

Exon 8 *c-kit* gene mutation in GISTs

- Cosman D, Lyman SD. Identification of a ligand for the *c-kit* proto-oncogene. *Cell* 1990; 63: 167-174.
- [9] Flanagan JG, Leder P. The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell* 1990; 63: 185-194.
- [10] Zsebo KM, Williams DA, Geissler EN, Broudy YC, Martin FH, Atkins HL, Hsu RY, Birkitt NC, Okino KH, Murdock DC, Jacobson FW, Langley KE, Smith KA, Takeishi T, Cattaneach BM, Galli SJ, Suggs SV. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the *c-kit* tyrosine kinase receptor. *Cell* 1990; 63: 213-224.
- [11] Nagata H, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, Metcalfe DD. Identification of a point mutation in the catalytic domain of the protooncogene *c-kit* in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci U S A* 1995; 92: 10560-10564.
- [12] Longley BJ, Tyrrell L, Lu SZ, Ma YS, Langley K, Ding TG, Duffy T, Jacobs P, Tang LH, Modlin I. Somatic *c-kit* activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm. *Nat Genet* 1996; 12: 312-314.
- [13] Tian Q, Frierson HF Jr, Krystal GW, Moskaluk CA. Activating *c-kit* gene mutations in human germ cell tumors. *Am J Pathol* 1999; 154: 1643-1647.
- [14] Went PT, Dirnhofer S, Bundi M, Mirlacher M, Schraml P, Mangialaio S, Dimitrijevic S, Kononen J, Lugli A, Simon R, Sauter G. Prevalence of KIT expression in human tumors. *J Clin Oncol* 2004; 22: 4514-4520.
- [15] Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol* 2006; 24: 4340-4346.
- [16] Rubin BP, Singer S, Tsao C, Duensing A, Lux ML, Ruiz R, Hibbard MK, Chen CJ, Xiao S, Tuveson DA, Demetri GD, Fletcher CD, Fletcher JA. KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. *Cancer Res* 2001; 61: 8118-8121.
- [17] Kinoshita K, Isozaki K, Hirota S, Nishida T, Chen H, Nakahara M, Nagasawa Y, Ohashi A, Shinomura Y, Kitamura Y, Matsuzawa Y. *C-kit* gene mutation at exon 17 or 13 is very rare in sporadic gastrointestinal stromal tumors. *J Gastroenterol Hepatol* 2003; 18: 147-151.
- [18] Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Haley A, Town A, Demetri GD, Fletcher CD, Fletcher JA. PDGFRA Activating Mutations in Gastrointestinal Stromal Tumors. *Science* 2003; 299: 708-710.
- [19] Hirota S, Ohashi A, Nishida T, Isozaki K, Kinoshita K, Shinomura Y, Kitamura Y. Gain-of-function mutations of platelet-derived growth factor receptor alpha gene in gastrointestinal stromal tumors. *Gastroenterology* 2003; 125: 660-667.
- [20] Nishida T, Hirota S, Taniguchi M, Hashimoto K, Isozaki K, Nakamura H, Kanakura Y, Tanaka T, Takabayashi A, Matsuda H, Kitamura Y. Familial gastrointestinal stromal tumours with germline mutation of the KIT gene. *Nat Genet* 1998; 19: 323-324.
- [21] Isozaki K, Terris B, Belghiti J, Schiffmann S, Hirota S, Vanderwinden JM. Germline-activating mutation in the kinase domain of KIT gene in familial gastrointestinal stromal tumors. *Am J Pathol* 2000; 157: 1581-1585.
- [22] Hirota S, Nishida T, Isozaki K, Taniguchi M, Nishikawa K, Ohashi A, Takabayashi A, Obayashi T, Okuno T, Kinoshita K, Chen H, Shinomura Y, Kitamura Y. Familial gastrointestinal stromal tumors associated with dysphagia and novel type germline mutation of KIT gene. *Gastroenterology* 2002; 122: 1493-1499.
- [23] Hartmann K, Wardelmann E, Ma Y, Merkelbach-Bruse S, Preussner LM, Woolery C, Baldus SE, Heinicke T, Thiele J, Buettner R, Longley BJ. Novel germline mutation of KIT associated with familial gastrointestinal stromal tumors and mastocytosis. *Gastroenterology* 2005; 129: 1042-1046.
- [24] Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, Lydon NB. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by *c-kit* and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 2000; 295: 139-145.
- [25] Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002; 347: 472-480.
- [26] Chen H, Isozaki K, Kinoshita K, Ohashi A, Shinomura Y, Matsuzawa Y, Kitamura Y, Hirota S. Imatinib inhibits various types of activating mutant kit found in gastrointestinal stromal tumors. *Int J Cancer* 2003; 105: 130-135.
- [27] Heinrich MC, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, McGreevey LS, Chen CJ, Van den Abbeele, Druker BJ, Kiese B, Eisenberg B, Roberts PJ, Singer S, Fletcher CDM, Silberman S, Dimitrijevic S, Fletcher JA. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol* 2003; 21: 4342-4349.

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- [28] Joensuu H, Eriksson M, Hall KS, Hartmann JT, Pink D, Schütte J, Ramadori G, Hohenberger P, Duyster J, Al-Batran SE, Schlemmer M, Bauer S, Wardelmann E, Sarlomo-Rikala M, Nilsson B, Sihto H, Monge OR, Bono P, Kallio R, Vehtari A, Leinonen M, Alvegård T, Reichardt P. One vs three years of adjuvant imatinib for operable gastrointestinal stromal tumor: randomized trial. *JAMA* 2012; 307: 1265-1272.
- [29] Demetri GD, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, Verweij J, McArthur G, Judson IR, Heinrich MC, Morgan JA, Desai J, Fletcher CD, George S, Bello CL, Huang X, Baum CM, Casali PG. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet* 2006; 368: 1329-1338.
- [30] Demetri GD, Reichardt P, Kang YK, Blay JY, Rutkowski P, Gelderblom H, Hohenberger P, Leahy M, von Mehren M, Joensuu H, Badalamenti G, Blackstein M, Le Cesne A, Schöffski P, Maki RG, Bauer S, Nguyen BB, Xu J, Nishida T, Chung J, Kappeler C, Kuss I, Laurent D, Casali PG; GRID study investigators. Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): an international multicentre randomised placebo-controlled phase 3 trial. *Lancet* 2013; 381: 295-302.
- [31] Bodemer C, Hermine O, Palmérini F, Yang Y, Grandpeix-Guyodo C, Leventhal PS, Hadj-Rabia S, Nasca L, Georgin-Lavialle S, Cohen-Akenine A, Launay JM, Barete S, Feger F, Arock M, Catteau B, Sans B, Stalder JF, Skowron F, Thomas L, Lorette G, Plantin P, Bordigoni P, Lortholary O, de Prost Y, Moussy A, Sobol H, Dubreuil P. Pediatric mastocytosis is clonal disease associated with D816V and other activating *c-KIT* mutations. *J Invest Dermatol* 2010; 130: 804-805.
- [32] Huss S, Kunstlinger H, Wardelmann E, Kleine MA, Binot E, Merkelbach-Bruse S, Rüdiger T, Mittler J, Hartmann W, Büttner R, Schildhaus HU. A subset of gastrointestinal stromal tumors previously regarded as wild-type tumors carries somatic activating mutations in KIT exon 8 (p.D419del). *Mod Pathol* 2013; 26: 1004-1012.
- [33] Gari M, Goodeve A, Wilson G, Winship P, Langabeer S, Linch D, Vandenberghe E, Peake I, Reilly J. *c-kit* proto-oncogene exon 8 in-frame deletion plus insertion mutations in acute myeloid leukaemia. *Br J Haematol* 1999; 105: 894-900.
- [34] Goemans BF, Zwaan ChM, Miller M, Zimmermann M, Harlow A, Meshinchi S, Loonen AH, Hählen K, Reinhardt D, Creutzig U, Kaspers GJ, Heinrich MC. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. *Leukemia* 2005; 19: 1536-1542.

Clinicopathological Significance of Leucine-Rich α 2-Glycoprotein-1 in Sera of Patients With Pancreatic Cancer

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Objectives: Leucine-rich α 2-glycoprotein-1 (LRG-1) is an inflammatory protein. Serum LRG-1 levels can reportedly be used as a cancer biomarker for several types of carcinoma. In the present study, we investigated the clinical usefulness of serum LRG-1 levels as a biomarker of pancreatic cancer.

Methods: A total of 124 patients with pancreatic cancer, 35 patients with chronic pancreatitis (CP), and 144 healthy volunteers were enrolled in the study. Serum LRG-1 levels were assayed by enzyme-linked immunosorbent assay. Immunohistochemistry was used to examine LRG-1 expression in pancreatic cancer tissues.

Results: Serum LRG-1 levels were significantly increased in patients with pancreatic cancer compared with CP patients and healthy volunteers. The LRG-1 levels increased with progressive clinical stages of pancreatic cancer. Receiver operator curve analysis showed that a combination of carbohydrate antigen 19-9 and LRG-1 resulted in a higher area under the curve for the diagnosis of pancreatic cancer. Positive staining was observed in all cases of pancreatic cancer, but positive signal was scarcely detected in tissues from CP patients or normal surrounding tissue.

Conclusions: These results suggest that serum LRG-1 is a promising biomarker for pancreatic cancer.

Key Words: leucine-rich α 2-glycoprotein-1, pancreatic cancer, biomarker (*Pancreas* 2015;44: 93–98)

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States.¹ Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. In Japan, the mortality rate for PDAC has increased over the past decade, currently ranking fifth.² Pancreatic ductal adenocarcinoma has an extremely poor prognosis with a 5-year survival rate of less than 5%. Early detection of PDAC remains clinically challenging because of its asymptomatic nature, and surgery offers the only potential cure. However, the 5-year survival of PDAC patients after curative resection (surgery alone) is estimated to be 10% to 20%.³ Curative surgery with adjuvant chemotherapy improves

overall survival,⁴ but the clinical outcome of pancreatic cancer has not been markedly improved. One strategy that could improve PDAC survival is to detect the cancer in its early clinical stages. Thus, there is an urgent need to develop biomarkers for the stratification of patients for current treatment modalities and the development of novel therapeutic strategies.

Molecules that are specifically overexpressed in tumors not only serve as useful diagnostic markers, but also as potential therapeutic targets.⁵ The development of methods that are sensitive and specific enough to permit an early diagnosis of PDAC may facilitate the detection and subsequent treatment of this disease. However, an important factor adds another level of complexity to this already demanding task. A number of overlapping symptomatological features are known to link pancreatic adenocarcinoma to the inflammatory disease chronic pancreatitis (CP), often obscuring the distinction between these 2 pathological conditions.

Carcinoembryonic antigen, carbohydrate antigen 19-9 (CA19-9), and DUPAN-2 are the only serum biomarkers currently available for PDAC detection and have shown some utility as diagnostic adjuncts and prognostic markers in Japan.⁶ However, these markers are not widely used in routine clinical practice in Europe and America due to low sensitivity, specificity, or reproducibility and therefore cannot be routinely used to diagnose PDAC. Although CA19-9 is not a specific marker, identifying characteristic CA19-9 carrier proteins may allow higher specificity (molecular proteomics). Although the detection of CA19-9 carrier proteins is established, it will be very difficult to detect the early stage of pancreatic cancer.⁷ To establish a next-generation biomarker for pancreatic cancer, combination assays of several cancer biomarkers or a novel type of cancer biomarker involved in CP will be required.

