchester DP, Ocwieja KC, Karnell JH, Clive RE, Menck HR. National patterns of care for pancreatic cancer: results of a survey by the Commission on Cancer. Ann Surg 1996;223:261–72.
4. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643–7.
5. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, Miki T, Rosenberg SA. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci U S A 1994;91:3515–9.
6. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, Yannelli JR, Adema GJ, Miki T, Rosenberg SA. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc Natl Acad Sci U S A 1994;91:6458–62.
7. Kiniwa Y, Fujita T, Akada M, Ito K, Shofuda T, Suzuki Y, Yamamoto A, Saida T, Kawakami Y. Tumor antigens isolated from a patient with vitiligo and T-cell-infiltrated melanoma. Cancer Res 2001;61:7900–7.
8. Ueda R, Iizuka Y, Yoshida K, Kawase T, Kawakami Y, Toda M. Identification of a human glioma antigen, SOX6, recognized by patients' sera. Oncogene 2004;23:1420–7.
9. Ishikawa T, Fujita T, Suzuki Y, Okabe S, Yuasa Y, Iwai T, Kawakami Y. Tumor-specific immunological recognition of frameshift-mutated peptides in colon cancer with microsatellite instability. Cancer Res 2003;63:5564–72.
10. Ito K, Fujita T, Akada M, Kiniwa Y, Tsukamoto M, Yamamoto A, Matsuzaki Y, Matsushita M, Asano T, Nakashima J, Tachibana M, Hayakawa M, et al. Identification of bladder cancer antigens recognized by IgG antibodies of a patient with metastatic bladder cancer. Int J Cancer 2004;108:712–24.
11. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci U S A 1995;92:11810–3.
12. Casciola-Rosen LA, Pluta AF, Plotz PH, Cox AE, Morris S, Wigley FM, Petri M, Gelber AC, Rosen A. The DNA mismatch repair enzyme PMS1 is a myositis-specific autoantigen. Arthritis Rheum 2001;44:389–96.
13. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Gladhaug I, Soreide O, Eriksen JA, Moller M, Baksaas I, Lothe RA, Saeterdal I, Gaudernack G. Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: Clinical and immunological responses in patients with pancreatic adenocarcinoma. Int J Cancer 2001;92:441–50.
14. Gjertsen MK, Saeterdal I, Saeboe-Larssen S, Gaudernack G. HLA-A3 restricted mutant ras specific cytotoxic T-lymphocytes induced by vaccination with T-helper epitopes. J Mol Med 2003;81:43–50.
15. Jaffee EM, Hruban RH, Biedrzycki B, Laheru D, Schepers K, Sauter PR, Goemann M, Coleman J, Grochow L, Donehower RC, Lillemoe KD, O'Reilly S, et al. Novel allogeneic granulocyte-macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. J Clin Oncol 2001; 19:145–56.
16. Peiper M, Goedegebuure PS, Linehan DC, Ganguly E, Douville CC, Eberlein TJ. The HER2/neu-derived peptide p654–662 is a tumor-associated antigen in human pancreatic cancer recognized by cytotoxic T lymphocytes. Eur J Immunol 1997;27:1115–23.
17. Tordsson J, Lavasani S, Ohlsson L, Karlstrom P, Svedberg H, Abrahmsen L, Brodin T. A3: a novel colon and pancreatic cancer reactive antibody from a primate phage library selected using intact tumour cells. Int J Cancer 2000;87:559–68.
18. Roose J, Korver W, de Boer R, Kuipers J, Hurenkamp J, Clevers H. The Sox-13 gene: structure, promoter characterization, and chromosomal localization. Genomics 1999;57:301–5.
19. Liu X, Peralta EA, Ellenhorn JD, Diamond DJ. Targeting of human p53-overexpressing tumor cells by an HLA A*0201-restricted murine T-cell receptor expressed in Jurkat T cells. Cancer Res 2000;60:693–701.

