Characterization of SEZ6L2 cell-surface protein as a novel prognostic marker for lung cancer

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To identify molecules that might serve as biomarkers or targets for development of novel molecular therapies, we have been screening genes encoding transmembrane/secretory proteins that are up-regulated in lung cancers, using cDNA microarrays coupled with purification of tumor cells by laser microdissection. A gene encoding seizure-related 6 homolog (mouse)-like 2 (SEZ6L2) protein, was chosen as a candidate for such molecule. Semi-quantitative RT-PCR and western-blot analyses documented increased expression of *SEZ6L2* in the majority of primary lung cancers and lung-cancer cell lines examined. SEZ6L2 protein was proven to be present on the surface of lung-cancer cells by flow cytometrical analysis using anti-SEZ6L2 antibody. Immunohistochemical staining for tumor tissue microarray consisting of 440 archived lung-cancer specimens detected positive SEZ6L2 staining in 327 (78%) of 420 non-small cell lung cancers (NSCLCs) and 13 (65%) of 20 small-cell lung cancers (SCLCs) examined. Moreover, NSCLC patients whose tumors revealed a higher level of SEZ6L2 expression suffered shorter tumor-specific survival compared to those with no SEZ6L2 expression. These results indicate that SEZ6L2 should be a useful prognostic marker of lung cancers. (*Cancer Sci* 2006; 97: 737–745)

Lung cancer is the leading cause of cancer deaths worldwide, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of those cases.[1] Regardless to histological subtypes, the 5-year survival rate of lung-cancer patients is around 10–15%[1,2] and even that of patients diagnosed at stage IA is less than 80%.[2,3] Within the last decade several newly developed cytotoxic agents such as paclitaxel, docetaxel, gemcitabine, and vinorelbine have begun to offer multiple choices for treatment to patients with advanced lung cancer, but each of these regimens confers only a modest survival benefit compared with cisplatin-based therapies.[4,5] Hence, novel therapeutic strategies such as molecular-targeted drugs, siRNAs and immunotherapies (antibodies and cancer vaccines) are eagerly expected. Although the precise pathways involved in lung tumorigenesis still remain unclear,[6] some evidences indicate that tumor cells express cell-surface markers unique to each histological type at particular stages of differentiation. Since cell-surface proteins or secretory autocrine-growth factors are considered to be more accessible to immune mechanisms and drug-delivery systems, identification of cancer-specific cell-surface and/or secretory proteins is likely to be an effective approach to develop novel diagnostic markers and therapeutic strategies.

We have been screening genes encoding molecules that are up-regulated in lung cancers, using cDNA microarrays and tumor cells purified by laser-capture microdissection.[7–15] To verify the biological and clinicopathological significance of the respective gene-products, we have been performing tissue microarray analysis of clinical lung-cancer materials.[11–15] This systematic approach combined with the search of cell-surface and/or secretory proteins by bioinformatics tools identified that SEZ6L2, seizure related 6 homolog (mouse)-like 2 (alias PSK-1), was frequently transactivated in a large population of lung cancers.

Application of multiple strategies for the identification of genes that encode secreted and transmembrane molecules, termed the Secreted Protein Discovery Initiative (SPDI), indicated SEZ6L2 to be one of novel transmembrane proteins.[16] *SEZ6L2* was also identified as a highly homologous gene to mouse *SEZ6* that had been first identified in the course of differential screening of mRNA from cortical neurons treated with pentylentetrazole (PTZ), a drug known to induce epileptic seizures.[17] The *SEZ6L2* encodes a 92.5-kDa protein with a N-terminal signal peptide, five SUSHI domains (SCR repeat), three CUB (initials of the first three identified proteins containing such domains: *c*omplement factor C1r/C1s, embryonic sea urchin protein *u*EGF, and *b*one morphogenetic protein 1) domains, and a C-terminal transmembrane domain. Although there is little information about the function of proteins including SUSHI and CUB domains, they have been postulated that they would be mainly involved in developmental process, cell–cell interaction, and cell adhesion. A previous study using cDNA microarray combined with bioinformatics analysis demonstrated that *SEZ6L2* is one of the 703 genes that are highly expressed in human hepatocellular carcinoma, although its physiological significance in carcinogenesis or its clinicopathological importance has not been clarified.[18]

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In this study, we reveal over-expression of SEZ6L2 protein in a considerable portion of lung cancers, and suggest that SEZ6L2 could be a novel prognostic marker and also a potential target for development of therapeutic antibodies for treatment of the majority of lung tumor.

Materials and Methods

Cell lines and clinical tissue samples

The 23 human lung-cancer cell lines used in this study included nine adenocarcinomas (ADCs; A427, A549, LC319, NCI-H1373, PC-3, PC-9, PC-14, NCI-H1666, and NCI-H1781), nine squamous-cell carcinomas (SCCs; EBC-1, LU61, NCI-H520, NCI-H1703, NCI-H2170, RERF-LC-AI, SK-MES-1, NCI-H226, and NCI-H647), one large-cell carcinoma (LCC; LX1), and four small-cell lung cancers (SCLCs; DMS114, DMS273, SBC-3, and SBC-5). A human bronchial epithelial cell line, BEAS2B (American Type Culture Collection; ATCC) was also included in the panel of the cells used in this study. All cells were grown in monolayers in appropriate media supplemented with 10% fetal calf serum (FCS) and were maintained at 37°C in an atmosphere of humidified air with 5% CO_2. Surgically resected primary NSCLC samples had been obtained earlier with informed consent.[7] A total of 420 formalin-fixed samples of primary NSCLCs (stage I-IIIA) including 263 ADCs, 116 SCCs, 28 LCCs, 13 adenosquamous carcinomas (ASCs) and adjacent normal lung tissues, had been obtained earlier along with clinicopathological data from patients undergoing surgery at Saitama Cancer Center (Saitama, Japan). ADCs were also classified into two groups: 129 mixed subtypes with bronchioloalveolar-cell carcinoma (BAC) components and 134 unmixed subtypes without BAC (non-BAC). SCLCs from postmortem materials (20 individuals) obtained from Hiroshima University (Hiroshima, Japan), were used in this study. NSCLC specimen and five tissues (heart, liver, lung, kidney, and pancreas) from postmortem materials (2 individuals with ADC) were also obtained from Hiroshima University. This study and the use of all clinical materials obtained with written informed consent were approved by the Institutional Research Ethics Committees. The histological classification of the tumor specimens was performed by the WHO criteria.[19] The postsurgical pathologic tumor-node-metastasis stage was determined according to the guidelines of the American Joint Committee on Cancer.[20]

Semi-quantitative RT-PCR analysis

Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc. Gaithersburg, MD, USA) according to the manufacturer's protocol. Extracted RNAs and normal human-tissue polyA RNAs were treated with DNase I (Roche Diagnostics, Basel, Switzerland) and then reversely transcribed using oligo $(dT)_{12-18}$ primer and Super-Script II reverse transcriptase (Life Technologies, Inc.). Semi-quantitative RT-PCR experiments were carried out with synthesized *SEZ6L2* gene-specific primers (5′-GGGAGTAT-GAAGTTTCCATCTG-3′ and 5′-GGATGCTGGTTTATTTA-CTGTAGG-3′), or with beta-actin (*ACTB*)-specific primers (5′-ATCAAGATCATTGCTCCTCCT-3′ and 5′-CTGCGCAA-GTTAGGTTTTGT-3′) as an internal control. All PCR reactions involved initial denaturation at 94°C for 2 min followed by 22 (for *ACTB*) or 30 cycles (for *SEZ6L2*) of 94°C for 30 s, 54–60°C for 30 s, and 72°C for 60 s on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

Northern-blot analysis

Human multiple-tissue blot (16 normal tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte; BD Biosciences Clontech, Palo Alto, CA, USA) was hybridized with a ^{32}P-labeled PCR product of *SEZ6L2*. The cDNA probes of *SEZ6L2* were prepared by RT-PCR using primers, 5′-GCTATGAGGG-CTTTGAGCTTATC-3′ and 5′-AGAAGCAAAGGTGGAGA-GACTGT-3′. Pre-hybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at −80°C for one week.