Leucine-rich α 2-glycoprotein-1 (LRG-1) was first identified in 1977 as an inflammatory protein in human serum.⁸ Recent studies have demonstrated that serum LRG-1 can be used as a biomarker in several kinds of cancer, including ovarian, lung, biliary tract, and hepatocellular carcinoma related to hepatitis B virus infection.^{9–13} Increased serum LRG-1 levels in patients with pancreatic cancer were first reported using multidimensional liquid chromatography followed by 2-dimensional difference gel electrophoresis in plasma proteomics.¹⁴ The authors used Western blot to confirm the increase in serum LRG-1 patients with pancreatic cancer compared with healthy volunteers (HVs), and they showed that patients with CP tended to express lower serum levels of LRG-1 than patients with PDAC. However, they did not find significant differences between patients with CP and patients with PDAC due, at least in part, to the limited number of patients in the study. The study did not investigate the relationships between the marker and the clinical stages of pancreatic cancer, and no immunological study of LRG-1 in pancreatic cancer was performed. As

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LRG-1 has also been reported to be part of an acute inflammatory response in ulcerative colitis and acute appendicitis, LRG-1 may be increased as a result of inflammation.^{15–18}

In the present study, we determined serum LRG-1 levels in patients with CP and PDAC and assessed the clinicopathological significance of the increase in LRG-1 in terms of clinical parameters and immunohistochemistry.

MATERIALS AND METHODS

Patients and Sample Collection

Peripheral blood samples from patients with pancreatic disease and apparently HVs were obtained from Ogaki Municipal Hospital, Japan Community Health Care Organization Osaka Hospital, and Osaka University Hospital. Written informed consent was obtained from all patients and HVs. The study was performed in accordance with the guidelines issued by the local institutional review board of Osaka University. Sera from 124 patients with PDAC and 35 patients with CP were evaluated. All blood sampling was done at the time of diagnosis (pretreatment). A total of 144 serum samples from HVs were used as controls. Detailed information on clinical background was available for 71 PDAC patients, 20 CP patients, and 91 HVs. The collection, processing, and storage of all blood samples were standardized as follows: blood samples were collected in a vacutainer tube, allowed to clot at room temperature for 30 minutes, and then centrifuged at $2500 \times g$ for 10 minutes. The serum was removed and immediately divided into 100- μ L and 1-mL aliquots and stored at -80°C until use.

Formalin-fixed, paraffin-embedded tissue blocks from patients with PDAC were obtained from surgical cases at Osaka University Hospital in 2011 and 2012. Eleven PDAC, 1 CP, and 3 noncystic lesions of intraductal papillary mucinous neoplasm (IPMN; control for normal pancreas) were randomly selected.

Quantification of Serum LRG-1

Serum LRG-1 levels were determined using the human LRG assay kit (IBL, Fujioka, Japan) according to the manufacturer's protocol. Briefly, each serum sample was diluted (1:100) with buffer from the kit and the assays performed in triplicate. The LRG-1 levels were determined from a standard curve created using control samples. If the concentration of serum LRG-1 was less than 1.56 ng/mL or greater than 100 ng/mL, the dilution rate was changed.

Serum CA19-9 levels were measured in the hospital laboratory at the time of diagnosis.

Statistical Analysis

All statistical analyses were performed using JMP (version 9.0; SAS Institute Inc, Japan). Continuous data between clinical groups were compared by the Mann-Whitney *U* test for nonparametric data and the Student *t* test for normally distributed data. The Pearson product-moment correlation coefficient was used to examine associations between 2 continuous variables. Associations between categorical variables were examined using Fisher exact test. Normal cutoffs were defined for LRG-1 as the optimum point at which sensitivity and specificity was maximized. Receiver operating characteristic (ROC) curves were generated, and the areas under the curve (AUCs) were defined.

Immunohistochemical Staining

To analyze LRG-1 expression by immunohistochemistry, formalin-fixed, paraffin-embedded tissue blocks from patients

with pancreatic cancer were deparaffinized in xylene and rinsed in a series of 100%, 95%, 90%, 80%, 70%, and 60% ethanol solutions. The antigen was recovered by incubating the slides in 10 mmol/L citrate buffer (pH 6.0) for 40 minutes in boiling water. To quench endogenous peroxidase activity, the slides were incubated with 0.3% H_2O_2 in methanol for 30 minutes and then rinsed extensively in phosphate-buffered saline. The slides were incubated with blocking serum for 20 minutes followed by a 1:400 dilution of rabbit anti-LRG monoclonal antibody (Cat# 13224-1-AP; ProteinTech Group, Chicago, Ill) overnight at 4°C . Endogenous biotin activity was blocked using an avidin D–biotin blocking solution before in situ localization of the antigen using a biotin-avidin antigen detection method (R.T.U. Vectastain kit, Vector Laboratories, Burlingame, Calif). After extensive washing in phosphate-buffered saline, sections were incubated in a diaminobenzidine solution (Stable DAB, Invitrogen, Carlsbad, Calif) and counterstained with a hematoxylin solution. The slides were then dehydrated by washing in ethanol and cleared in xylene. Cover slips were placed on the slides, which were evaluated for LRG-1 staining. The intensity of LRG-1 staining was scored on a scale of 0 to 2 (0, no staining; 1, moderate staining; 2, strong staining).

RESULTS

Quantification of Serum LRG-1 Levels

Serum LRG-1 levels were significantly elevated in patients with PDAC (7.99 [5.07] $\mu\text{g}/\text{mL}$) compared with HVs (3.51 [1.42] $\mu\text{g}/\text{mL}$) and patients with CP (4.96 [2.11] $\mu\text{g}/\text{mL}$) ($P < 0.001$; Fig. 1, Table 1).

When 71 patients with pancreatic cancer were investigated in terms of clinical stage, the mean (SD) LRG-1 concentrations significantly increased with the progression of clinical stage as follows: 3.33 (0.66) $\mu\text{g}/\text{mL}$ in stage I, 7.07 (4.18) $\mu\text{g}/\text{mL}$ in stage II, 8.74 (5.86) $\mu\text{g}/\text{mL}$ in stage III, and 8.72 (4.35) $\mu\text{g}/\text{mL}$ in stage IV (Fig. 2). Among them, significant difference of the LRG-1 concentrations was observed between stage I and the other stages, but the differences between stage II and III, III and IV, and II and IV were not significant. The mean (SD) LRG-1 concentration was

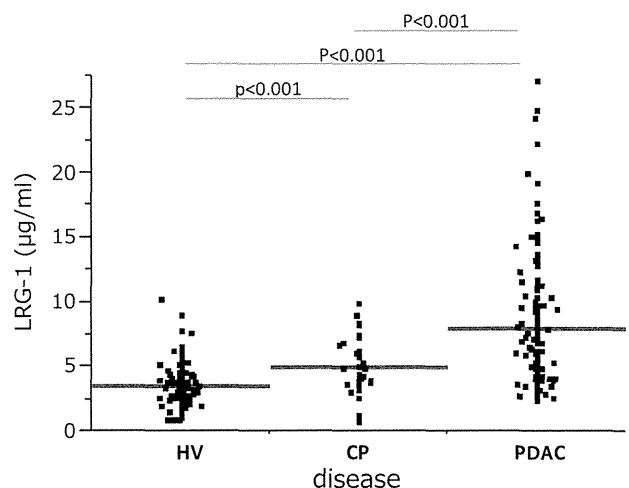


FIGURE 1. Detection of serum LRG-1 by enzyme-linked immunosorbent assay. Serum LRG-1 concentrations were determined for 124 patients with PDAC, 35 patients with CP, and 144 HVs. The mean (SD) LRG-1 concentration for PDAC patient sera was 7.99 (5.07) $\mu\text{g}/\text{mL}$ compared with 3.51 (1.42) $\mu\text{g}/\text{mL}$ for HV sera ($P < 0.001$) and 4.96 (2.11) $\mu\text{g}/\text{mL}$ for CP patients ($P < 0.001$).

TABLE 1. Clinicopathological Characteristics of Patients With PDAC, HVs, and Patients With CP

	PDAC (n = 124)	HV (n = 144)	CP (n = 35)	PDAC vs HV	PDAC vs CP
Age, y*	66.4 (7.83)	44.9 (12.5)	66.7 (10.6)	$P < 0.01$	NS
Sex, % male	70.4	59.3	60.0	NS	NS
LRG-1, $\mu\text{g/mL}^*$	7.99 (5.07)	3.51 (1.42)	4.96 (2.11)	$P < 0.01$	$P < 0.01$
CA19-9, U/mL*	213.7 (208.1)	13.3 (9.61)	7.88 (8.88)	$P < 0.01$	$P < 0.01$
Tumor location (head/body, tail)	42/29				
Lymph node metastasis (positive/negative)	31/40				
Clinical stage (1/2/3/4) [†]	6/28/21/16				

*Data are presented as mean (SD).

[†]General Rules for the Study of Pancreatic Cancer, 6th edition (Japan Pancreas Society).¹⁹

NS indicates not significant.

significantly higher in the lymph node metastasis–positive group than the lymph node metastasis–negative group (9.23 [4.68] $\mu\text{g/mL}$ and 6.51 [4.69] $\mu\text{g/mL}$, respectively; $P < 0.001$). These results clearly demonstrate that in patients with PDAC, LRG-1 can be associated with local progression or lymph node metastasis of the primary tumor, but not with distant progression, such as liver metastasis. In addition, serum LRG-1 levels positively correlated with CA19-9 ($R^2 = 0.088$, $P < 0.001$), but not with carcinoembryonic antigen ($R^2 = 0.017$, $P = 0.27$).

ROC Curve Analysis and Diagnostic Performance

The ROC curve analysis was performed to determine the clinical usefulness of LRG-1 for diagnosing pancreatic cancer. As shown in Figure 3A, CA19-9 was useful for differentiating patients with PDAC from HVs with AUC of 0.869. At a cutoff value of 37 U/mL for CA19-9, the optimal sensitivity and specificity were 71.9% and 96.4%, respectively. Similar analysis indicated that the AUC for LRG-1 was 0.850. At a cutoff value of

4.81 $\mu\text{g/mL}$ for LRG-1, the optimal sensitivity and specificity were 67.7% and 88.2%, respectively. The combination of LRG-1 and CA19-9 was analyzed by fixing the cutoff value at that of CA19-9. The combination of LRG-1 and CA19-9 improved the differential diagnosis between patients with PDAC and HVs, with an increase in AUC of 0.881 and 75.6% sensitivity and 91.7% specificity.

The ability of LRG-1 to make a differential diagnosis between patients with PDAC and patients with CP was also examined. The ROC curve analysis showed that AUC for CA19-9 was 0.913 (Fig. 3B). At a cutoff value of 37 U/mL for CA19-9, the optimal sensitivity and specificity were 73.7% and 100.0%, respectively. Similar analysis for LRG-1 showed AUC of 0.686. At a cutoff value of 4.81 $\mu\text{g/mL}$ for LRG-1, the optimal sensitivity and specificity were 67.7% and 54.3%, respectively. The combination of LRG-1 and CA19-9 enhanced the differentiating power between patients with PDAC and patients with CP, with an increase in AUC to 0.942 with 75.6% sensitivity and 100.0% specificity.

In CA19-9–negative cases, the AUC for LRG-1 was 0.785. At a cutoff value of 4.81 $\mu\text{g/mL}$ for LRG-1, the sensitivity and specificity in differentiating patients with PDAC from HVs were 68.8% and 86.3%, respectively. In regards to differentiating patients with PDAC from patients with CP, the AUC was 0.65, and the sensitivity and specificity were 68.8% and 50.0%, respectively.

LRG-1 Immunohistochemistry

Serum cancer biomarkers can be produced by both cancer cells and the tissues surrounding them. Because LRG-1 is produced in the liver and white blood cells, it is important to identify which cells produce LRG-1 in pancreatic cancer tissue. To address this issue, the pattern of LRG-1 protein expression was assessed by immunohistochemistry using morphological cancer lesions and noncancerous lesions from resection tissue. As shown in Figure 4, pancreatic cancer cells stained positively on the cell surface with a plasma membrane staining pattern, but pancreatic duct cells from noncancer cases, including CP and IPMN, did not stain. However, in CP cases, inflammatory cells such as lymphocytes exhibited weak staining. Positive staining was observed in all cases of pancreatic cancer, but a positive signal was scarcely detected in tissue from CP patients or normal surrounding tissue. Serum LRG-1 levels did not correlate with the intensity of immunostaining ($P = 0.327$).