20. Scanlan MJ, Chen YT, Williamson B, Gure AO, Stockert E, Gordan JD, Tureci O, Sahin U, Pfreundschuh M, Old LJ. Characterization of human colon cancer antigens recognized by autologous antibodies. Int J Cancer 1998;76:652–8.
21. Scanlan MJ, Gout I, Gordon CM, Williamson B, Stockert E, Gure AO, Jager D, Chen YT, Mackay A, O'Hare MJ, Old LJ. Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. Cancer Immun 2001;1:4.
22. Diesinger I, Bauer C, Brass N, Schaefers HJ, Comtesse N, Sybrecht G, Meese E. Toward a more complete recognition of immunoreactive antigens in squamous cell lung carcinoma. Int J Cancer 2002;102:372–8.
23. Scott HS, Antonarakis SE, Lalioti MD, Rossier C, Silver PA, Henry MF. Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2). Genomics 1998;48:330–40.
24. Scorilas A, Black MH, Talieri M, Diamandis EP. Genomic organization, physical mapping, and expression analysis of the human protein arginine methyltransferase 1 gene. Biochem Biophys Res Commun 2000;278:349–59.
25. Friedrich M, Villena-Heinsen C, Meyberg R, Woll-Hermann A, Reitnauer K, Schmidt W, Tilgen W, Reichrath J. Immunohistochemical analysis of DNA "mismatch-repair" enzyme human Mut-S-Homologon-2 in ovarian carcinomas. Histochem J 1999;31:717–22.
26. Wilson TM, Ewel A, Duguid JR, Eble JN, Lescoe MK, Fishel R, Kelley MR. Differential cellular expression of the human hMSH2 repair enzyme in small and large intestine. Cancer Res 1995;55:5146–50.
27. Tureci O, Schmitt H, Fadle N, Pfreundschuh M, Sahin U. Molecular definition of a novel human galectin which is immunogenic in patients with Hodgkin's disease. J Biol Chem 1997;272:6416–22.
28. Cardillo M, Yankelevich B, Mazumder A, Lupu R. Heregulin induces increase in sensitivity of an erbB-2-overexpressing breast cancer cell type to lysis by lymphokine-activated killer cells. Cancer Immunol Immunother 1996;43:19–25.
29. Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M, Nishimura Y. Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. Biochem Biophys Res Commun 2001;281:936–44.
30. Yamamoto H, Itoh F, Nakamura H, Fukushima H, Sasaki S, Perucho M, Imai K. Genetic and clinical features of human pancreatic ductal adenocarcinomas with widespread microsatellite instability. Cancer Res 2001;61:3139–44.
31. Kondo E, Furukawa T, Yoshinaga K, Kijima H, Semba S, Yatsuoka T, Yokoyama T, Fukushige S, Horii A. Not hMSH2 but hMLH1 is frequently silenced by hypermethylation in endometrial cancer but rarely silenced in pancreatic cancer with microsatellite instability. Int J Oncol 2000;17:535–41.
32. Jager E, Nagata Y, Gnjatic S, Wada H, Stockert E, Karbach J, Dunbar P R, Lee SY, Jungbluth A, Jager D, Arand M, Ritter G, et al. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. Proc Natl Acad Sci U S A 2000;97:4760–5.
33. Nishimura MI, Avichezer D, Custer MC, Lee CS, Chen C, Parkhurst MR, Diamond RA, Robbins PF, Schwartzentruber DJ, Rosenberg SA. MHC class I-restricted recognition of a melanoma antigen by a human CD4+ tumor infiltrating lymphocyte. Cancer Res 1999;59:6230–8.
34. Ayyoub M, Rimoldi D, Guillaume P, Romero P, Cerottini JC, Valmori D, Speiser D. Tumor-reactive, SSX-2-specific CD8+ T cells are selectively expanded during immune responses to antigen-expressing tumors in melanoma patients. Cancer Res 2003;63:5601–6.
35. Ayyoub M, Hesdorffer CS, Montes M, Merlo A, Speiser D, Rimoldi D, Cerottini JC, Ritter G, Scanlan M, Old LJ, Valmori D. An immunodominant SSX-2-derived epitope recognized by CD4+ T cells in association with HLA-DR. J Clin Invest 2004;113:1225–33.
36. Jager E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jager D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ, Knuth A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J Exp Med 1998;187:265–70.
37. Nakatsura T, Senju S, Ito M, Nishimura Y, Itoh K. Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur J Immunol 2002;32:826–36.
38. Shichijo S, Nakao M, Imai Y, Takasu H, Kawamoto M, Niiya F, Yang D, Toh Y, Yamana H, Itoh K. A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. J Exp Med 1998;187:277–88.
39. Ralhan R, Arora S, Chattopadhyay TK, Shukla NK, Mathur M. Circulating p53 antibodies, p53 gene mutational profile and product accumulation in esophageal squamous-cell carcinoma in India. Int J Cancer 2000;85:791–5.
40. Sigurgeirsson B, Lindelof B, Edhag O, Allander E. Risk of cancer in patients with dermatomyositis or polymyositis. A population-based study. N Engl J Med 1992;326:363–7.
41. Airio A, Pukkala E, Isomaki H. Elevated cancer incidence in patients with dermatomyositis: a population based study. J Rheumatol 1995;22:1300–3.
42. Chen YJ, Wu CY, Shen JL. Predicting factors of malignancy in dermatomyositis and polymyositis: a case-control study. Br J Dermatol 2001;144:825–31.
43. Hill CL, Zhang Y, Sigurgeirsson B, Pukkala E, Mellemkjaer L, Airio A, Evans SR, Felson DT. Frequency of specific cancer types in dermatomyositis and polymyositis: a population-based study. Lancet 2001;357:96–100.