Preparation of anti-SEZ6L2 polyclonal antibody

Rabbit antibodies specific for extracellular portion of SEZ6L2 were raised by immunizing rabbits with 6-histidine fused human SEZ6L2 protein (codons 737–787; accession No. NM_012410), and purified with standard protocols using affinity columns (Affi-gel 10; Bio-Rad Laboratories, Hercules, CA, USA) conjugated with the 6-histidine fused protein. On Western blots we confirmed that the antibody was specific to SEZ6L2, using lysates from NSCLC tissues and cell lines as well as normal lung tissues.

Western-blot analysis

Cells and tissues were lyzed in lysis buffer; 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 0.5% deoxycholate-Na, 0.1% SDS, plus protease inhibitor (Protease Inhibitor Cocktail Set III; Calbiochem Darmstadt, Germany). We used an ECL western-blotting analysis system (GE Healthcare Bio-sciences, Piscataway, NJ), as previously described.[12,13] SDS-PAGE was performed in 7.5% polyacrylamide gels. PAGE-separated proteins were electroblotted onto nitrocellulose membranes (GE Healthcare Bio-sciences) and incubated with a rabbit polyclonal antihuman SEZ6L2 antibody. A goat antirabbit IgG-HRP antibody (GE Healthcare Bio-sciences) was served as the secondary antibodies for these experiments.

Flow-cytometric analysis

Lung-cancer cells (1×10^6 cells) were incubated with a rabbit polyclonal antihuman SEZ6L2 antibody for detecting the extracellular domain of the protein (0.34 mg/mL) or control rabbit IgG (0.34 mg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 1 h. The cells were washed in PBS and then incubated with AlexaFluor 488-conjugated antirabbit IgG (Molecular Probes, Eugene, OR, USA) at 4°C for 30 min. The cells were washed in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson Labware, Bedford, MA, USA) and analyzed by ModFit software (Verity Software House, Inc. Topsham, ME, USA).

Immunohistochemistry and tissue microarray

Tumor-tissue microarrays were constructed using 440 formalin-fixed primary lung cancers (420 NSCLCs and 20 SCLCs), according to the method published previously.[21–23] The tissue

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area for sampling was selected by visual alignment with the corresponding HE-stained section on a slide. Three, four, or five tissue cores (diameter 0.6 mm; height 3–4 mm) taken from a donor tumor block were placed into a recipient paraffin block using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). A core of normal tissue was punched from each case, and 5-μm sections of the resulting microarray block were used for immunohistochemical analysis.

To investigate the presence of SEZ6L2 protein in clinical samples that had been embedded in paraffin blocks, we stained the sections as previously described.[11–15] Briefly, 16.25 μg/mL of a rabbit polyclonal antihuman SEZ6L2 antibody was added after blocking of endogenous peroxidase and proteins. The sections were incubated with HRP-labeled antirabbit IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin.

Three independent investigators assessed SEZ6L2 positivity semiquantitatively without prior knowledge of clinicopathological data. The intensity of SEZ6L2 staining was evaluated using following criteria: strong positive (2+), dark brown staining in more than 50% of tumor cells completely obscuring membrane and cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell membrane and cytoplasm; absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted only as strongly positive if reviewers independently defined them as such.

Statistical analysis

Statistical analyses were performed using the StatView statistical program (SaS, Cary, NC, USA). We used contingency tables to analyze the relationship between SEZ6L2 expression and clinicopathological variables in NSCLC patients. Tumor-specific survival curves were calculated from the date of surgery to the time of death related to NSCLC, or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for SEZ6L2 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were performed with the Cox proportional-hazard regression model to determine associations between clinicopathological variables and cancer-related mortality. First, we analyzed associations between death and possible prognostic factors including age, gender, histological type, pT-classification, and pN-classification, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong SEZ6L2 expression into the model, along with any and all variables that satisfied an entry level of a P-value less than 0.05. As the model continued to add factors, independent factors did not exceed an exit level of $P < 0.05$.

RNA interference assay

Using the vector-based RNA interference (RNAi) system, psiH1BX3.0, which we had established earlier to direct the synthesis of siRNAs in mammalian cells,[10,12,13,15] we transfected 10 μg of siRNA-expression vector with 30 μL of Lipofectamine 2000 (Invitrogen) into two NSCLC cell lines (A549, LC319) that endogenously over-expressed *SEZ6L2*. The transfected cells were cultured for five days in the presence of appropriate concentrations of geneticin (G418). Cell numbers and viability were measured by Giemsa staining and MTT assay in triplicate. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control-1 (EGFP: enhanced green fluorescent protein (GFP) gene, a mutant of *Aequorea victoria* GFP), 5′-GAAGCAGCACGACTTCTTC-3′; control-2 (LUC, luciferase gene from *Photinus pyralis*), 5′-CGTACGCGGAATACTTCGA-3′; control-3 (Scramble: Chloroplast *Euglena gracilis* gene coding for the 5S and 16S rRNA), 5′-GCGCGCTTTGTAGGATTCG-3′; siRNA-SEZ6L2-1 (si-1), 5′-CCAACCGGCTGCTTCTGCA-3′; siRNA-SEZ6L2-2 (si-2), 5′-CTGGAAGTGACCCAGACCA-3′; siRNA-SEZ6L2-3 (si-3), 5′-GCTTCAGGGAAAGTCCCTT-3′. To validate our RNAi system, individual control siRNAs were tested by semiquantitative RT-PCR to confirm the decrease in expression of the corresponding target genes that had been transiently transfected to COS-7 cells. Down-regulation of *SEZ6L2* expression by functional siRNA, but not by controls, was also confirmed in the cell lines used for this assay.

Results

SEZ6L2 expression in lung tumors, cell lines, and normal tissues

To search for novel target molecules for development of therapeutic agents and/or diagnostic markers for NSCLC, we first screened genes that showed more than a 3-fold higher level of expression in cancer cells than in normal cells, in half or more of the 37 NSCLCs analyzed by cDNA microarray.[7] Among 23 040 genes screened, we identified the *SEZ6L2* transcript as a good candidate (3-fold or higher expression in 81% of the NSCLC cases), and confirmed its transactivation by semiquantitative RT-PCR experiments in 12 of 15 additional lung-cancer tissues and in 19 of 23 lung-cancer cell lines (NSCLC and SCLC samples), while its expression in normal lung tissue cells or a human bronchial epithelial cell line, BEAS2B, was hardly detectable (Fig. 1a,b).

We subsequently generated rabbit polyclonal antibody specific to human SEZ6L2 and confirmed by western-blot analysis an expression of SEZ6L2 protein in 4 cancer cell lines of lung, in which the *SEZ6L2* transcript had been detected at a high level (Fig. 1c). We found no band in two cell lines, which expressed no *SEZ6L2* transcript. As SEZ6L2 was suggested to be a type I membrane protein, we attempted to validate SEZ6L2 expression on the surfaces of lung-cancer cells using flow-cytometry with anti-SEZ6L2 polyclonal antibody. This analysis indicated that the antibody bound to A549 and EBC-1 cells, in which *SEZ6L2* transcript had been detected at a high level, but not to NCI-H647 cells, which had not expressed *SEZ6L2* (Fig. 1d). We also examined expression of SEZ6L2 protein in NSCLC tissues using the same antibody. Western-blot analysis revealed the increased SEZ6L2 protein expression in tumor tissues in representative pairs of ADC samples analyzed (Fig. 2a). Immunohistochemical analysis of tumor tissues detected positive staining for SEZ6L2 specifically in cancer cells in 7 of the 10 NSCLC cases examined, but the staining was hardly detectable in surrounding normal lung epithelial cells (Fig. 2b–d). Interestingly, the invasive border of the tumor adjacent to the non-cancerous cells showed the tendency of strong staining. SEZ6L2 localized at the plasma membrane as well as in the cytoplasm of tumor cells (Fig. 2e–g).