DISCUSSION

We demonstrated that preoperative serum LRG-1 levels are elevated in patients with PDAC compared with HVs and patients with CP. The LRG-1 level is associated with disease progression and

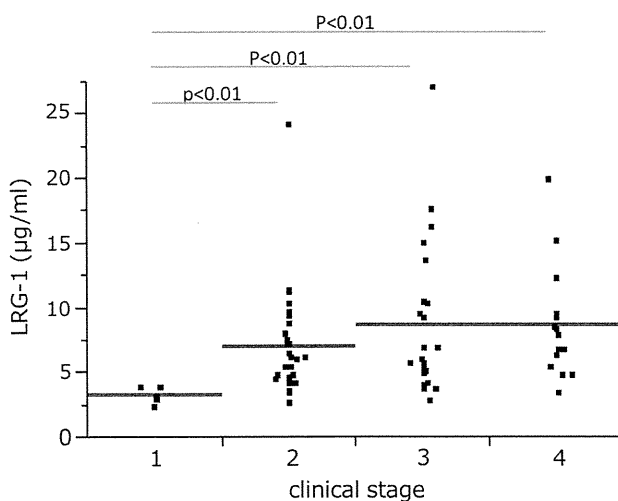


FIGURE 2. Detection of serum LRG-1 in pancreatic cancer patients by enzyme-linked immunosorbent assay. The mean (SD) LRG-1 concentration increased with the progression of clinical stage as follows: stage I, 3.33 (0.66) $\mu\text{g/mL}$; stage II, 7.07 (4.18) $\mu\text{g/mL}$; stage III, 8.74 (5.86) $\mu\text{g/mL}$; and stage IV, 8.72 (4.35) $\mu\text{g/mL}$. A significant difference was observed between stage I and the other stages, but the differences between stage II and III, III and IV, and III and IV were not significant.

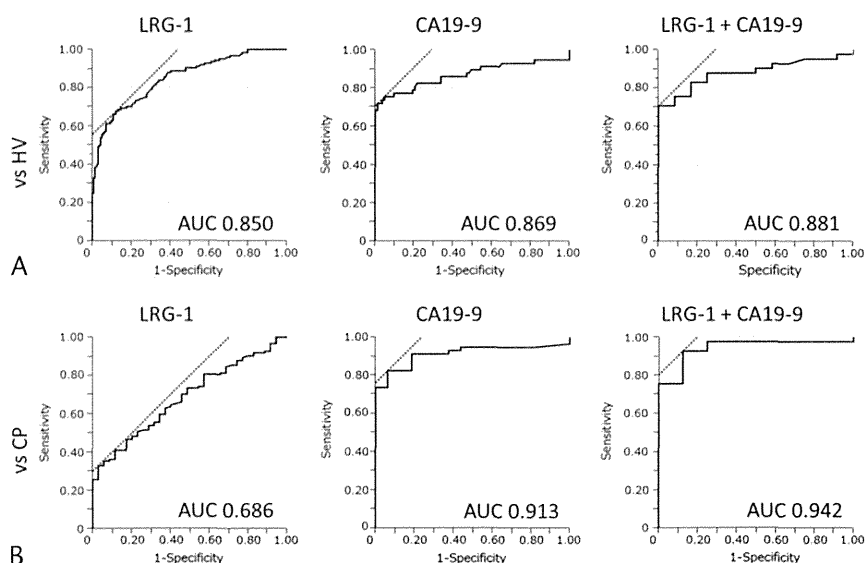


FIGURE 3. Receiver operating characteristic curve analysis for PDAC patients versus HVs (A) and PDAC patients versus CP patients (B). A, Power of LRG-1, CA19-9, and the combination of LRG-1 and CA19-9 in differentiating PDAC patients from HVs. The AUC was 0.850 for LRG-1 alone, 0.869 for CA19-9 alone, and 0.881 for LRG-1 and CA19-9 together. B, Power of LRG-1, CA19-9, and the combination of LRG-1 and CA19-9 in differentiating PDAC patients from CP patients. The AUC was 0.686 for LRG-1 alone, 0.913 for CA19-9 alone, and 0.942 for LRG-1 and CA19-9 together.

lymph node metastasis. Although previous studies have suggested that LRG-1 is induced by both tumor cells and acute/chronic inflammation, serum LRG-1 levels were not as high in the CP patients in our study as previously reported, due in part to the mild/moderate levels of CP we investigated. Thus, the diagnosis of CP with mild inflammation may be difficult. In contrast, immunohistochemical staining clearly revealed that pancreatic cancer cells, but not noncancer lesions from CP and IPMN cases, were positive for LRG-1. This result suggests that high levels of LRG-1 in pancreatic cancer may be derived from cancer cells or a change in the micro-environment, such as local inflammation associated with cancer progression. However, serum LRG-1 levels did not correlate with staining intensity in tissues, suggesting that serum LRG-1 levels may be dependent on the cleavage of LRG-1 on the cell surface. Andersen et al⁹ reported an association between LRG-1 and ovarian cancer. The mean serum LRG-1 concentration was higher in ovarian cancer patients than in healthy women, and highest among stage III/IV patients. Ovarian cancer cells secrete LRG-1, which may contribute directly to the elevated levels of LRG-1 observed in the serum of ovarian cancer patients. Interestingly, the time course is quite different for plasma LRG-1 and carbohydrate antigen 125 (CA125). Suboptimal debulking surgery results in a substantial decrease in CA125, whereas LRG-1 levels remain elevated. Thus serum LRG-1 levels seem to be more directly related to tumor burden than CA125.

Initially, LRG-1 was classified as a marker of acute phase inflammatory responses. Serada et al¹⁵ showed that serum LRG-1 concentrations correlate better with disease activity in ulcerative colitis than C-reactive protein. Kawakami et al²⁰ reported that LRG-1 levels increase after radiofrequency ablation for hepatocellular carcinoma, suggesting a possible role in the acute stress reaction. We confirmed an association between LRG-1 and C-reactive protein in patients with pancreatic cancer ($R^2 = 0.774$, $P < 0.001$). These dual functions of LRG-1 as a tumor marker and acute phase protein prompted us to use it as a marker of tumor recurrence after an acute phase.

We conducted this study to confirm that LRG-1 can be used as a diagnostic marker for PDAC. We found that LRG-1 can

distinguish between patients with PDAC and HVs or patients with CP, but we were not able to demonstrate its effectiveness as an early diagnostic marker. However, we confirmed that serum LRG-1 levels are associated with disease progression and lymph node metastasis, and that pancreatic cancer cells express LRG-1 on the cell surface. An analysis of LRG-1 glycosylation may be a promising approach to distinguish cancer-associated LRG-1 from inflammation-associated LRG-1. In the future, if glycan changes in LRG-1 can be used to identify the pancreatic cancer-specific LRG-1, which is different from the LRG-1 protein made by normal cells such as hepatocytes, it may be a more specific biomarker in PDAC.

Leucine-rich α 2-glycoprotein-1 was purified more than 30 years ago. The protein contains 8 typical 24-residue leucine-rich repeats with the consensus sequence.²¹ However, the physiological function of LRG-1 is still largely unknown. Shirai et al²² showed that autologous cytochrome *c* (cyt *c*) is an endogenous ligand of LRG-1 and functions in the detoxification of neurotoxins from snake venom. The expression of LRG-1 is up-regulated during neutrophilic granulocyte differentiation in response to granulocyte-colony stimulating factor, suggesting that LRG-1 is involved in granulopoiesis. Leucine-rich α 2-glycoprotein-1 functions as one of the pattern recognition receptors of polymorphonuclear neutrophils and modulates neutrophil function through innate immunity. Serum LRG-1 concentrations correlate well with disease activity in ulcerative colitis. Recent reports have also demonstrated the diagnostic value of LRG-1 in the urine of children with acute appendicitis. These results support the role of LRG-1 in inflammatory responses. Leucine-rich α 2-glycoprotein-1 and apoptotic protease activating factor 1 (Apaf-1) have some amino acid sequence homology, and LRG-1 binding to cyt *c* is similar to that of Apaf-1.²³ However, in contrast to Apaf-1, LRG-1 may clear potentially dangerous cyt *c*. This report suggests a role of LRG-1 in preventing lymphocyte death. When bound to extracellular cyt *c* released from apoptotic cells, serum LRG-1 acts as a survival factor for lymphocytes, and possibly other cells. Moreover, aberrant neovascularization contributes to diseases, such as cancer, and is the consequence of inappropriate angiogenic signaling. The epithelial growth factor receptor and vascular

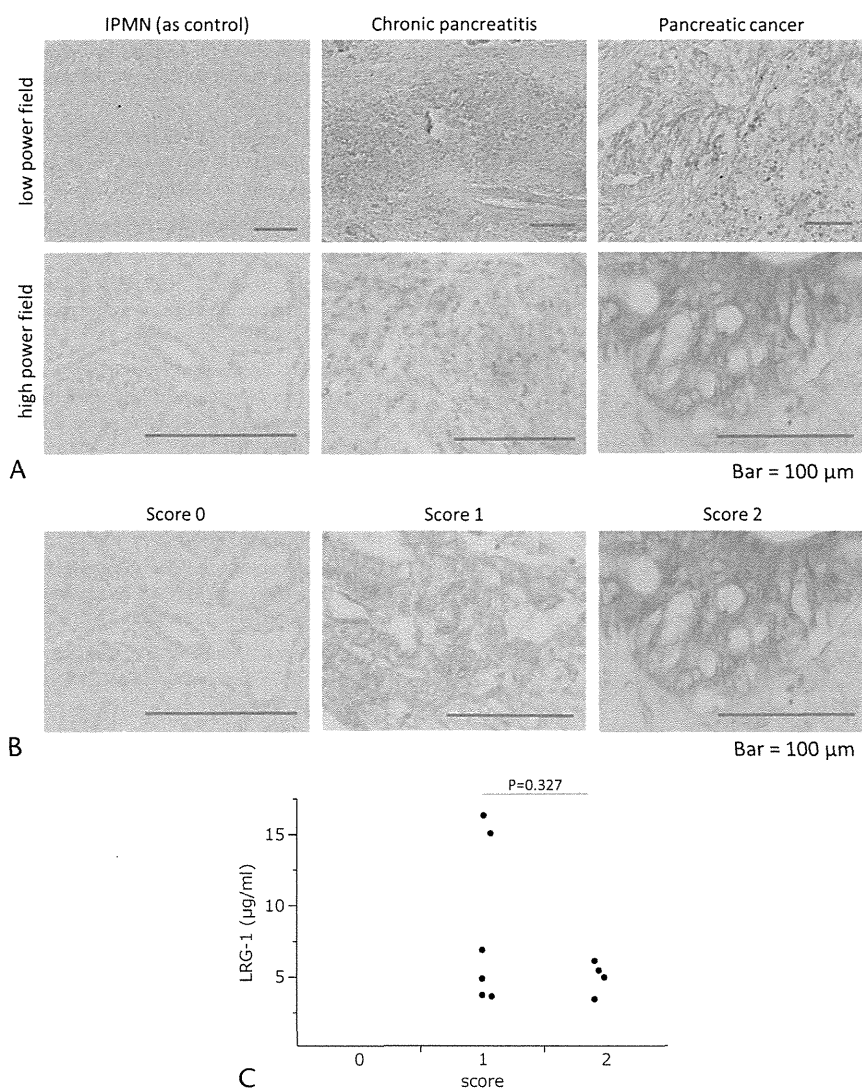


FIGURE 4. Immunohistochemistry of LRG-1 to assess the expression. A, In IPMN cases, the pancreatic duct cells were not stained. In the CP case, the pancreatic duct cells were not stained, but inflammatory cells, such as lymphocytes, stained lightly. In all PDAC cases, the pancreatic cancer cells were stained. Scale bar = 100 μm. B, The intensity of LRG-1 was scored on a scale from 0 to 2 (0, no or faint staining; 1, moderate staining; and 2, strong staining). Scale bar = 100 μm. C, No correlation was found between serum LRG-1 levels and staining intensity ($P = 0.327$).

endothelial growth factor are related to angiogenesis, and targeted agents that are capable of inhibiting signal transduction through epithelial growth factor receptor and vascular endothelial growth factor have already been developed and are used clinically.²⁴ More recently, LRG-1 was reported to promote angiogenesis by modulating endothelial transforming growth factor β signaling.²⁵ These results imply that LRG-1 may be a therapeutic target for controlling pathogenic angiogenesis in cancer.