European Journal of Neurology 2005, **12:** 566–570

SHORT COMMUNICATION

Myasthenia gravis accompanied by alopecia areata: clinical and immunogenetic aspects

S. Suzuki[a], M. Shimoda[b], M. Kawamura[c], H. Sato[a], S. Nogawa[a], K. Tanaka[a], N. Suzuki[a] and M. Kuwana[d]

Departments of [a]Neurology and [b]Pathology, [c]Division of General Thoracic Surgery, and [d]Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan

Keywords:
alopecia areata, HLA class II, myasthenia gravis, thymoma

Received 5 July 2004
Accepted 15 July 2004

The purpose of this study was to evaluate the clinical characteristics of patients who had both myasthenia gravis (MG) and alopecia areata (AA). Clinical information was retrospectively collected for 159 Japanese patients with MG. Human leukocyte antigen (HLA)-DQB1 and DRB1 alleles were determined by genotyping. Of 159 MG patients, six (3.7%) developed AA after the onset of MG and thymectomy. The prevalence of AA in MG patients was higher than that reported in Caucasians. The frequencies of bulbar involvement, myasthenic crisis, and thymoma were significantly higher in MG patients with AA than in those without ($P = 0.007$, 0.004, and 0.006, respectively). All but one patient with AA had advanced stage thymoma. Three patients with a severe form of AA (alopecia totalis) had additional autoimmune diseases: myocarditis, myositis, and pure red cell aplasia. DRB1*0901 and DQB1*0303 tended to be more frequently detected in the six MG patients with AA than in the 82 patients without it. In conclusion, a subset of MG patients who have severe neuromuscular symptoms and thymoma develop AA several years after thymectomy.

Introduction

Autoimmune skin disorders are known complications in the course of myasthenia gravis (MG) (Drachman, 1994). Alopecia areata (AA), characterized by a sudden and patchy loss of the scalp hair, is a T-cell-mediated autoimmune disorder that targets hair follicles (Odom *et al.*, 2000; Randall, 2001). Hair loss may reverse completely, become chronic, or progress to a total loss of scalp hair (a severe form of AA termed alopecia totalis; AT) (Odom *et al.*, 2000). Coexistence of MG and AA has been reported a few times (Kanda *et al.*, 1986; Kubota *et al.*, 1997), but the clinical and immunogenetic features in MG patients that lead to AA are unknown. To clarify these points, we retrospectively evaluated our cohort of MG patients.

Patients and methods

We studied 159 unrelated Japanese MG patients (75 men and 84 women) evaluated at the Keio University Hospital during 1990–2002. The mean age at onset was 41.7 ± 19.1 years (range 3–87), and the mean observation period was 11.0 ± 7.8 years (range 1–31). The diagnosis of MG was based on clinical, electrophysiological, and immunological criteria (Drachman, 1994). Clinical and laboratory findings were retrospectively collected by reviewing clinical charts, and clinical classification was assessed according to the recommendations of the Myasthenia Gravis Foundation of America (Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America and coauthors 2000). Transsternal extended thymectomy was performed for 89 patients. The extent of thymoma at surgery was graded according to the Masaoka classification (Masaoka *et al.*, 1981) and thymic tumor histology was categorized according to the World Health Organization classification (Rosai and Sobin, 1999). Diagnosis of AA was based on typical dermatologic findings and exclusion of the effects of irradiation and/or chemotherapy for thymoma. In some patients, AA was confirmed by the typical histologic finding of lymphocyte infiltration in the peribulbar area, obtained from skin biopsy. Human leukocyte antigen (HLA)-DQB1 and DRB1 alleles were determined by polymerase chain reaction followed by analysis of restriction fragment length polymorphisms (Inoko and Ota, 1993).