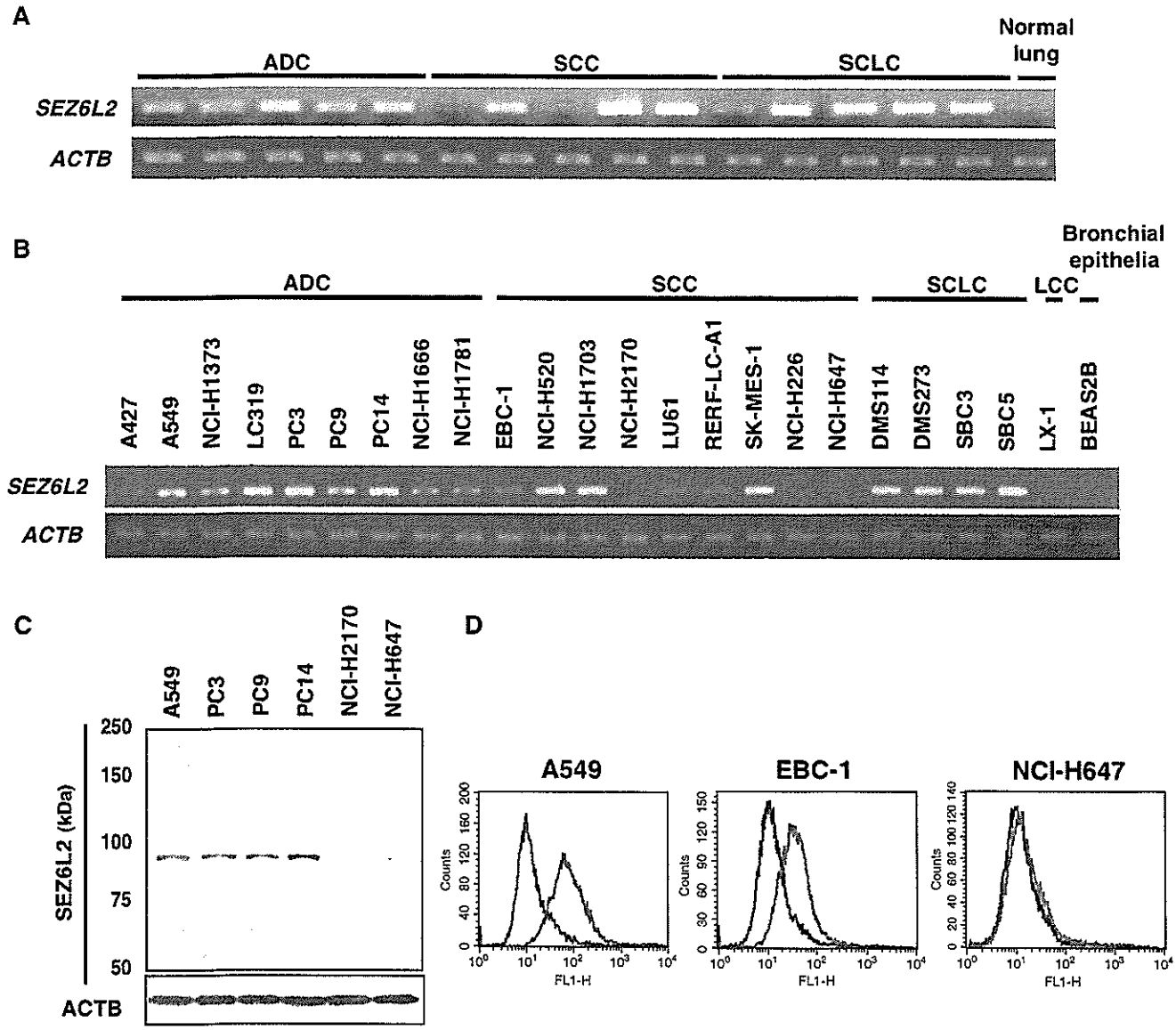

Fig. 1. Validation of SEZ6L2 expression and localization in lung cancers. (a) Expression of *SEZ6L2* in 15 clinical lung-cancer samples, examined by semiquantitative RT-PCR. (b) Expression of *SEZ6L2* in 23 lung-cancer cell lines, examined by semiquantitative RT-PCR. (c) Expression of SEZ6L2 protein in 6 lung-cancer cell lines, examined by western-blot analysis. (d) Expression of SEZ6L2 protein on cell surfaces in lung-cancer lines A549, EBC-1, and NCI-H647, evaluated by flow-cytometric analysis. Signal intensity values (*Y*-axis) of cells treated with antihuman SEZ6L2 polyclonal antibody (red) or cells treated with rabbit IgG (control; black) were shown.

Northern-blot analysis using human *SEZ6L2* cDNA as a probe detected a 3.2-kb transcript of weak signal only in brain, pancreas, prostate, and testis among the 16 normal human tissues (data not shown). We also examined expression of SEZ6L2 protein with anti-SEZ6L2 antibody on five normal tissues (heart, liver, lung, kidney, and pancreas), and found that it was hardly detectable in these tissues (Fig. 3a–e) while positive SEZ6L2 staining appeared in lung tumor tissues (Fig. 3f).

Association of SEZ6L2 expression with poor prognosis of NSCLC patients

To verify the biological and clinicopathological significance of SEZ6L2, we additionally examined the expression of SEZ6L2 protein by means of tissue microarrays containing lung-cancer tissues from 440 patients. We classified a pattern of SEZ6L2 expression on the tissue array ranging from absent (scored as 0) to weak/strong positive (scored as 1+ ~ 2+) (Fig. 4a–d). Of the 420 NSCLC cases examined, SEZ6L2 was strongly stained in 31 (7.4%; score 2+), weakly stained in 296 (70.5%; score 1+), and not stained in 93 cases (22.1%; score 0) (details are shown in Table 1). Weak positive staining (score 1+) was observed in 65% (13 of 20) of SCLC cases examined. As shown in Table 1, gender (higher in female; $P = 0.007$ by Fisher's exact test) and hisotological type (higher in ADC; $P < 0.001$ by Fisher's exact test) were significantly associated with the SEZ6L2 positivity (score 1+ ~ 2+). The median survival time of NSCLC patients was significantly related to the expression levels of SEZ6L2 (3172 days in score 0

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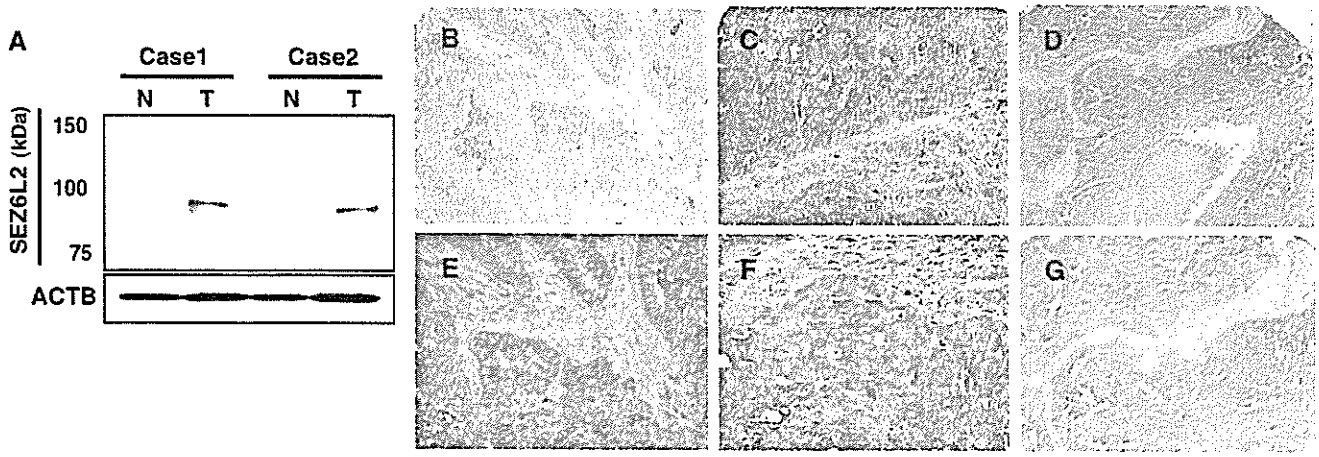