In conclusion, the present study clearly shows that LRG-1 has promise as a novel tumor biomarker for PDAC.

REFERENCES

1. Jemal A, Siegel R, Xu J, et al. Cancer Statistics, 2010. *CA Cancer J Clin.* 2011;61:133–134.
2. Ministry of Health, Labour and Welfare (2010). The Dynamic Statistics of the Population in 2010. Available at: http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/kakutei11/d/11_h7.pdf. Accessed December 12, 2012.
3. Wagner M, Redaelli C, Lietz M, et al. Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. *Br J Surg.* 2004;91:586–594.
4. Neoptolemos JP, Stocken DD, Bassi C, et al. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. *JAMA.* 2010;304:1073–1081.
5. Jamieson NB, Carter CR, McKay CJ, et al. Tissue biomarkers for prognosis in pancreatic ductal adenocarcinoma: a systematic review and meta-analysis. *Clin Cancer Res.* 2011;17:3316–3331.
6. Humphris JL, Chang DK, Johns AL, et al. The prognostic and predictive value of serum CA19-9 in pancreatic cancer. *Ann Oncol.* 2012;23:1713–1722.
7. Yachida S, Jones S, Bozic I. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature.* 2010;467:1114–1117.
8. Haupt H, Baudner S. Isolation and characterization of an unknown, leucine-rich 3.1-S-alpha2-glycoprotein from human serum. *Hoppe Seylers Z Physiol Chem.* 1977;358:639–646.

9. Andersen JD, Boylan K, Jemmerson R, et al. Leucine-rich alpha-2-glycoprotein-1 is upregulated in sera and tumors of ovarian cancer patients. *J Ovarian Res.* 2010;3:21.
10. Okano T, Kondo T, Kakisaka T, et al. Plasma proteomics of lung cancer by a linkage of multi-dimensional liquid chromatography and two-dimensional difference gel electrophoresis. *Proteomics.* 2006;6:3938–3948.
11. Sandanayake NS, Sinclair J, Andreola F, et al. A combination of serum leucine-rich α -2-glycoprotein-1, CA19-9 and interleukin-6 differentiate biliary tract cancer from benign biliary strictures. *Br J Cancer.* 2011;105:1370–1378.
12. Sarvari J, Mojtahedi Z, Kuramitsu Y, et al. Differential expression of haptoglobin isoforms in chronic active hepatitis, cirrhosis and HCC related to HBV infection. *Oncol Lett.* 2011;2:871–877.
13. Ladd JJ, Busald T, Johnson MM, et al. Increased plasma levels of the APC-interacting protein MAPRE1, LRG1, and IGFBP2 preceding a diagnosis of colorectal cancer in women. *Cancer Prev Res (Phila).* 2012;5:655–664.
14. Kakisaka T, Kondo T, Okano T, et al. Plasma proteomics of pancreatic cancer patients by multi-dimensional liquid chromatography and two-dimensional difference gel electrophoresis (2D-DIGE): up-regulation of leucine-rich alpha-2-glycoprotein in pancreatic cancer. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2007;852:257–267.
15. Serada S, Fujimoto M, Terabe F, et al. Serum leucine-rich alpha-2 glycoprotein is a disease activity biomarker in ulcerative colitis. *Inflamm Bowel Dis.* 2012;18:2169–2179.
16. Kharbanda AB, Raj AJ, Cosme Y, et al. Novel serum and urine markers for pediatric appendicitis. *Acad Emerg Med.* 2012;19:56–62.
17. Ai J, Druhan LJ, Hunter MG, et al. LRG-accelerated differentiation defines unique G-CSFR signaling pathways downstream of PU.1 and C/EBPepsilon that modulate neutrophil activation. *J Leukoc Biol.* 2008;83:1277–1285.
18. Kentsis A, Ahmad S, Kurek K, et al. Detection and diagnostic value of urine leucine-rich α -2-glycoprotein in children with suspected acute appendicitis. *Ann Emerg Med.* 2012;60:78–83.
19. Japan Pancreas Society. *General Rules for the Study of Pancreatic Cancer* 6th ed. Japan: Kanehara & Co Ltd; 2009.
20. Kawakami T, Hoshida Y, Kanai F, et al. Proteomic analysis of sera from hepatocellular carcinoma patients after radiofrequency ablation treatment. *Proteomics.* 2005;5:4287–4295.
21. Takahashi N, Takahashi Y, Putnam FW. Periodicity of leucine and tandem repetition of a 24-amino acid segment in the primary structure of leucine-rich alpha 2-glycoprotein of human serum. *Proc Natl Acad Sci.* 1985;82:1906–1910.
22. Shirai R, Gotou R, Hirano F, et al. Autologous extracellular cytochrome c is an endogenous ligand for leucine-rich α 2-glycoprotein and β -type phospholipase A2 inhibitor. *J Biol Chem.* 2010;285:21607–21614.
23. Codina R, Vanasse A, Kelekar A, et al. Cytochrome c-induced lymphocyte death from the outside in: inhibition by serum leucine-rich alpha-2-glycoprotein-1. *Apoptosis.* 2010;15:139–152.
24. Kim ST, Park KH, Shin SW, et al. Dose KRAS mutation status affect on the effect of VEGF therapy in metastatic colon cancer patients? *Cancer Res Treat.* 2014;46:48–54.
25. Wang X, Abraham S, McKenzie JA, et al. LRG1 promotes angiogenesis by modulating endothelial TGF- β signaling. *Nature.* 2013;499:306–311.

Original article

Proteomic identification of heterogeneous nuclear ribonucleoprotein K as a novel cold-associated autoantigen in patients with secondary Raynaud's phenomenonLingli Yang^{1,2}, Minoru Fujimoto², Hiroyuki Murota¹, Satoshi Serada², Manabu Fujimoto³, Hiromi Honda², Kohji Yamada^{2,4}, Katsuya Suzuki⁵, Ayumi Nishikawa⁵, Yuji Hosono⁶, Yoshihiro Yoneda⁷, Kazuhiko Takehara³, Yoshitaka Imura⁶, Tsuneyo Mimori⁶, Tsutomu Takeuchi⁵, Ichiro Katayama¹ and Tetsuji Naka²**Abstract****Objective.** The aim of this study was to identify cold-associated autoantibodies in patients with RP secondary to CTDs.**Methods.** Indirect immunofluorescence staining was performed on non-permeabilized cold-stimulated normal human dermal microvascular endothelial cells (dHMVECs), using patients' sera. Cold-induced alterations in cell surface proteomes were analysed by isobaric tag for relative and absolute quantitation (iTRAQ) analysis. Serological proteome analysis (SERPA) was applied to screen cold-associated autoantigens. The prevalence of the candidate autoantibody was determined by ELISA in 290 patients with RP secondary to CTDs (SSc, SLE or MCTD), 10 patients with primary RP and 27 healthy controls.**Results.** Enhanced cell surface immunoreactivity was detected in cold-stimulated dHMVECs when incubated with sera from patients with secondary RP. By iTRAQ analysis, many proteins, including heterogeneous nuclear ribonucleoprotein K (hnRNP-K), were found to be increased on the cell surface of dHMVECs after cold stimulation. By the SERPA approach, hnRNP-K was identified as a candidate autoantigen in patients with secondary RP. Cold-induced translocation of hnRNP-K to the cell surface was confirmed by immunoblotting and flow cytometry. By ELISA analysis, patients with secondary RP show a significantly higher prevalence of anti-hnRNP-K autoantibody (30.0%, 61/203) than patients without RP (9.2%, 8/87, $P=0.0001$), patients with primary RP (0%, 0/10, $P=0.0314$) or healthy controls (0%, 0/27, $P=0.0001$).**Conclusion.** By comprehensive proteomics, we identified hnRNP-K as a novel cold-associated autoantigen in patients with secondary RP. Anti-hnRNP-K autoantibody may potentially serve as a biomarker for RP secondary to various CTDs.**Key words:** proteomics, Raynaud's phenomenon, autoantibody, heterogeneous nuclear RNP-K.¹Department of Dermatology, Osaka University Graduate School of Medicine, ²Laboratory of Immune Signal, National Institute of Biomedical Innovation, ³Department of Dermatology, Kanazawa University, Kanazawa, ⁴Biomolecular Dynamics Group, Graduate School of Frontier Biosciences, Osaka University, Osaka, ⁵Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, ⁶Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, KyotoUniversity, Kyoto and ⁷National Institute of Biomedical Innovation, Osaka, Japan.

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Introduction

Advances in proteomics technologies have enabled us to identify proteins extracted from various clinical samples. Recently an innovative multiplexed quantitative proteomic technology called isobaric tag for relative and absolute quantification (iTRAQ) has been successfully used to detect disease-related proteins from cultured cells and clinical samples [1]. This novel technology can not only identify proteins in samples, but also compare relative expression levels of detected proteins from between four to eight different samples [2]. In addition, when samples enriched with cell surface proteins are analysed, iTRAQ technology can characterize differences in cell surface proteomes between samples [3].

RP is an exaggerated vasoconstrictive response of the fingers and toes to external stress such as cold temperatures [4]. RP is characterized classically as the episodic colour change of blanching, cyanosis and rubor in response to external stress [5]. In most patients, this colour change is a benign, reversible condition and no underlying disease is detectable (i.e. primary RP) [4]. However, in some patients RP is the early manifestation of underlying disease (i.e. secondary RP), most frequently of CTDs, including SSc [4], SLE [6] and MCTD [7]. RP in CTD patients is more severe than primary RP and causes tissue damage, including digital ulcers and gangrene, presumably due to endothelial abnormalities [8]. Moreover, given the substantial morbidity in patients with CTDs, RP patients developing CTDs should be promptly identified and managed. Thus it is important to distinguish between primary and secondary RP, but the diagnostic methods have not been established.

Because autoantibody production is a common hallmark of CTDs, RP secondary to CTDs might have a similar autoimmune pathogenesis. We therefore hypothesized that cold stimulation, as an environmental cue, may contribute to the triggering of local autoimmune reactions in patients with CTD-related secondary RP. In this study, taking advantage of iTRAQ technology, we detected cold-induced alteration in cell surface proteomes of endothelial cells. We then obtained candidate autoantigens in RP by serological proteome analysis (SERPA) [9–11] combining two-dimensional electrophoresis and western blotting using patients' sera. Our analyses collectively suggest that in patients with secondary RP, heterogeneous nuclear ribonucleoprotein K (hnRNP-K) is a cold-associated autoantigen that translocates onto the cell surface by cold stimulation.

Materials and methods

Human serum samples

Between March 2003 and January 2012, Japanese patients referred to hospitals of Osaka University (Osaka, Japan), Kyoto University (Kyoto, Japan), Kanazawa University (Kanazawa, Japan) and Keio University (Tokyo, Japan) were newly evaluated for RP as well as for SSc according to ACR criteria [12], SLE

according to ACR criteria [13, 14] or MCTD according to the criteria proposed by the Ministry of Health and Welfare in Japan [15]. RP was defined by repeated episodes of biphasic or triphasic colour change on cold exposure and non-RP was defined by no colour changes on cold exposure, as described previously [16]. The possible RP of unilateral or uniphasic colour change was not included in this study. After these exclusions, randomly selected patients, including 155 well-defined SSc with RP [SSc-RP(+)] patients, 54 SLE without RP [SLE-RP(-)] patients, 1 MCTD without RP [MCTD-RP(-)] patient and 10 MCTD with RP [MCTD-RP(+)] patients, were included in this study. SSc without RP [SSc-RP(-)] patients ($n=32$) and SLE with well-defined RP [SLE-RP(+)] ($n=38$) patients, the frequencies of which are substantially low, were intentionally recruited and included in the study. Sera and characteristics (demographic characteristics, autoantibody profile) of CTD patients were collected at the time of diagnosis. Ten patients with primary RP who had well-defined RP for >2 years and had normal findings on laboratory tests, negative findings on autoantibody assays and no objective clinical signs of CTD or other diseases were also included. These patient characteristics are summarized in Table 1. As controls, healthy individuals with no evidence of any physical disorder were included [$n=27$; male:female ratio 3:24, average age 51.0 years (s.d. 13.2)]. All patients gave written informed consent prior to inclusion and this study was approved by the Medical Ethics Committees of the National Institute of Biomedical Innovation (Osaka, Japan), Osaka University Hospital, Kyoto University Hospital, Kanazawa University Hospital and Keio University Hospital.