Clinical information and blood samples were obtained after the patients gave their written informed consent, as approved by the Keio University Institutional Review Board. Statistical differences were analyzed by Fisher's two-tailed exact test or Student's *t*-test

Correspondence: Shigeaki Suzuki, Department of Neurology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160–8582, Japan (tel.: +81 3 5363 3788; fax: +81 3 3353 1272; e-mail: shigeaki714@aol.com).

as appropriate. The odds ratio (OR) with a 95% confidence interval (95% CI) was calculated to determine significant differences.

Results

Of 159 patients with MG, six (3.7%) had a history of AA and three of these had AT, a severe form of AA. In all cases, AA developed after the diagnosis of MG. Demographic and clinical findings were compared between MG patients with AA and those without it (Table 1). The mean age of MG onset tended to be younger in patients with AA ($P = 0.051$). All patients with AA had generalized disease, and their maximum symptom was severe: grade V for three patients, grade IIIb for two, and grade IIa for one. In fact, the frequencies of bulbar involvement and myasthenic crisis were significantly higher in patients with AA than in those without it ($P = 0.007$ and 0.004, respectively). Serum anti-acetylcholine receptor (AChR) antibodies were positive in 126 patients, and all patients with AA had the high level of antibodies (mean 236.9 ± 230.4 nM, range 28–650). Of 89 patients who received extended thymectomy, 44 had histologically confirmed thymoma. All but one patient with AA had thymoma, and this frequency was significantly higher than in patients without AA ($P = 0.006$). In patients with thymoma, advanced stage (stage III and IVa in Masaoka's classification) was more common in patients with AA than in those without ($P = 0.03$). All five patients with AA and thymoma had type B thymoma (type B1 for one, type B2 for 2, and type B3 for 2), and two of these patients experienced recurrence of thymoma. Skin biopsy was performed in three patients with AA showing lymphocytes infiltration in the peribulber area. Additional autoimmune diseases were common in the patients with AA. In particular, all three patients with AT had autoimmune diseases in addition to MG and AA: one had giant cell myocarditis and myositis, and the other two had pure red cell aplasia (PRCA) (Suzuki *et al.*, 2003).

The clinical courses of the six MG patients with AA are summarized in Fig. 1. Three patients (MG14, MG72, and MG104) had AT. It was noted that all patients developed AA after the onset of MG (7.6 ± 6.9 years, range 2–18) and thymectomy (6.7 ± 5.0 years, range 2–14). All the patients except MG142 were confirmed to have thymoma by histology. MG36 and MG90 developed AA in parallel with MG activity, and the remaining patients developed AA when the MG was quiescent. The AA of three patients (MG36, MG90, and MG142) improved within several months of treatment with topical corticosteroids, but extensive immunosuppressive therapy (oral cortico-

Table 1 Demographic and clinical features and HLA class II alleles in myasthenia gravis (MG) patients with and without alopecia areata (AA)

	MG with AA ($n = 6$)	MG without AA ($n = 153$)	*P*-value	OR (95% CI)
Demographic features				
Men	67%	46%	0.4	–
Mean age at MG onset (years)	32.1 ± 9.6	42.1 ± 19.3	0.051	–
Follow-up period (years)	14.5 ± 6.0	10.9 ± 7.6	0.2	–
Clinical features				
Generalized	100%	64%	0.3	–
Bulbar involvement	83%	26%	0.007*	14.1 (2.6–76.5)
Myasthenic crisis	50%	5%	0.004*	18.1 (4.7–67.9)
High anti-AChR Ab level[a]	50%	18%	0.09	–
Thymoma[b]	83%	25%	0.006*	14.2 (2.7–75.8)
Stage III + IVa[c]	80% (4/5)	26% (10/39)	0.03*	11.6 (1.6–82.4)
Type B[d]	100% (5/5)	71% (24/34)	0.3	–
Recurrence	40% (2/5)	10% (4/39)	0.1	–
Additional autoimmune disease[e]	50%	16%	0.06	–
HLA class II alleles				
DRB1*0901	50%	20% (16/82)	0.1	–
DQB1*0301	0%	18% (15/82)	0.5	–
DQB1*0303	50%	20% (16/82)	0.1	–
DQB1*0402	0%	15% (12/82)	0.6	–