Fig. 2. Expression and localization of SEZ6L2 in clinical lung cancer tissues. (a) Western-blot analysis of SEZ6L2 protein in two representative pairs of lung adenocarcinoma samples. (b–g) Representative images of immunohistochemical analysis of SEZ6L2 protein in lung adenocarcinoma tissues. Magnification, ×100 (b, c, d) and ×200 (e, f, g).

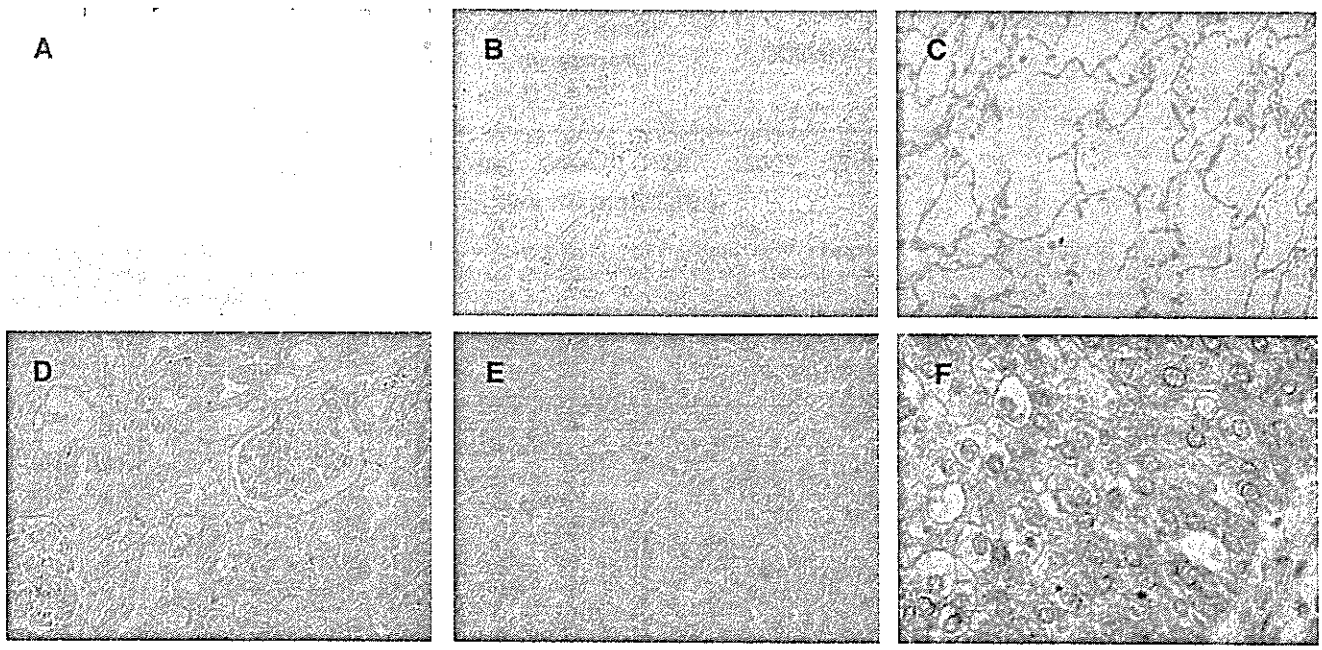

Fig. 3. Expression of SEZ6L2 protein in normal organ tissues.(a–f) Immunohistochemical evaluation of SEZ6L2 protein in representative normal tissues; adult heart (a), liver (b), lung (c), kidney (d), and pancreas (e), as well as lung adenocarcinoma tissues (f). Magnification, ×200.

cases, 2346 days in 1+, and 1134 days in 2+; $P = 0.0209$ by log-rank test; Fig. 4e). By univariate analysis, pT stage (T3, T4 *versus* T1, T2), pN stage (N1, N2 *versus* N0), age (≥ 65 *versus* < 65), gender (Male *versus* Female), histological classification (ADC *versus* other histological types), and strong SEZ6L2 positivity (score 2+ *versus* 0, 1+) were all significantly related to poor tumor-specific survival among NSCLC patients ($P = < 0.0001, < 0.0001, 0.0038, 0.0027, 0.0102$, and 0.0138, respectively; Table 2). In multivariate analysis of the prognostic factors, pT stage, pN stage, age, and strong SEZ6L2 expression were indicated to be an independent prognostic factor ($P = 0.0001, < 0.0001, < 0.0001, 0.0144$, respectively; Table 2).

Inhibition of endogenous *SEZ6L2* expression by siRNA in NSCLCs

To assess whether up-regulation of SEZ6L2 plays a role in growth or survival of lung cancer cells, we constructed three independent plasmids that were designed to express siRNA against *SEZ6L2* (si-1, si-2, and si-3), along with three different control plasmids (siRNAs for EGFP, LUC and Scramble). The treatment of NSCLC cells with the three effective and specific siRNAs could reduce expression of *SEZ6L2*, but did not suppress cell growth significantly (data not shown), suggesting that up-regulation of SEZ6L2 is not directly related to growth or survival of cancer cells.