Cell culture and cold stimulation

Normal human dermal microvascular (MV) endothelial cells (dHMVECs, passages 4–6) were purchased from Takara Bio (Otsu, Japan). dHMVECs were cultured on type 1 collagen-coated plates (Iwaki Glass, Tokyo, Japan) in endothelial basal medium 2 supplemented with endothelial cell growth medium 2 MV SingleQuots (Clonetics, San Diego, CA, USA). Confluent dHMVECs cells were incubated in serum-containing medium at 10°C (cold stress) or 37°C (control) in a 5% CO₂ humidified incubator for the periods indicated. Cells were maintained at 10°C or 37°C during the washing/detachment procedure. Suspended cells were then harvested by centrifugation at room temperature. Before and after cold stimulation, cell viability was verified by trypan blue exclusion assay. In some experiments, cells were further fixed with 4% paraformaldehyde at room temperature.

Indirect immunofluorescence staining

dHMVECs were seeded on six-well plates precoated with type 1 collagen (5×10^4 cells/well) and grown to confluence. Cells were fixed with 4% paraformaldehyde. Non-permeabilized cells were then incubated with sera from patients and healthy controls (HCs; 1:500 dilution) for 1 h at room temperature, followed by incubation with FITC-conjugated rabbit anti-human IgG (1:1000 dilution;

TABLE 1 Patient characteristics (n = 300 with CTDs or primary RP)

Variable	SSc (n = 187)	SLE (n = 92)	MCTD (n = 11)	Primary RP (n = 10)
Age, mean (s.d.), years	55.3 (13.8)	40.3 (14.9)	59.5 (16.3)	25.4 (3.1)
Gender (F:M), n:n	156:31	86:6	11:0	9:1
Smoking, % (n/N)	44.2 (50/163)	25.8 (16/46)	0 (0/11)	0 (0/10)
ANA positivity, % (n/N)	91.4 (167/183)	96.7 (89/92)	100 (10/10)	0 (0)
Anti-Scl-70, % (n/N)	31.0 (58/187)	NA	NA	0 (0/10)
Anti-centromere, % (n/N)	33.7 (63/187)	NA	9.1 (1/11)	0 (0/10)
Anti-Sm, % (n/N)	NA	34.5 (30/87)	9.1 (1/11)	NA
Anti-dsDNA, % (n/N)	NA	65.1 (54/83)	0 (0/3)	NA
Anti-U1-RNP, % (n/N)	7.5 (14/187)	50.0 (43/86)	100.0 (11/11)	0 (0/10)
Anti-SSA/Ro, % (n/N)	7.5 (14/187)	57.8 (52/87)	45.5 (5/11)	NA

Scl-70: topoisomerase I; Sm: Smith; U1-RNP: U1 ribonucleoprotein; N: the number of available patients (varies according to the number of available observations); NA: not available.

Dako Immunoglobulins, Copenhagen, Denmark). Cells counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) were visualized using a Biozero microscope (Keyence, Itasca, IL, USA).

Capture of cell surface proteins

After cold stimulation (10°C) for the indicated periods (0 min, 30 min, 1 h, 3 h), dHMVEC cells were washed with pre-warmed (37°C) or precooled (10°C) PBS three times and cell surface proteins were then labelled by biotinylation and pulled down by avidin-agarose resin as described previously [3]. The isolated cell surface proteins were kept at -20°C until use.

iTRAQ analysis

Cell surface proteins described above were digested by trypsin and separately labelled with the iTRAQ reagent (Applied Biosystems, Foster City, CA, USA) as described previously [3]. Briefly, iTRAQ reagents 114–117 were used to label cells without cold stimulation (baseline), cells with cold stimulation at 10°C for 30 min, cells with cold stimulation at 10°C for 1 h and cells with cold stimulation at 10°C for 3 h, respectively. Samples were then pooled and fractionated by strong cation exchange chromatography [3]. Nano liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses were performed on an LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA) as described previously [3]. Protein identification and quantification for iTRAQ analysis were carried out using Proteome Discoverer software (version 1.1; Thermo Fisher Scientific) against the Swiss Prot protein database [SwissProt 2010_10 (521 016 sequences)] as described previously [3] with modifications as follows: carbamidomethylation and iTRAQ4plex (Lys, N-terminal) were specified as static modifications, whereas CAMthiopropionyl (Lys, N-terminal), iTRAQ4plex (Tyr) and oxidation (Met) were specified as variable modifications in the database search. The mass spectrometry raw data and the data of peptide identifications were uploaded to PeptideAtlas (<http://www.peptideatlas.org/PASS/PASS00387>). Information about the subcellular

localization of detected proteins was obtained by using UniprotKB (<http://www.uniprot.org/>).

Serological proteome analysis

Proteins were extracted from cultured dHMVEC cells using the Complete Mammalian Proteome Extraction Kit (Calbiochem, La Jolla, CA, USA) and stored at -80°C until use. Two-dimensional electrophoresis and immune blotting analysis using patient or HC sera were performed as previously described [10, 11]. Protein spots in a silver-stained gel, corresponding to positive spots on western blot membranes, were excised from the gel and digested in gel as described previously [17].

Preparation of recombinant human hnRNP-K

Full-length hnRNP-K cDNA was amplified from total cDNA of dHMVEC using KOD-plus (Toyobo, Osaka, Japan) with the following primers: 5'-TGGAAGTGAACAGCCAGAAG AA-3' (forward) and 5'-GCATTAGAATCCTTCAACATCTG C-3' (reverse). Full-length hnRNP-K cDNA was subcloned into a pET28 vector (Novagen, Madison, WI, USA), resulting in expression of hnRNP-K with a 6 × His tag. The DNA sequence was confirmed using the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Recombinant protein was produced in *Escherichia coli* as described previously [10, 11].

ELISA

The ELISA assay was performed using MaxiSorp plates (Nunc A/S, Roskilde, Denmark) coated with 1 µg/well of recombinant human full-length hnRNP-K protein. After dilution (1:500) with *E. coli* cell lysates to block the non-specific reactivity of sera with bacterial proteins, sera were incubated with plate-bound hnRNP-K for 1 h, followed by detection of antigen–antibody complexes by horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Dako, Carpinteria, CA, USA) as described previously [10, 11]. Optical density (OD) was read at 450 nm on a Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). The antibody titre was expressed using arbitrary binding units calculated

according to the following formula: binding units of sample = $[OD_{\text{sample}} / (\text{mean } OD_{\text{HC sera}} + 3 \text{ s.d.}_{\text{HC sera}})] \times 100$. Based on this formula, 100 binding units was used as the cut-off point.

Western blot analysis

Extracted proteins were subjected to western blot analysis as previously described [18] with the following antibodies: serum at 1:200 dilution; anti-hnRNP-K (1:500 dilution; Cell Signaling Technology, Danvers, MA, USA); anti-VE-cadherin (1:500 dilution; BD Transduction Laboratories, San Jose, CA, USA); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), followed by rabbit anti-human IgG, donkey anti-rabbit or sheep anti-mouse HRP-conjugated secondary antibodies (1:5000 dilution; GE Healthcare, Piscataway, NJ, USA) and visualized with Western Lightning Plus ECL reagent (Perkin-Elmer, Boston, MA, USA).

Fluorescence-activated cell sorting

After stimulation, cells fixed with 4% paraformaldehyde were incubated with rabbit anti-human hnRNP-K antibody at 1:100 dilution and labelled with FITC-conjugated goat anti-rabbit immunoglobulin (BD Biosciences, San Jose, CA, USA). Normal rabbit IgG was used as a control. Stained cells were analysed using a FACS Canto II cytometer (Becton-Dickinson, Mountain View, CA, USA) and the results were analysed using FlowJo software (TreeStar, Ashland, OR, USA).

Statistical analysis

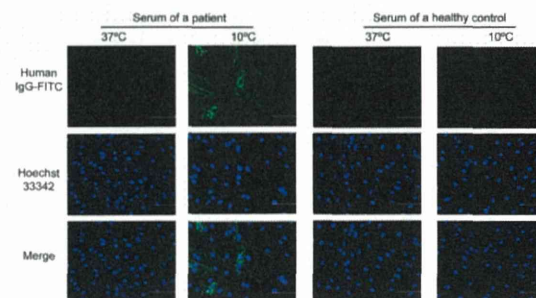
Continuous variables are expressed as mean (s.d.) and proportions were used for categorical variables. Means were compared with Student's *t*-test, highly skewed distributions were compared using the Mann-Whitney *U* test and proportions were compared using Fisher's exact test. Differences between groups were compared using the Kruskal-Wallis test followed by Steel's test. The data were entered and analysed using Excel statistical software (Microsoft, Redmond, WA, USA). Significance was defined as $P < 0.05$.

Results

Cold-induced autoimmune reactions on the cell surface

To determine whether cold can affect autoimmune reactions on the surface of vascular endothelial cells, non-permeabilized dHMVECs pretreated with or without cold stimulation were incubated with sera from patients with RP secondary to SSc or HCs. By indirect immunofluorescence staining using sera from patients, cell surface immunoreactivity was strongly induced in cold-stimulated dHMVECs (eight of nine patients with SSc-related RP; Fig. 1), but not in unstimulated cells. In contrast, no distinct immunostaining was observed in cold-stimulated or unstimulated cells when incubated with sera from HCs ($n=3$; Fig. 1). These results indicate that

Fig. 1 Indirect immunofluorescence staining



Non-permeabilized cultured dHMVECs with or without cold stimulation were incubated with sera (1:500 dilution) from patients with SSc-related secondary RP ($n=9$) or healthy controls ($n=3$) ($\times 100$ magnification). Representative images are shown here. FITC-conjugated rabbit anti-human IgG (green); Hoechst 33342 (blue); bar: 100 μm . dHMVECs: dermal human microvascular endothelial cells.

pretreatment of dHMVECs with cold is critical in inducing the interaction between cell surface autoantigens and sera from patients.

Identification of cold-induced surface proteome alterations in dHMVECs by iTRAQ analysis

The results above suggest that proteins including autoantigens in dHMVECs might be exposed to the cell surface in response to cold stimulation. Therefore we next investigated cold-induced cell surface proteome alterations in dHMVECs. Cells were stimulated with cold (10°C for 0 min, 30 min, 1 h and 3 h) and cell surface proteins enriched by a biotinylation-based approach were quantitatively analysed by iTRAQ technology using nano LC-MS/MS analysis. As listed in supplementary Table S1, available at *Rheumatology* Online, 1581 proteins were identified. According to the annotation from UniprotKB, 451 proteins (28.5%) are located mainly in the plasma membrane, 503 proteins (31.8%) in the cytoplasm, 238 proteins (15.1%) in the nucleus and the remaining proteins (24.6%) in other subfractions such as the mitochondrion and the endoplasmic reticulum. The expression of ~50% of the proteins increased >2 -fold after cold stimulation at 10°C for 3 h (see supplementary Table S1, available at *Rheumatology* Online). The top 30 highly increased proteins are listed in Table 2.