[a]High anti-AChR Ab level = anti-acetylcholine receptor antibody level > 100 nM.
[b]Histologically confirmed.
[c]According to the Masaoka classfication (Masaoka *et al.*, 1981).
[d]According to the World Health Organization (Rosai and Sobin, 1999).
[e]Myocarditis ($n = 1$) and pure red cell aplasia (PRCA) ($n = 2$) in MG with AA. Graves' disease ($n = 10$), Hashimoto's thyroiditis ($n = 6$), rheumatoid arthritis ($n = 3$), PRCA ($n = 2$), myocarditis ($n = 2$), ulcerative colitis ($n = 1$), and neuromyotonia ($n = 1$) in MG without AA.
*Statistically significant ($P < 0.05$).

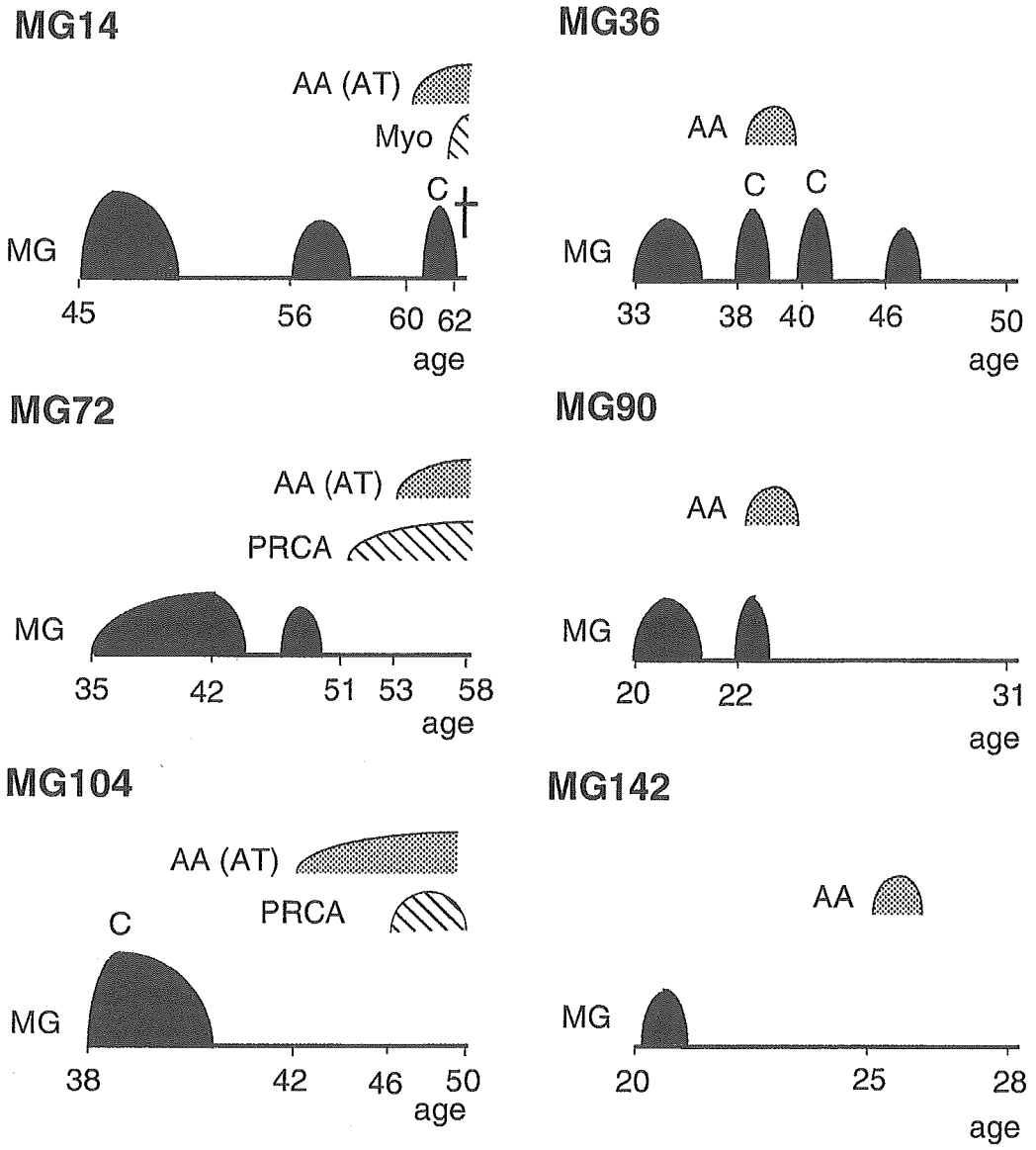

Figure 1 Individual clinical courses of six myasthenia gravis (MG) patients who developed alopecia areata (AA). Alopecia totalis (AT) is a severe form of AA. Three patients had an episode of myasthenic crisis (C). MG14 died of myocarditis (Myo); the remaining patients are alive. PRCA, pure red cell aplasia.

steroids with or without immunosuppressants) had no benefit for AT (MG14, MG72, and MG104). Interestingly, the additional autoimmune diseases experienced by these patients also developed after the onset of MG and thymectomy. MG14 died of myocarditis and resultant intractable ventricular arrhythmia; the other two are still alive, 4–12 years after the onset of AA.

Both MG and AA are reported to be associated with particular HLA class II alleles (Drachman, 1994; Odom *et al.*, 2000; Randall, 2001). To investigate the immunogenetic backgrounds associated with the development of AA in MG patients, we compared the relative frequencies of DRB1 and DQB1 alleles between the six MG patients with AA and the 82 patients without it. Although we failed to detect significant differences in the frequencies of HLA class II alleles between the groups, DRB1*0901 and DQB1*0303, which are in linkage disequilibrium, tended to be more frequent in patients with AA (Table 1). None of the patients with AA had DQB1*0301, the allele associated with AA in Caucasians (Odom *et al.*, 2000; Randall, 2001), or DQB1*0402, the allele associated with MG complicated by thymoma in the Japanese (Suzuki *et al.*, 2001).

Discussion