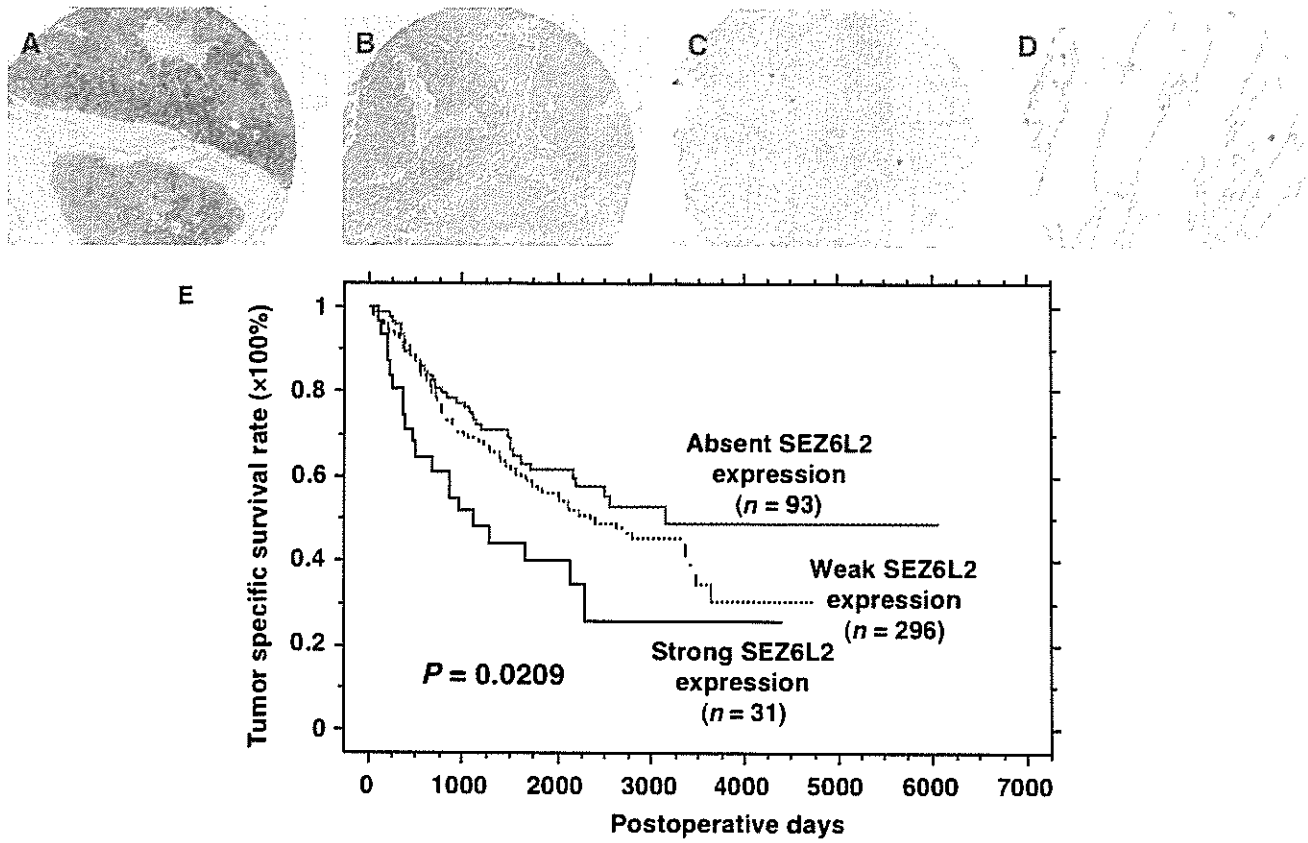

Fig. 4. Association of increased SEZ6L2 expression with poorer clinical outcomes among NSCLC patients. (a–d) Immunohistochemical evaluation of SEZ6L2 protein expression on tissue microarrays. Examples are shown for strong (a), weak (b), or absent (c) SEZ6L2 expression in lung SCCs, and for no expression in normal lung (d). Magnification, ×100. (e) Kaplan-Meier analysis of tumor-specific survival in 420 patients with NSCLCs according to the level of SEZ6L2 expression ($P = 0.0209$; log-rank test).

Table 1. Associabon between SEZ6L2-positivity in NSCLC tissues and patients' characteristics ($n = 420$)

	Total $n = 420$	SEZ6L2 strong positive $n = 31$	SEZ6L2 weak positive $n = 296$	SEZ6L2 absent $n = 93$	P value strong/weak vs absent
Gender					
Male	290	20	195	75	
Female	130	11	101	18	0.007†
Age (years)					
< 65	207	15	147	45	
≥ 65	213	16	149	48	NS
Histological type					
ADC	263	24	198	41	
SCC	116	3	77	36	< 0.001*†
Others	41	4	21	16	
pT factor					
T1+T2	301	22	212	67	
T3+T4	119	9	84	26	NS
pN factor					
N0	259	14	180	65	
N1+N2	161	17	116	28	NS
Smoking history					
Never smoker	129	10	96	23	
Smoker	291	21	200	70	NS

ADC, adenocarcinoma; SCC, squamous cell carcinoma.
Others, large cell carcinoma plus adenosquamous cell carcinoma.
*ADC versus other histology.
†$P < 0.05$ (Fisher's exact test).
NS, no significance.

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Table 2. Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

Variables	Hazards ratio	95% CI	Unfavorable/Favorable	*P*-value
Univariate analysis				
SEZ6L2	1.789	1.126–2.841	Strong(+)/ Weak(+) or (–)	0.0138*
Age (years)	1.520	1.145–2.018	65 ≥ / < 65	0.0038*
Gender	1.640	1.187–2.265	Male / Female	0.0027*
Histological type	1.444	1.091–1.912	others/ADC†	0.0102*
pT factor	1.889	1.411–2.528	T3+T4/T1+T2	< 0.0001*
pN factor	2.930	2.197–3.908	N1+N2/N0	< 0.0001*
Multivariate analysis				
SEZ6L2	1.814	1.126–2.922	Strong(+)/ Weak(+) or (–)	0.0144*
Age (years)	1.930	1.442–2.581	65 ≥ / < 65	< 0.0001*
Gender	1.413	0.989–2.019	Male / Female	0.0572
Histological type	1.184	0.861–1.628	others ADC†	0.2982
pT factor	1.787	1.329–2.404	T3+T4/T1+T2	0.0001*
pN factor	2.356	1.761–3.153	N1+N2/N0	< 0.0001*

†ADC, adenocarcinorna.
*$P < 0.05$.

Discussion

Molecular-targeted therapies are expected to be highly specific to malignant cells, with minimal adverse reactions due to their well-defined mechanisms of action. Equally desirable prospects are minimally invasive, and highly sensitive and specific new diagnostic methods that would adapt readily to clinical settings. From this point of view, tumor-specific transmembrane/secretory proteins should have significant advantages because they are located either at the cell surface or within the extracellular space; if they are present in serum, it makes them easily accessible as molecular markers. Some tumor-specific markers already available, such as CYFRA or Pro-GRP, are transmembrane/secretory proteins.[24,25] In addition, an example of rituximab (Rituxan), a chimeric monoclonal antibody against CD20-positive lymphomas, provides proof of the concept that targeting specific cell-surface molecules can result in significant clinical benefits.[26] During the course of our attempt to identify novel cancer-specific cell-surface or secretory proteins, we have been exploiting the power of genome-wide expression analysis to select genes that are over-expressed on the surface of cancer cells.[11,14] Functional and immunohistochemical analysis of candidate molecules on tissue microarray has revealed that SEZ6L2 is likely to be a potential target for development of novel tools for diagnosis and treatment of lung cancer.

SEZ6L2 was indicated to be a type I transmembrane protein with extracellular SUSHI and CUB domains by the bioinformatics tools.[16] SUSHI domains are known as complement control protein (CCP) modules, which exist in a wide variety of complements and adhesion proteins,[27] and are involved in protein-protein or protein–ligand interactions.[28] On the other hand, CUB domains are structurally related to immunoglobulins and play important roles in cell adhesion.[29] Some members of the proteins with these domains that include a number of serine protein kinases, complement components, cubulin, spermadhesin, bone morphogenetic protein 1, and others involved in cell adhesion or interaction with extracellular matrix components, were shown to have key functions in embryonic development.[30–34] In this study, we demonstrated that SEZ6L2 protein was expressed in a great majority of surgically resected NSCLC specimens, whereas it was scarcely expressed in normal tissues. Furthermore, the higher SEZ6L2 expression level was associated with shorter cancer-specific survival periods. This is, to our best knowledge, the first study to show prognostic value of SEZ6L2 expression in human cancers. Our observations implied that over-expressed SEZ6L2 may be associated with further malignant progression or unique phenotype in a subset of NSCLCs. However, reduction of SEZ6L2 expression by siRNA against *SEZ6L2*, did not suppress cell growth significantly, suggesting that up-regulation of SEZ6L2 is unlikely to be essential to growth or survival of cancer cells.