Identification of autoantigens by SERPA in patients with secondary RP

We then used a SERPA approach to screen autoantigens associated with secondary RP. At first, proteins from dHMVEC lysates were separated on two-dimensional gels and were visualized by silver staining (Fig. 2A) or were transferred to membranes for immunoblotting. These membranes were incubated with sera from nine

TABLE 2 List of differentially expressed proteins on the cell surface of dHMVECs after cold stimulation

Accession	Protein name	0.5h	1h	3h
Q96RS6	NudC domain-containing protein 1	3.25	4.24	6.18
P10301	Ras-related protein R-Ras	1.35	3.90	5.95
Q92797	Symplekin	2.94	3.81	5.54
Q643R3	Lysophospholipid acyltransferase LPCAT4	3.06	3.00	5.45
Q99808	Equilibrative nucleoside transporter 1	2.62	3.69	5.21
Q9Y6I9	Testis-expressed sequence 264 protein	2.66	4.11	5.12
Q8TEQ6	Gem-associated protein 5	4.71	6.03	5.08
Q2TAL8	Glutamine-rich protein 1	3.03	4.56	4.99
Q08379	Golgin subfamily A member 2	2.41	4.10	4.74
P49916	DNA ligase 3	2.52	2.94	4.74
P35659	Protein DEK	2.04	3.65	4.67
O60264	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	1.87	4.38	4.52
P60709	Actin, cytoplasmic 1	3.03	3.67	4.28
P55265	Double-stranded RNA-specific adenosine deaminase	2.22	3.86	4.27
Q16643	Drebrin	2.60	3.75	4.24
Q6DD88	Atlastin-3	2.09	3.83	4.14
P29966	Myristoylated alanine-rich C-kinase substrate	1.89	3.61	4.10
Q9ULT8	E3 ubiquitin-protein ligase HECTD1	1.74	3.63	4.08
P61019	Ras-related protein Rab-2A	2.04	3.63	4.03
P61978	hnRNP-K	2.12	3.75	4.03
P01112	GTPase HRas	1.68	2.01	3.95
Q07666	KH domain-containing, RNA-binding, signal transduction-associated protein 1	1.74	3.54	3.92
Q9NZ01	Trans-2,3-enoyl-CoA reductase	1.77	2.08	3.89
Q9NQW6	Actin-binding protein anillin	3.78	5.01	3.86
O00483	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	2.12	2.94	3.86
Q9NP72	Ras-related protein Rab-18	1.96	3.20	3.83
O94901	Protein unc-84 homolog A	2.04	2.77	3.79
P38646	Stress-70 protein, mitochondrial	1.83	3.39	3.76
Q96S97	Myeloid-associated differentiation marker	2.49	3.10	3.75
P49585	Choline-phosphate cytidylyltransferase A	2.68	3.62	3.74

Values are shown as relative expression levels (at 10°C for 0.5, 1 and 3 h) compared with baseline (at 37°C). dHMVECs: dermal human microvascular endothelial cells; hnRNP-K: heterogeneous nuclear ribonucleoprotein K.

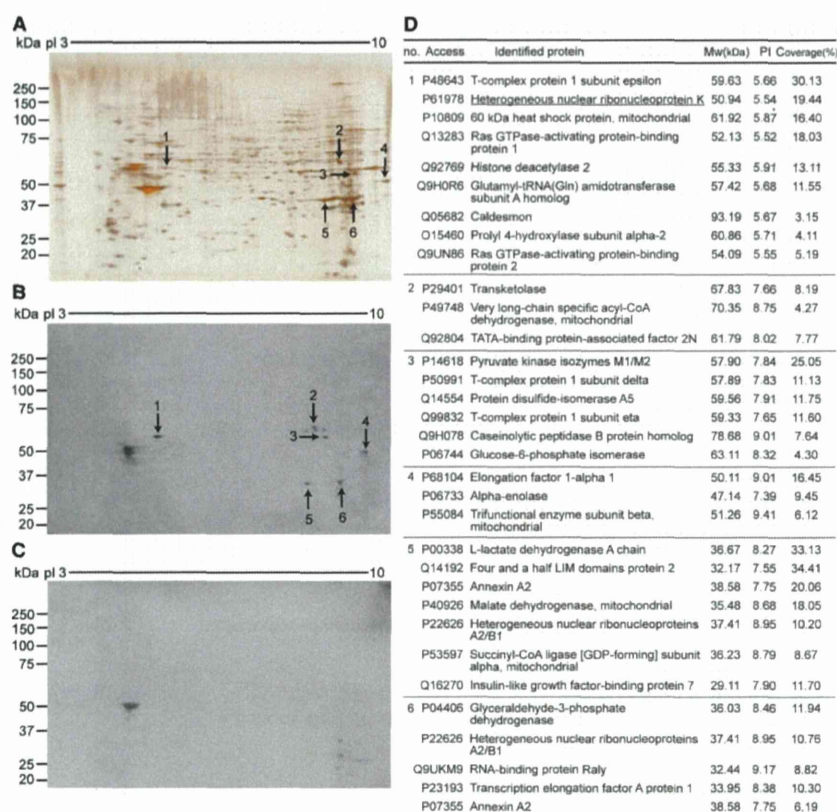
patients with RP secondary to SSC (Fig. 2B) or three HCs (Fig. 2C). Six specific spots, which were recognized by sera from >75% of patients, but not by those from HCs, were selected (Fig. 2B). Proteins extracted from the corresponding spots on the silver-stained gel (Fig. 2A) were then subjected to LC-MS/MS analysis and candidate autoantigens were identified by a database search as detailed in Fig. 2D. Among identified candidates, hnRNP-K, a member of the nuclear proteins involved in nucleic acid metabolism [19], was also found in the list of increased proteins on the cell surface under cold stimulation (Table 2), suggesting that hnRNP-K might be a cold-related autoantigen in patients with secondary RP.

Cell surface expression of hnRNP-K under cold stimulation was confirmed by western blot and FACS analysis

The hnRNP proteins are among the most abundant proteins in the nucleus [20]. Indeed, hnRNP-K in dHMVECs under physiological culture conditions was stained

intensely in the nucleus by immunofluorescence analysis (see supplementary Fig. S1A and B, available at *Rheumatology* Online). Cold stimulation (10°C for 3 h) induced extranuclear localization of hnRNP-K (see supplementary Fig. S1C and D, available at *Rheumatology* Online), which was nonetheless overwhelmed by intensive staining of nuclear hnRNP-K. To investigate further the appearance of hnRNP-K on the cell surface after cold stimulation, we next performed western blot and FACS analysis. By western blot analysis using concentrated cell surface proteins, hnRNP-K clearly increased in dHMVECs after cold stimulation, but VE-cadherin did not (Fig. 3A). In contrast, the expression level of total hnRNP-K was found to be unchanged during cold stimulation, suggesting that hnRNP-K was not newly synthesized after cold stimulation (Fig. 3A). By FACS analysis, cold-induced expression of hnRNP-K on the cell surface was further confirmed (Fig. 3B). These results collectively suggest that hnRNP-K is a protein that translocates to the cell surface on cold stimulation.

Fig. 2 Identification of autoantigens by SERPA



(A–C) Total protein extracts of dHMVECs were separated by two-dimensional PAGE followed by silver staining analysis (A) or by western blot analysis with diluted sera from SSc patients with RP (B) or from healthy controls (C). Arrows and numbers indicate protein spots that were recognized only by the patients' sera. Representative images are shown here. (D) List of proteins identified by LC-MS/MS analysis (Mw: molecular weight; pI: isoelectric point). dHMVECs: dermal human microvascular endothelial cells; LC-MS/MS: liquid chromatography–tandem mass spectrometry.

Detection of anti-hnRNP-K antibody in sera from patients with secondary RP by western blot and ELISA analysis

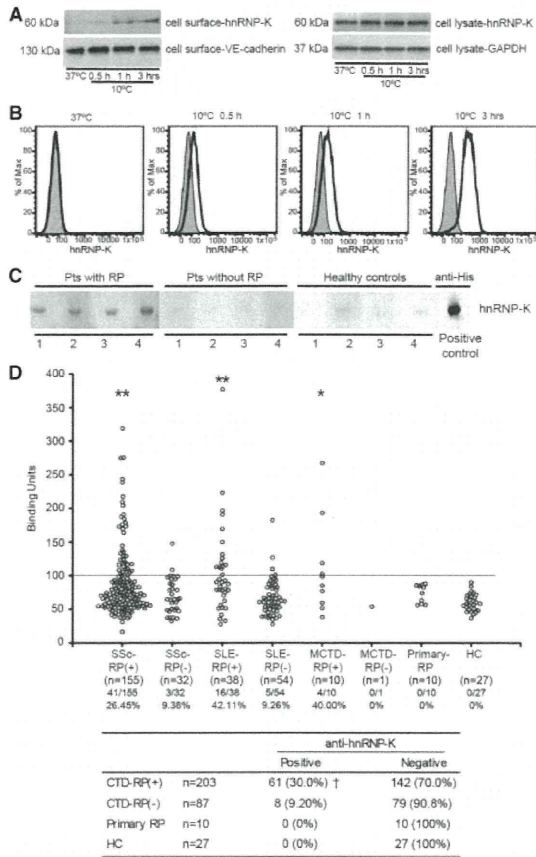
To confirm that hnRNP-K is the autoantigen recognized by sera from patients with secondary RP, full-length recombinant human hnRNP-K protein was prepared and subjected to western blot analysis. As disease controls, SSc patients without RP were intentionally recruited to the study and their sera were investigated. Notably, intense reactivity against recombinant hnRNP-K protein was visualized in sera from SSc patients with RP, but not sera from SSc patients without RP or from HCs (Fig. 3C). This result implies that anti-hnRNP-K antibody is relevant to RP rather than to SSc itself.

We then developed an ELISA system using recombinant hnRNP-K protein to screen anti-hnRNP-K antibody in various CTD patients (SSc, SLE and MCTD) with or without RP and in patients with primary RP (Table 1). As shown in Fig. 3D (top), ELISA analysis revealed that

significant elevations of anti-hnRNP-K antibody levels were observed in patients with SSc-, SLE- and MCTD-related RP, but not in patients without RP or those with primary RP compared with HCs (Fig. 3D, top). The prevalence of autoantibodies against hnRNP-K was 26.5% in SSc patients with RP, 42.1% in SLE patients with RP and 40.0% in MCTD patients with RP. In contrast, anti-hnRNP-K positivity was markedly low in CTD patients without RP and was 0% in primary RP patients and HCs (Fig. 3D, top). These results suggest that anti-hnRNP-K antibody is relevant to CTD-related secondary RP rather than to a single CTD.

Of all the CTDs (SSc, SLE and MCTD) in this study, a 30.0% prevalence of anti-hnRNP-K antibody was observed in patients with RP, much higher than that in patients without RP (9.20%, $P=0.0001$), in patients with primary RP (0%, $P=0.0314$) or in HCs (0%, $P=0.0003$) (Fig. 3D, bottom). Anti-hnRNP-K-positive CTD patients had a significantly higher prevalence of RP (88.4%) than

Fig. 3 Cell surface expression of hnRNP-K after cold stimulation and the presence of anti-hnRNP-K autoantibody in sera from patients with secondary RP



(A) Biotinylated surface proteins from dHMECs incubated at 37°C or 10°C (indicated periods) were precipitated with streptavidin-conjugated agarose beads and analysed by western blot analysis using anti-hnRNP-K. Levels of VE-cadherin surface protein (left panel) and GAPDH expression in total cell lysates (right panel) are shown as controls. (B) Cell surface hnRNP-K expression in dHMECs was analysed by flow cytometry. Shaded histogram indicates staining with control IgG. (C) His-tagged recombinant hnRNP-K protein was subjected to western blot analysis using diluted sera from SSc patients with RP, without RP and healthy controls or with anti-6 × His antibody. (D) (Top) Serum anti-hnRNP-K antibody levels in indicated CTD patients (with or without RP), primary RP patients and healthy controls were determined by ELISA using recombinant human hnRNP-K. The y-axis denotes binding units. The solid horizontal line indicates the positive cut-off limit as described in Materials and methods. Note that this cohort contains a relatively high number of SSc without RP patients and SLE with RP patients, because those patients were intentionally recruited to this study. **P* < 0.05, ***P* < 0.01 vs HCs. (Bottom) Anti-hnRNP-K positivity in patients is summarized. CTD

anti-hnRNP-K-negative patients (64.3%, *P* = 0.0001; Table 3). Anti-hnRNP-K antibody showed no correlation with age, gender, smoking or antibodies to U1 ribonucleoprotein (U1-RNP), centromere or SSA/Ro in CTD patients (Table 3). In addition, anti-hnRNP-K showed no correlation with autoantibodies against topoisomerase I (Scl-70) in SSc patients or those against dsDNA or Sm in SLE patients (data not shown). Thus anti-hnRNP-K antibody might be a novel class of biomarker for a subgroup of patients with CTD-related secondary RP.