The frequency of AA in our cohort of Japanese MG patients was 3.7% (six of 159). This frequency was similar to that reported by the study of Kubota *et al.* (1997), also conducted in Japan (3.0%, six of 202). When patients with thymoma were selected, the frequency rose to 11% (five of 44) in our study and 17% (six of 35) in the study by Kubota et al (Kubota *et al.*, 1997). In contrast, the frequency of AA in MG patients with thymoma was only 0.5% (one of 207) in the largest cohort in Caucasians (Evoli *et al.*, 2002). Although there are no accurate data comparing prevalence of AA in the general population between the Japanese and Caucasians (McDonagh and Tazi-Ahnini, 2002), the prevalence of AA in MG patients is probably to be higher in the Japanese than in Caucasians. This ethnic difference might be explained by the variability in immunogenetic backgrounds. In this regard, we found a trend toward a correlation between the DRB1*0901-DQB1*0303 haplotype and AA in MG patients, although this difference did not reach statistical significance, probably because of the small number of patients with AA in our cohort. As the DRB1*0901-DQB1*0303 haplotype is relatively specific to Orientals, this haplotype may be responsible for the higher frequency of AA in Japanese MG patients. In the present study, we have identified a subset of MG patients who later develop AA after thymectomy. Clinical characteristics of this subset included younger onset, a severe form of MG complicated by bulbar involvement and an episode of myasthenic crisis, and thymoma (especially advanced stage). Another interesting feature is that MG and thymectomy always preceded the onset of AA. It was also noted that MG patients with AA, especially AT, had additional autoimmune disorders, including myocarditis, myositis, and PRCA. In this regard, there is a report describing a patient with MG who had both AT and Hashimoto's thyroiditis (Kanda *et al.*, 1986). Taken together, this case report, our previous report describing the development of PRCA in MG patients with thymoma after thymectomy (Suzuki *et al.*, 2003), and our current data suggest that MG patients with thymoma who received thymectomy are susceptible to certain types of autoimmune diseases. Special attention to the development of AA and other autoimmune disorders should be paid to MG patients with severe neuromuscular symptoms and thymoma after extended thymectomy.