Recently, CUB domain-containing protein 1 (CDCP1), a novel member of CUB family proteins, was described as a marker for metastatic tissues over-expressed in colorectal cancer as well as in breast and lung carcinomas.[35] In mice models, expression levels of CDCP1 protein correlated with the metastatic ability of human epidermoid carcinoma cell line, HEp3 variant.[36] The CDCP1 molecule also contains intracellular binding sites for SH2 and SH3 domains and was phosphorylated at tyrosine residues by an Src kinase family member.[36] Since SEZ6L2 has a short C-terminal cytoplasmic domain with the sequence of Asn-Pro-X-Tyr, this motif is a potential target for tyrosine phosphorylation by Src family proteins.[37] A combination of our results and possible biological functions of the proteins with similar domains, suggest that activation of SEZ6L2 in cancer cells may modulate the cell adhesion, or the interaction and communication of cancer cells with the extracellular matrix or ligands, and may also function in some signal transduction, which possibly result in the promotion of tumor cell motility or invasion, and their subsequent highly malignant phenotype. In fact, immunohistochemical study demonstrated that SEZ6L2 localized at the plasma membrane as well as in the cytoplasm of tumor cells. Furthermore, SEZ6L2 protein expression was likely to increase at the invasive border of the tumor adjacent to the non-cancerous cells, thus partly supporting our hypothesis (Fig. 2b–g). Further elucidation of the mechanism implied by these observations should reveal important new information

about cell-cell communication, differentiation, and cancer progression.

Tumor tissue microarray is a powerful method to validate clinicopathological significance of candidate molecular markers using a large number of clinical samples, however, considerable heterogeneity exists within lung cancer with respect to morphology and the expression of biomarkers. We took 3–5 tissue-cores from each donor tumor block, all of which were carefully reviewed by the pathologists to cover most of histological subtypes within each tumor, which enabled us to correlate more detailed tumor subtypes with SEZ6L2 expression. Lung ADC has been classified in five histological subtypes; BAC, acinar, papillary, solid with mucin, and ADC with mixed subtypes. BAC is the subtype showing no invasive features, and good prognosis can be expected for patients with non-invasive BAC, although most lung ADC have mixed subtypes, which have invasive components.[38–40] We divided the 263 ADC cases into two subgroups with or without BAC component. Of the 129 cases with BAC subtype, 16 (12.4%) revealed strong positive staining of SEZ6L2 and 92 (71.3%) showed weakly positive staining. Of the 134 cases with non-BAC subtype, 8 (6.0%) were judged to be strong positive and 101 (75%) were to be weak positive, suggesting that SEZ6L2 staining was not associated with these BAC histology.

In summary, we have shown that over-expressed SEZ6L2 is likely to be an essential contributor to malignant features of NSCLCs. The data reported here imply the possibility of SEZ6L2 as a potential prognostic marker for lung cancers. Moreover, this molecule is a possible target for development of therapeutic approaches such as molecular-targeted antibodies to any types of cancers over-expressing this molecule on the cell surface.

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Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer

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Gastric cancer (GC) is one of the most common malignancies worldwide. Genes expressed only in cancer tissue will be useful molecular markers for diagnosis and may also be good therapeutic targets. However, little is known about cancer-specific genes, at least in GC. In this study, we searched for GC-specific genes by serial analysis of gene expression (SAGE) data analysis and quantitative reverse transcription (RT)–PCR. Comparing GC SAGE libraries with those of various normal tissues in the SAGEmap database, we identified 54 candidate GC-specific genes. Quantitative RT–PCR analysis of these candidates revealed that *APin protein* (*APIN*), *taxol resistance-associated gene 3* (*TRAG3*), *cytochrome P450, family 2, subfamily W, polypeptide 1* (*CYP2W1*), *melanoma inhibitory activity* (*MIA*), *matrix metalloproteinase-10* (*MMP-10*), *dickkopf homolog 4* (*DKK4*), *GW112, regenerating islet-derived family, member 4* (*REGIV*), and *HORMA domain-containing 1* (*HORMAD1*) were expressed much more highly in GC than in 14 kinds of normal tissues. Immunohistochemical staining for MIA, MMP-10, and DKK4 was found in 47 (31.1%), 68 (45.0%), and two (1.3%) of 151 GCs, respectively, and staining for both MIA and MMP-10 was correlated with poor prognosis in advanced GC ($P=0.0001$ and 0.0141, respectively). Moreover, enzyme-linked immunosorbent assay showed high levels of MMP-10 (65/69, 94.2%) in serum samples from patients with GC. Levels of MIA were raised in a small proportion of serum samples from patients with GC (4/69, 5.8%). In Boyden chamber invasion assays, MIA-transfected GC cells were up to three times more invasive than cells transfected with empty vector. Taken together, these results suggest that MMP-10 is a good marker for the detection of GC and that MIA and MMP-10 are prognostic factors for GC. As expression of MIA and MMP-10 is narrowly restricted in cancer, these two molecules may be good therapeutic targets for GC.
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Keywords: gastric cancer; MIA; MMP10; DKK4; SAGE; tumor serum marker

Introduction

According to the World Health Organization, gastric cancer (GC) is the fourth most common malignancy worldwide, with approximately 870 000 new cases occurring yearly. Mortality due to GC is second only to that due to lung cancer (Ohgaki and Matsukura, 2003). Cancer develops as a result of multiple genetic and epigenetic alterations (Yasui *et al.*, 2000; Ushijima and Sasako, 2004). Better knowledge of changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment is a major goal in this field (Yasui *et al.*, 2004). Genes encoding transmembrane/secretory proteins expressed specifically in cancers may be ideal biomarkers for cancer diagnosis (Buckhaults *et al.*, 2001). If the function of the gene product is involved in the neoplastic process, this gene may constitute a therapeutic target.

We previously performed serial analysis of gene expression (SAGE) on four primary GC samples (Gene Expression Omnibus accession number GSE 545; SAGE Hiroshima GC tissue) and identified several genes and tags that are potentially involved in invasion, metastasis, and carcinogenesis (Oue *et al.*, 2004; Yasui *et al.*, 2004). In this study, to identify potential molecular markers for diagnosis of GC and molecular therapeutic targets, we systematically searched for GC-specific genes in SAGE libraries. Comparing GC SAGE libraries with those of various normal tissues in the SAGEmap

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database (Lal *et al.*, 1999), we identified 54 candidate GC-specific genes in GC libraries but not in libraries from 14 normal tissues, including brain, lung, and heart. We also performed quantitative reverse transcription (RT)–PCR to investigate the specificity of these candidate GC-specific genes. We show that *APin protein* (*APIN*), *taxol resistance-associated gene 3* (*TRAG3*), *cytochrome P450, family 2, subfamily W, polypeptide 1* (*CYP2W1*), *melanoma inhibitory activity* (*MIA*), *matrix metalloproteinase-10* (*MMP-10*), *dickkopf homolog 4* (*DKK4*), *GW112, regenerating islet-derived family, member 4* (*REGIV*), and *HORMA domain-containing 1* (*HORMAD1*) were much more highly expressed in GC than in normal tissues. Among these genes, overexpression of *REGIV* and *GW112* in GC has been reported (Oue *et al.*, 2004, 2005; Zhang *et al.*, 2004). Immunohistochemical analysis of MIA, MMP-10, and DKK4 in 151 GC samples revealed that MIA and MMP-10 are frequently overexpressed in GC. We also measured MIA and MMP-10 in serum and peritoneal wash fluid from patients with GC to investigate the potential utility of these measurements in cancer diagnosis.