Discussion

Environmental factors are implicated in the pathogenesis of autoimmune diseases. Previously translocation of intracellular autoantigens to the cell surface was described in ultraviolet radiation-stimulated keratinocytes [21–23]. In the present study, using comprehensive proteomics approaches, we identified hnRNP-K as a novel autoantigen that translocates to the cell surface of dHMECs after cold stimulation. ELISA analysis revealed that anti-hnRNP-K antibody was significantly elevated in patients with RP secondary to CTDs, but not in patients with primary RP. Thus our data imply hnRNP-K is a putative secondary RP-associated autoantigen that is exposed on the cell surface by cold stimulation.

iTRAQ analysis indicated that cold stimulation in dHMECs could induce cell surface expression of a variety of proteins, including intracellular proteins, without an overt effect on cell viability, as seen by trypan blue exclusion assay (data not shown). Previous lines of evidence indicate that cytoplasmic and nuclear proteins can reach the cell surface by Golgi-independent non-conventional transport pathways [24–26]. However, it is unknown how cold stimulation induces the translocation of hnRNP-K and other intracellular proteins. Further study is currently under way in our laboratory.

Among many up-regulated proteins identified by iTRAQ analysis, hnRNP-K was the only protein simultaneously identified by SERPA screening. However, our study does not exclude the possibility that there remain other cold-associated autoantigens among these proteins. In particular, since the SERPA approach has limitations in detecting native antigens, antibodies that recognize only native-form autoantigens might have been missed in our study. A screening strategy other than SERPA is necessary to clarify this issue.

hnRNP-K is a 464 amino acid nuclear protein with three K homology domains that mediate DNA and RNA binding [27]. Autoantibodies to hnRNP-K have previously been detected in sera from patients with aplastic anaemia and

patients are combined here. Values are the number (%) of patients. †*P* = 0.00001 vs CTD-RP(-), *P* = 0.0314 vs primary RP, *P* = 0.0001 vs HCs by Fisher's exact test.

dHMECs: dermal human microvascular endothelial cells; hnRNP-K: heterogeneous nuclear ribonucleoprotein K; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

TABLE 3 Basic information of 290 patients with CTDs according to the presence of anti-hnRNP-K antibodies

Variable	Positive (n = 69)	Negative (n = 221)	P value ^a
Age, mean (s.d.), years	52.4 (15.6)	53.3 (15.4)	0.3613
Gender (F:M), n:n	61:8	192:29	0.8383
Smoking, % (n/N)	18.9 (10/53)	31.6 (56/177)	0.0841
RP, % (n)	88.4 (61)	64.3 (142)	0.0001
ANA positivity, % (n/N)	94.0 (63/67)	93.1 (203/218)	1.0000
Anti-U1-RNP, % (n/N)	26.5 (18/68)	23.1 (50/166)	0.6255
Anti-SSA/Ro positivity, % (n/N)	27.9 (19/68)	23.6 (52/220)	0.5202

^aUnivariate analysis using the Mann-Whitney U test or Fisher's exact test. N: the number of available patients (varies according to the number of available observations). hnRNP-K: heterogeneous nuclear ribonucleoprotein K.

RA (31% and 24%, respectively), although the co-morbidity of RP in these patients was not described in this report [28]. Autoantibodies to other hnRNPs such as hnRNP-H1 [29], -B1 and -F [30] have also been reported as possible diagnostic markers of CTDs, but their relevance to RP has not been examined.

Although anti-hnRNP-K antibody was identified initially using sera from patients with SSc-related RP, this autoantibody was also detected in sera from RP patients with other CTDs. Notably, anti-hnRNP-K antibody levels in RP patients secondary to SSc, SLE and MCTD were significantly higher than in HCs. In addition, the prevalence of anti-hnRNP-K antibody in SSc patients with RP (26.5%), SLE patients with RP (42.1%) and MCTD patients with RP (40.0%) was markedly higher than in their RP-negative counterparts (9.4%, 9.3% and 0%, respectively) and in primary RP patients (0%). Moreover, anti-hnRNP-K autoantibody was not associated with smoking, which was believed to be a cause of blood vessel narrowing [31], nor with the presence of anti-U1-RNP and ACAs, which are highly related to MCTD and SSc, respectively [32, 33]. Thus these results suggest that anti-hnRNP-K antibody is particularly relevant to CTD-related secondary RP, but not to a single CTD or primary RP. Our findings should be replicated in a larger study.

The pathogenesis of RP likely involves various abnormalities in the vascular, neural and intravascular systems [4]. At present, it remains to be determined whether anti-hnRNP-K autoantibody detected by our ELISA system can bind to hnRNP-K on vascular endothelial cells *in vivo*. In addition, it is unknown whether this autoantibody contributes to the pathogenesis. Because our cohort of patients includes only a limited number of severe RP patients who experienced hospitalization and/or digit loss, it is unknown whether anti-hnRNP-K antibody correlates with the severity of RP. However, it is tempting to speculate that following cold stimulation, anti-hnRNP-K autoantibody may promote antibody-dependent pathogenesis by inducing vascular endothelial damage. As previously reported, anti-endothelial cell antibodies (AECAs) are a heterogeneous group of autoantibodies against

various cell surface proteins in endothelial cells and can contribute leucocyte adhesion by inducing adhesion molecules and cytokines in endothelial cells [34–36]. Anti-hnRNP-K antibody may thus be a class of AECA that becomes active under cold stimulation in patients with secondary RP. Further analyses are needed to elucidate the correlation of anti-hnRNP-K autoantibodies with the pathogenesis of secondary RP.

In summary, we provide the first evidence of cold-associated autoantibodies in patients with secondary RP. Anti-hnRNP-K antibody may be a potential biomarker for RP secondary to CTDs. Longitudinal studies are warranted to determine whether the evaluation of anti-hnRNP-K antibody in RP patients may aid in the detection of CTDs in these patients.

Rheumatology key messages

- Cold-associated autoantibody in patients with secondary RP is successfully identified by a proteomic approach.
- hn-RNP-K translocates to the endothelial cell surface upon cold stimulation.
- Anti-hnRNP-K autoantibody may serve as a biomarker for secondary RP.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

References

- Unwin RD, Griffiths JR, Whetton AD. Simultaneous analysis of relative protein expression levels across multiple samples using iTRAQ isobaric tags with 2D nano LC-MS/MS. *Nat Protoc* 2010;5:1574–82.
- Serada S, Fujimoto M, Ogata A *et al*. iTRAQ-based proteomic identification of leucine-rich alpha-2 glycoprotein as a novel inflammatory biomarker in autoimmune diseases. *Ann Rheum Dis* 2010;69:770–4.
- Yokoyama T, Enomoto T, Serada S *et al*. Plasma membrane proteomics identifies bone marrow stromal antigen 2 as a potential therapeutic target in endometrial cancer. *Int J Cancer* 2013;132:472–84.
- Herrick AL. The pathogenesis, diagnosis and treatment of Raynaud phenomenon. *Nat Rev Rheumatol* 2012;8:469–79.
- Bakst R, Merola JF, Franks AG Jr, Sanchez M. Raynaud's phenomenon: pathogenesis and management. *J Am Acad Dermatol* 2008;59:633–53.
- Dimant J, Ginzler E, Schlesinger M *et al*. The clinical significance of Raynaud's phenomenon in systemic lupus erythematosus. *Arthritis Rheum* 1979;22:815–9.
- Grader-Beck T, Wigley FM. Raynaud's phenomenon in mixed connective tissue disease. *Rheum Dis Clin North Am* 2005;31:465–81, vi.
- Maricq HR, Harper FE, Khan MM, Tan EM, LeRoy EC. Microvascular abnormalities as possible predictors of disease subsets in Raynaud phenomenon and early connective tissue disease. *Clin Exp Rheumatol* 1983;1:195–205.
- Srivastava R, Aslam M, Kalluri SR *et al*. Potassium channel KIR4.1 as an immune target in multiple sclerosis. *N Engl J Med* 2012;367:115–23.
- Serada S, Fujimoto M, Takahashi T *et al*. Proteomic analysis of autoantigens associated with systemic lupus erythematosus: anti-aldolase A antibody as a potential marker of lupus nephritis. *Proteomics Clin Appl* 2007;1:185–91.
- He P, Naka T, Serada S *et al*. Proteomics-based identification of alpha-enolase as a tumor antigen in non-small lung cancer. *Cancer Sci* 2007;98:1234–40.
- Masi AT. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. *Arthritis Rheum* 1980;23:581–90.
- Tan EM, Cohen AS, Fries JF *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271–7.
- Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;40:1725.
- Kasukawa R, Tojo T, Miyawaki S. Preliminary diagnostic criteria for classification of mixed connective tissue disease. In: Kasukawa R, Sharp GC, eds. *Mixed Connective Tissue Diseases and Antinuclear Antibodies*. Amsterdam, the Netherlands: Elsevier, 1987:41–7.
- Brennan P, Silman A, Black C *et al*. Validity and reliability of three methods used in the diagnosis of Raynaud's phenomenon. The UK Scleroderma Study Group. *Br J Rheumatol* 1993;32:357–61.
- Umegaki-Arao N, Tamai K, Nimura K *et al*. Karyopherin alpha2 is essential for rRNA transcription and protein synthesis in proliferative keratinocytes. *PLoS One* 2013;8:e76416.
- Yang L, Serada S, Fujimoto M *et al*. Periostin facilitates skin sclerosis via PI3K/Akt dependent mechanism in a mouse model of scleroderma. *PLoS One* 2012;7:e41994.
- Han SP, Tang YH, Smith R. Functional diversity of the hnRNPs: past, present and perspectives. *Biochem J* 2010;430:379–92.
- Dreyfuss G, Matunis MJ, Pinol-Roma S, Burd CG. hnRNP proteins and the biogenesis of mRNA. *Annu Rev Biochem* 1993;62:289–321.
- Furukawa F, Kashiwara-Sawami M, Lyons MB, Norris DA. Binding of antibodies to the extractable nuclear antigens SS-A/Ro and SS-B/La is induced on the surface of human keratinocytes by ultraviolet light (UVL): implications for the pathogenesis of photosensitive cutaneous lupus. *J Invest Dermatol* 1990;94:77–85.
- Wang B, Dong X, Yuan Z, Zuo Y, Wang J. SSA/Ro antigen expressed on membrane of UVB-induced apoptotic keratinocytes is pathogenic but not detectable in supernatant of cell culture. *Chin Med J* 1999;112:512–5.
- Golan TD, Elkon KB, Gharavi AE, Krueger JG. Enhanced membrane binding of autoantibodies to cultured keratinocytes of systemic lupus erythematosus patients after ultraviolet B/ultraviolet A irradiation. *J Clin Invest* 1992;90:1067–76.
- Nickel W, Seedorf M. Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. *Annu Rev Cell Dev Biol* 2008;24:287–308.
- Nickel W, Rabouille C. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* 2009;10:148–55.
- Nickel W. Pathways of unconventional protein secretion. *Curr Opin Biotechnol* 2010;21:621–6.
- Bomsztyk K, Denisenko O, Ostrowski J. hnRNP K: one protein multiple processes. *Bioessays* 2004;26:629–38.
- Qi Z, Takamatsu H, Espinoza JL *et al*. Autoantibodies specific to hnRNP K: a new diagnostic marker for immune pathophysiology in aplastic anemia. *Ann Hematol* 2010;89:1255–63.
- Van den Bergh K, Hooijkaas H, Blockmans D *et al*. Heterogeneous nuclear ribonucleoprotein h1, a novel nuclear autoantigen. *Clin Chem* 2009;55:946–54.
- Op De Beeck K, Maes L, Van den Bergh K *et al*. Heterogeneous nuclear RNPs as targets of autoantibodies in systemic rheumatic diseases. *Arthritis Rheum* 2012;64:213–21.