As the majority of the patients developed AA independent of MG disease activity, the pathogenic processes of MG and AA may be different, although MG and AA are both thymoma-associated autoimmune diseases (Müller-Hermelink and Marx, 2000). In contrast to MG, which is an autoantibody-mediated disease, the infiltration of cytotoxic $CD8^+$ T cells into the target organ is crucial to the development of AA (Odom *et al.*, 2000; Randall, 2001). In fact, we confirmed that lymphocytes, which infiltrated in the peribulbar area, were more predominantly $CD8^+$ than $CD4^+$ T cells by the immunohistochemical analyses of skin biopsy. The autoimmune diseases observed in patients with MG and AA were myocarditis, myositis, PRCA, and Hashimoto's thyroiditis, all of which are mediated by cytotoxic T cells, rather than autoantibodies. As the phenotype of peripheral blood T-cell pools are affected by thymectomy in MG patients (Sempowski *et al.*, 2001), alteration of the T-cell repertoire after thymectomy may play a role in the development of AA and other cytotoxic T-cell-mediated autoimmune disorders in individuals susceptible to MG.

Acknowledgements

The authors thank Ms Yoko Tanaka for her expert technical assistance. This work was supported by a grant from the Japanese Ministry of Education, Science, Sports and Culture.

References

Drachman DB (1994). Myasthenia gravis. *N Engl J Med* **330**:1797–1810.

Evoli A, Minisci C, DiSchino C *et al.* (2002). Thymoma in patients with MG. Characteristics and long-term outcome. *Neurology* **59**:1844–1850.

Inoko H, Ota M (1993). PCR-RFLP. In: Hui KM, Bidwell J, eds. *Handbook of HLA Typing Techniques*. Boca Raton, FL: CRC Press, pp. 9–70.

Kanda F, Jinnai K, Baba H, Shundou F, Tujita T (1986). Myasthenia gravis with alopecia universalis and Hashimoto's disease: a case report. *Neurol Med (Tokyo)* **25**:556–558.

Kubota A, Komiyama A, Hasegawa O (1997). Myasthenia gravis and alopecia areata. *Neurology* **48**:774–775.

Masaoka A, Monden Y, Nakahara K, Tanioka T (1981). Follow-up study of thymomas with special reference to their clinical stages. *Cancer* **48**:2485–2492.

McDonagh AJG, Tazi-Ahnini R (2002). Epidemiology and genetics of alopecia areata. *Clin Exp Dermatol* **27**:405–409.

Müller-Hermelink HK and Marx A (2000). Thymoma. *Curr Opin Oncol* **12**:426–433.

Odom RB, James WD, Berger TG (2000). *Andrew's Diseases of the Skin*, 9th edn. Philadelphia, PA, W.B. Saunders Company.

Randall VA (2001). Is alopecia areata an autoimmune disease? *Lancet* **358**:1922–1923.

Rosai J, Sobin LH (1999). Histological typing of tumors of the thymus. *International Histologic Classification of Tumors*, 2nd edn. New York, Springer.

Sempowski GD, Thomasch JR, Gooding ME *et al.* (2001). Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. *J Immunol* **166**:2808–2817.

Suzuki S, Kuwana M, Yasuoka H, Tanaka K, Fukuuchi Y, Kawakami Y (2001). Heterogeneous immunogenetic background in Japanese adults with myasthenia gravis. *J Neurol Sci* **189**:59–64.

Suzuki S, Nogawa S, Tanaka K, Koto A, Fukuuchi Y, Kuwana M (2003). Initial predictors of development of pure red cell aplasia in myasthenia gravis after thymectomy. *Clin Neurol Neurosurg* **106**:16–18.

Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America, Jaretzki A III, Barohn RJ, Ernstoff RM, Kaminski HJ, Keesey JC, Penn AS, Sanders DB (2000). Myasthenia gravis. Recommendations for clinical research standards. *Neurology* **55**:16–23.