Results

Identification of genes expressed more highly in GC than in normal tissues

To identify genes expressed specifically in GC, we compared tags from each GC SAGE library to the normal SAGE libraries (white matter, cerebellum, thalamus, heart, lung, stomach, colon, liver, kidney, leukocyte, peritoneum, skeletal muscle, spinal cord, and lymph node) as described in Materials and methods. We obtained 24 candidates from W226T, 15 candidates from W246T, 27 candidates from S219T, and 13 candidates from P208T. In total, we identified 54 individual candidate genes in our GC libraries but not in the normal libraries (Table 1). To confirm that these candidates were GC-specific, quantitative RT–PCR was performed to measure the expression of these candidates in nine GC samples and in 14 normal tissues (heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain, and spinal cord). Representative results are shown in Figure 1. Expression of the 54 candidate genes was not necessarily specific for GC. However, several genes showed much higher expression in GC than in normal tissues. We then focused on cancer specificity. We calculated the specificity index for each gene. First, we identified the normal tissue in which the target gene expression was highest (mRNA expression levels are shown as A, Table 1). We then identified the GC among nine in which the target gene expression was highest (mRNA expression levels are shown as B, Table 1). The specificity index (B/A ratio) for each gene is shown in Table 1. Of the 54 candidates, nine genes: *APIN*, *TRAG3*, *CYP2W1*, *MIA*, *MMP-10*, *DKK4*, *GW112*, *REGIV*, and *HORMAD1* were found to show high specificity for GC.

mRNA expression of high-specificity genes for GC

Expression of the nine high-specificity genes for GC was analysed by quantitative RT–PCR in an additional 44 GC samples and corresponding non-neoplastic mucosa samples. We calculated the ratio of target gene mRNA expression levels between GC tissue (T) and corresponding non-neoplastic mucosa (N). T/N ratios >2-fold were considered to represent overexpression. Genes showing overexpression in >40% of the samples included *GW112* (25/44, 56.8%), *MMP-10* (23/44, 52.3%), *CYP2W1* (22/44, 50.0%), *HORMAD1* (20/44, 45.5%), and *TRAG3* (18/44, 40.9%). Other genes were overexpressed in <30% of the samples examined (*MIA*, 13/44, 29.5%; *APIN*, 12/44, 27.3%; *DKK4*, 11/44, 25.0%). mRNA expression levels of *GW112* were correlated with T grade (depth of tumor invasion, $P<0.0001$), N grade (degree of lymph node metastasis, $P=0.0089$), and tumor stage ($P=0.0019$; Table 2). Those of *MIA* were also correlated with T grade ($P=0.0007$), N grade ($P=0.0335$), and tumor stage ($P=0.0068$; Table 2).

Immunohistochemical staining for MIA, MMP-10, and DKK4 in GC and noncancerous tissues

To confirm overexpression of genes whose expression by RT–PCR was much higher in GC than in normal tissues, we performed immunohistochemical analysis of MIA, MMP-10, and DKK4 because antibodies against these three proteins are available. Immunohistochemical analysis was performed in noncancerous tissues with the highest mRNA expression to serve as positive controls. Immunostaining of MIA and MMP-10 in the lung, in which mRNA expression of both 2 genes was the highest, showed staining of chondrocytes in peribronchial cartilage, but not of epithelial components (Figure 2a, b). Both MIA and MMP-10 are reported to be expressed in cartilage (Dietz and Sandell, 1996; Kevorkian *et al.*, 2004). Little is known about DKK4 expression. *DKK4* mRNA is reported to be undetectable in all human adult and fetal tissues examined by Northern blotting (Krupnik *et al.*, 1999). As our quantitative RT–PCR showed the highest expression of *DKK4* in the duodenum, immunostaining for DKK4 was performed in the duodenum, and staining for DKK4 was observed in a small number of epithelial cells (Figure 2c). Staining was absent with antibody preincubated with DKK4 protein (data not shown).

Immunohistochemistry was then performed on 151 GC samples (Figure 2d–f). Of these, 47 (31.1%) were positive for MIA staining, 68 (45.0%) were positive for MMP-10 staining, and two (1.3%) were positive for DKK4 staining. We analysed the relation between staining for each of these three proteins and clinicopathologic characteristics. Staining for MIA was correlated with T grade ($P=0.0002$), N grade ($P=0.0015$), and tumor stage ($P<0.0001$) (Supplementary Table 1). Staining for MMP-10 was correlated with T grade ($P=0.0306$) (Supplementary Table 2). There was no clear correlation between DKK4 staining and clinical characteristics (data not shown). We also analysed the

Table 1 Summary of quantitative RT–PCR analysis of candidate genes specifically expressed in gastric cancer

Gene name	*Normal organ with highest expression*		*GC with highest expression mRNA expression level (B)*	*Specificity index (B/A)*	*GC case no. with two-fold mRNA expression over that of normal organ with highest expression*	*GC case no. with 10-fold mRNA expression over that of normal organ with highest expression*
	Organ name	*mRNA expression level (A)*				
High specificity						
APIN	Stomach	1.0[a]	202.3[a]	202.3	3	1
TRAG3	Spleen	27.3	1038.3	38.1	4	3
CYP2W1	Small intestine	17.4	604.7	34.8	3	2
MIA	Lung	1.0	29.7	29.7	3	1
MMP10	Lung	3.1	63.6	20.8	10	2
DKK4	Small intestine	4.6	89.9	19.6	3	1
GW112	Small intestine	8.7	147	16.9	2	2
REGIV	Pancreas	8.9	112.2	12.6	1	1
HORMAD1	Leukocyte	43.7	448.8	10.3	2	1
Low specificity						
PPARBP	Brain	5.7	52.7	9.3	1	
SULT1C1	Kidney	1.1	6	5.5	3	
BIRC5	Bone marrow	2.7	13.5	4.9	3	
FLJ10036	Pancreas	1.8	6.2	3.4	5	
SH3BGRL2	Brain	4.5	12	2.7	1	
FLJ36666	Pancreas	3.5	7.8	2.2	2	
No specificity						
ETS2	Skeletal muscle	6.5	12.5	1.9		
TD-60	Pancreas	1.9	3.3	1.7		
MGC20806	Brain	4.2	6.1	1.5		
MYBL2	Bone marrow	3.5	4.4	1.3		
alpha4GnT	Pancreas	1.2	1.4	1.2		
GPP34R	Pancreas	12.6	12.7	1		
MLL4	Pancreas	4.9	4.6	0.9		
AQR	Pancreas	5.6	5	0.9		
TAPBP	Leukocyte	4.8	4.2	0.9		
KIF4A	Bone marrow	5	4.2	0.8		
ALDH7A1	Liver	1.1	0.8	0.7		
MAPK13	Pancreas	7.9	5.5	0.7		
FXYD3	Colon	4.3	2.8	0.6		
THBS3	Pancreas	6.2	3.5	0.6		
CCT3	Pancreas	10.6	5.4	0.5		
STAT2	Liver	5.2	2.7	0.5		
LMO6	Skeletal muscle	32.0	14.7	0.5		
TMLHE	Heart	21.4	9.4	0.4		
SFRS9	Pancreas	7.4	2.7	0.4		
C4orf9	Skeletal muscle	8.5	3	0.4		
SEC31L2	Skeletal muscle	13.1	4.6	0.3		
TYRO3	Brain	25.3	8.7	0.3		
HOXA10	Skeletal muscle	6472	1937.5	0.3		
JUN	Skeletal muscle	12.9	3.7	0.3		
ATPIF1	Heart	28.4	7.4	0.3		
ATE1	Heart	11.9	2.8	0.2		
BRD4	Skeletal muscle	11.8	2.7	0.2		
PEGASUS	Heart	9.6	2.1	0.2		
IL16	Brain	9.6	2.1	0.2		
TPT1	Pancreas	17.8	3.6	0.2		
PRKAG1	Skeletal muscle	13	2.6	0.2		
RPL8	Pancreas	11.6	2.2	0.2		
GITA	Heart	31.8	4.7	0.1		
NEK9	Heart	15	2.1	0.1		
IFRD1	Pancreas	20.8	2.4	0.1		
NIPSNAP3B	Skeletal muscle	49.5	5.6	0.1		
CBFA2T3	Pancreas	34.1	1.7	0.1		
DEFA6	Small intestine	108.4	1.7	0		
DEFA5	Small intestine	280.1	3.5	0		

[a]The units are arbitrary. Target mRNA expression levels were standardized to 1.0 μg total RNA from normal stomach as 1.0.