- 31 D.S.G. Tobacco report: PHS Study Group, after 14-month survey, agrees that smoking is indeed harmful. *Science* 1964;143:227.
- 32 Buchanan RR, Riglar AG. The titre of anti-centromere antibodies: its relationship to Raynaud's phenomenon and vascular occlusion. *Br J Rheumatol* 1989;28:221-6.
- 33 Furtado RN, Pucinelli ML, Cristo VV, Andrade LE, Sato EI. Scleroderma-like nailfold capillaroscopic abnormalities are associated with anti-U1-RNP antibodies and Raynaud's phenomenon in SLE patients. *Lupus* 2002;11:35-41.
- 34 Carvalho D, Savage CO, Black CM, Pearson JD. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells in vitro. Induction of adhesion molecule expression and involvement of endothelium-derived cytokines. *J Clin Invest* 1996;97:111-9.
- 35 Del Papa N, Guidali L, Sironi M *et al.* Anti-endothelial cell IgG antibodies from patients with Wegener's granulomatosis bind to human endothelial cells in vitro and induce adhesion molecule expression and cytokine secretion. *Arthritis Rheum* 1996;39:758-66.
- 36 Lin CF, Chiu SC, Hsiao YL *et al.* Expression of cytokine, chemokine, and adhesion molecules during endothelial cell activation induced by antibodies against dengue virus nonstructural protein 1. *J Immunol* 2005;174:395-403.

Human herpesvirus 6 gM/gN complex interacts with v-SNARE in infected cells

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Human herpesvirus 6 (HHV-6) glycoprotein M (gM) is an envelope glycoprotein that associates with glycoprotein N (gN), forming the gM/gN protein complex, in a similar manner to the other herpesviruses. Liquid chromatography-MS/MS analysis showed that the HHV-6 gM/gN complex interacts with the v-SNARE protein, vesicle-associated membrane protein 3 (VAMP3). VAMP3 colocalized with the gM/gN complex at the *trans*-Golgi network and other compartments, possibly the late endosome in HHV-6-infected cells, and its expression gradually increased during the late phase of virus infection. Finally, VAMP3 was incorporated into mature virions and may be transported with the gM/gN complex.

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INTRODUCTION

Human herpesvirus 6 (HHV-6) belongs to the betaherpesvirus subfamily (Roizmann *et al.*, 1992). HHV-6 isolates can be classified as HHV-6A and HHV-6B (Ablashi *et al.*, 2013) based on genetic and antigenic differences, cell tropism, and pathogenesis (Ablashi *et al.*, 1991; Aubin *et al.*, 1991; Campadelli-Fiume *et al.*, 1993; Chandran *et al.*, 1992; Mori, 2009; Yamanishi *et al.*, 1988).

Herpesviruses encode several glycoproteins on the envelope of viral particles that work for entry, assembly and egress of the virus. Of these, glycoprotein M (gM) is a remarkable envelope glycoprotein as it is conserved among all herpesvirus subfamilies. Most herpesviruses, including herpes simplex virus type-1 (Baines & Roizman, 1991), pseudorabies virus (Dijkstra *et al.*, 1996) and equine herpesvirus 1 (Osterrieder *et al.*, 1996), do not require gM for replication. Marek's disease virus (Tischer *et al.*, 2002) and varicella-zoster virus (Yamagishi *et al.*, 2008) abolish virus growth *in vitro*. However, the gM protein of human cytomegalovirus (HCMV), which belongs to the betaherpesvirus subfamily, is essential for the production of infectious virus (Hobom *et al.*, 2000).

HHV-6 gM is a product of the U72 ORF and comprises 343 aa (Kawabata *et al.*, 2012; Lawrence *et al.*, 1995). Post-infection (*p.i.*), it is translated into a 47–63 kDa protein that is post-translationally glycosylated (Kawabata *et al.*, 2012). It is a type III transmembrane protein with seven membrane-spanning domains and a C-terminal cytoplasmic tail. HHV-6 gM interacts with the product of the U46 ORF, known as gN, to form a complex for transport to the *trans*-Golgi network (TGN) and endosomal compartments (Kawabata *et al.*, 2012). Finally, the gM/gN complex is

incorporated into mature virions. Interestingly, unlike in alpha herpesviruses, HHV-6 gM is essential for virus growth (Kawabata *et al.*, 2012).

To further examine the role of HHV-6A gM during HHV-6 infection, we performed liquid chromatography (LC)-MS/MS analysis to identify the cellular components that interact with the gM/gN complex. The results showed that the gM/gN complex interacts with VAMP3 (vesicle-associated membrane protein 3).

VAMP3 is a v-SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein that resides in recycling endosomes and endosome-derived transport vesicles. v-SNARE interacts with SNARE proteins on target membranes (t-SNAREs) to form *trans*-SNARE complexes, which draw the two membranes together and drive membrane fusion (Jahn & Scheller, 2006; Jahn *et al.*, 2003; Rothman, 1994; Söllner *et al.*, 1993). SNAREs are cytoplasmic-oriented type I membrane proteins that play a role in intracellular trafficking mechanisms during exocytosis by forming a complex that facilitates the transient fusion of the vesicular and plasma membrane lipid bilayers (Mohrmann & Sørensen, 2012). This membrane fusion is dependent on the formation of a complex between t-SNARE and v-SNARE proteins (Jahn & Scheller, 2006; Jahn *et al.*, 2003).

VAMP3 is localized to recycling endosomes (McMahon *et al.*, 1993) and plays a role in the fusion of recycling endosomes and the plasma membrane by forming a complex with the surface t-SNARE complex, Stx4/SNAP23 (Hu *et al.*, 2007). The VAMP3/Stx4/SNAP23 SNARE complex mediates the long-loop recycling pathway that delivers recycling endosomes and their cargo to the cell

surface, and plays an important role in regulating the ability of macrophages to effectively adhere and spread on fibronectin (Veale *et al.*, 2011). VAMP3 is also involved in integrin trafficking, cell migration and cell adhesion (Luftman *et al.*, 2009; Tayeb *et al.*, 2005). In addition, it plays a role in the exocytosis of α -granules in platelets (Polgár *et al.*, 2002), as well as in the recycling of endocytosed transferrin receptors to the cell surface (Galli *et al.*, 1994).

Here, we describe the interaction between the gM/gN complex and VAMP3 in HHV-6A-infected cells and discuss the potential for the association between the gM/gN complex and VAMP3 to modify its localization and mediate its incorporation into mature virions.

RESULTS

Identification of cellular proteins interacting with the gM/gN complex

A recent study showed that the cytoplasmic tail of HCMV gM interacts with the cellular protein FIP4, which is a Rab11-GTPase effector protein important for gM/gN trafficking and for accumulation of the envelope glycoprotein complex in the assembly compartment in HCMV-infected cells (Krzyszaniak *et al.*, 2009). To examine the function of HHV-6A gM/gN, we tried to identify the cellular protein(s) that interact with the gM/gN complex. First, gM and gN were cotransfected into 293T cells and gM was immunoprecipitated from the lysates with an anti-gM mAb. Silver staining of gels containing proteins separated from the lysates of the gM/gN-expressing cells revealed several specific bands at approximately 10 kDa; these proteins were not present in the lysates of cells expressing gM alone. One specific band (Fig. 1a, arrowhead) was excised from the gel and subjected to LC-MS/MS analysis. The results of this analysis identified VAMP3 as the interacting protein (Fig. 1b).

To confirm the interaction between VAMP3 and gM/gN, lysates from cells expressing both HA-tagged gM and FLAG-tagged gN were immunoprecipitated with an anti-HA antibody, followed by Western blotting with anti-gM, anti-FLAG or anti-VAMP3 antibodies. As shown in Fig. 1c, VAMP3 was coprecipitated when gM and gN were coexpressed. However, VAMP3 was not coprecipitated when gM or gN was expressed alone. CD63 was not coprecipitated even when gM and gN were coexpressed (Fig. 1c). These results indicate that VAMP3 interacts with the gM/gN complex.

Interaction between gM and VAMP3 in HHV-6A-infected cells

To confirm the interaction between the gM/gN complex and VAMP3 in HHV-6A-infected cells, lysates from HHV-6A-infected HSB-2 cells were immunoprecipitated with an anti-gM mAb or an anti-VAMP3 antibody, followed by Western blotting with anti-gM, anti-VAMP3, anti-CD63 or

anti-gB antibodies (Fig. 2). Endogenous VAMP3 coprecipitated with gM and vice versa; gB or endogenous CD63 coprecipitated with neither gM nor VAMP3 (Fig. 2b). These results indicate that gM also interacts with VAMP3 in HHV-6A-infected cells. Interestingly, although gM with a molecular mass of 15 kDa was detected in the lysates of HHV-6A-infected cells, it did not coprecipitate with the anti-VAMP3 antibody.

To examine the cellular localization of gM and VAMP3 in HHV-6A-infected cells, an indirect immunofluorescence assay (IFA) was performed using HHV-6A-infected HSB-2 cells at 96 h p.i. (Fig. 3). Confocal microscopy of HHV-6A-infected HSB-2 cells showed that gM and VAMP3 appear to partially colocalize to the same cellular compartment. In addition, gM and VAMP3 partially colocalized with TGN46, a marker of the TGN (Fig. 3b), and with CD63, a marker of late endosomes and multivesicular bodies (MVB) (Fig. 3a). The expression of endogenous VAMP3 was much lower in uninfected cells than infected cells [Fig. 3a(ii), b(ii)]. These results indicate that VAMP3 may localize with gM to the endosomal compartment in addition to TGN during the late stage of infection. Preimmune serum of guinea pig did not react with either HHV-6A-infected [Fig. 3d(i)] or uninfected cells [Fig. 3d(ii)], although anti-VAMP3 antibody obtained from the same guinea pig reacted with HHV-6A-infected cells [Fig. 3c(i)] but not uninfected cells [Fig. 3c(ii)].

VAMP3 is present in purified HHV-6A virions

Recently, we showed that HHV-6A virions are released through MVBs via the cellular exosomal pathway and that gB and gM are present on exosomes (Mori *et al.*, 2008). To examine whether VAMP3 expressed in HHV-6A-infected cells is present on virions and exosomes, we purified virions from the culture medium of HHV-6A-infected cells. As expected, VAMP3, gM and CD63 were detected by western blotting of the virion fractions (Fig. 4a). Virion fractions were confirmed with the presence of gB (Fig. 4a) and viral DNA (Fig. 4b). In addition, VAMP3 was detected on HHV-6A virions by immunogold labelling electron microscopy analysis [Fig. 4c(i)], but it was rarely detected on virions without primary antibody [Fig. 4c(ii)]. These results indicate that VAMP3 is incorporated into viral particles along with the gM/gN complex.

Intracellular localization of the gM/gN complex and VAMP3 in cells transiently expressing gM/gN

We next examined the intracellular localization of gM, gN and VAMP3 (Fig. 5). When plasmids expressing gM and gN were cotransfected into HeLa cells [Fig. 5a(i)], gM/gN colocalized with endogenous VAMP3 in the perinuclear region. However, when gM was expressed alone, it failed to colocalize with VAMP3 [Fig. 5a(ii)]. Because the gM/gN complex localized to the TGN in HHV-6A-infected cells, we hypothesized that the gM/gN complex would interact with