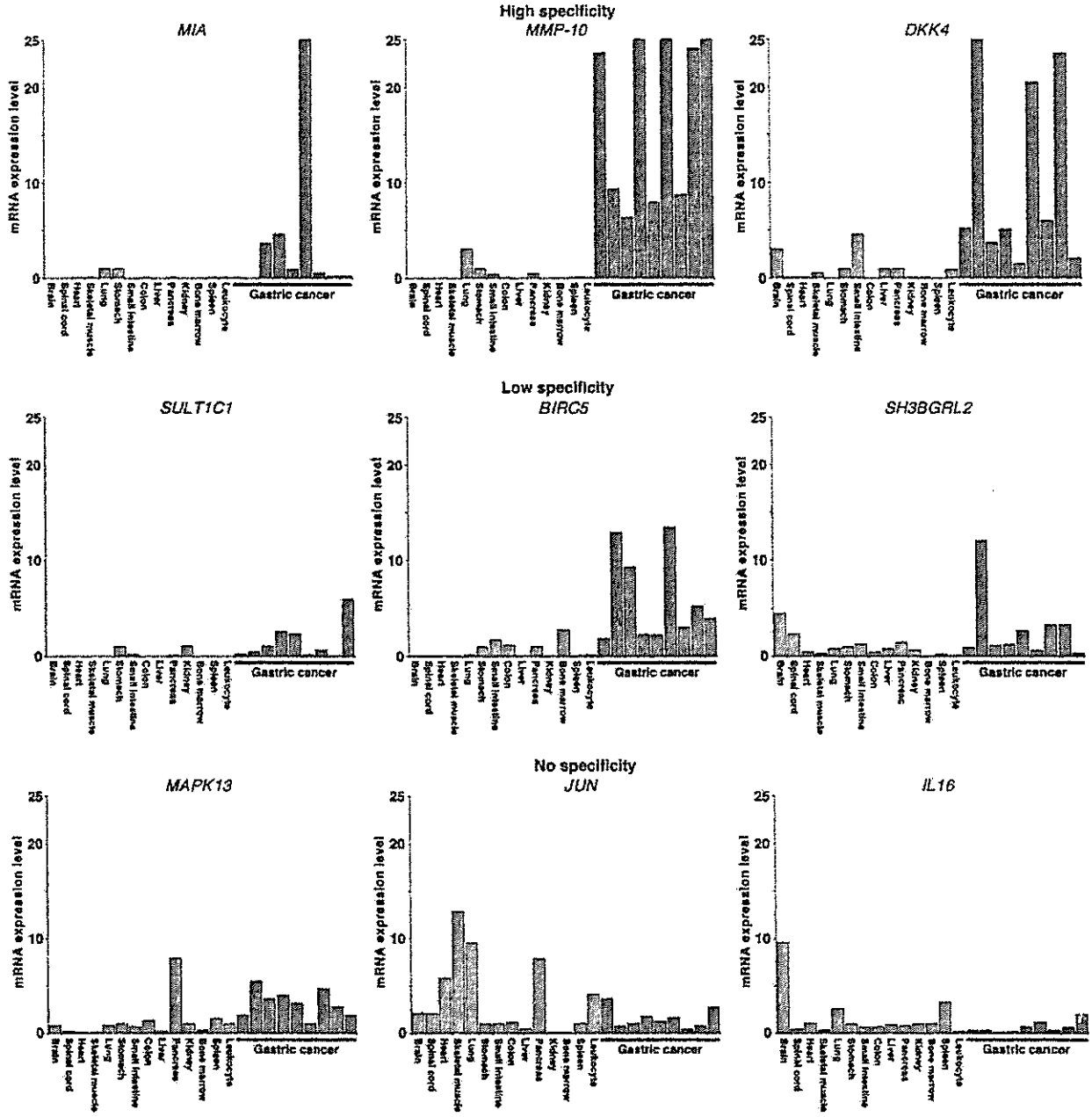


Figure 1 Quantitative RT–PCR analysis of candidate cancer (at least GC)-specific genes in 14 normal tissues and nine GC samples. Definitions of high specificity, low specificity, and no specificity are as described in Materials and methods. mRNA expression levels of *MIA*, *MMP-10*, and *DKK4* were much higher in GC samples than in normal tissues. In contrast, mRNA expression levels of *MAPK13*, *JUN*, and *IL16* were not significantly different between GC and normal tissues.

prognostic value of MIA and MMP-10 staining. The prognosis of patients with MIA- or MMP-10-positive tumors was significantly worse in the group of 58 advanced GC (invading through the muscularis propria into the serosa) patients ($P=0.0001$ and 0.0141, respectively, log-rank test) (Figure 2g). In corresponding non-neoplastic gastric mucosa from GC patients, staining for MIA and MMP-10 was weak or negative, whereas DKK4-positive cells were detected in intestinal metaplasia of the stomach (data not shown).

MIA and MMP-10 levels in serum and peritoneal wash fluid from patients with GC

MIA and MMP-10 are reported to be secreted (Blesch *et al*., 1994; Ramos *et al*., 2004). Therefore, we

Table 2 Relation between mRNA expression and clinicopathologic characteristics in gastric cancer

Gene name	*TNM stage*	*mRNA expression*		P-*value*[b]
		Overexpression[a]	*Not overexpression*	
GW112	T grade			
	T1/2	5 (23.8%)	16	<0.0001
	T3/4	20 (87.0%)	3	
	N grade			
	N0	4 (26.7%)	11	0.0089
	N1/2/3	21 (72.4%)	8	
	Stage			
	Stage I/II	7 (31.8%)	15	0.0019
	Stage III/IV	18 (81.8%)	4	
	Histologic type			
	Intestinal	13 (54.4%)	11	0.7662
	Diffuse	12 (60.0%)	8	
MIA	T grade			
	T1/2	1 (4.8%)	20	0.0007
	T3/4	12 (52.2%)	11	
	N grade			
	N0	1 (6.7%)	14	0.0335
	N1/2/3	12 (41.4%)	17	
	Stage			
	Stage I/II	2 (9.1%)	20	0.0068
	Stage III/IV	11 (50.0%)	11	
	Histologic type			
	Intestinal	7 (29.2%)	17	1.0000
	Diffuse	6 (30.0%)	14	

[a]We calculated the ratio of target gene mRNA expression levels between GC tissue (T) and corresponding non-neoplastic mucosa (N). T/N ratios >2-fold were considered to represent overexpression. [b]Fisher's exact test.

determined whether these proteins can be detected in sera from patients with GC by enzyme-linked immunosorbent assay (ELISA). Among the 151 GC cases analysed by immunohistochemistry, serum samples were available for ELISA from 69 GC cases. MIA is known to be a tumor marker to detect metastatic disease in patients with malignant melanomas (Bosserhoff *et al.*, 1997), but MIA levels in serum from patients with GC have not been investigated. MIA levels were significantly higher in four of 17 serum samples from patients with stage IV GC than in those of healthy individuals (Figure 3a). MIA serum levels were not significantly different between patients with stage I, II, or III GC and healthy individuals. Of 20 serum samples from patients with gastritis, one showed a high MIA level. But, levels of MIA in all 20 serum samples were below 15 ng/ml. When the cutoff level for MIA was set at 20 ng/ml, the sensitivity for detection of GC was only 5.8% (4/69), but specificity was 100.0% (60/60). We compared the protein expression status obtained by immunostaining with serum levels of the MIA measured by ELISA in 69 GC cases. Levels of MIA in serum samples from the patients with GC showing MIA-positive immunostaining ($n=29$, mean$\pm$s.e. 7.7 ± 2.1 ng/ml) did not differ significantly from those with GC showing MIA-negative immunostaining ($n=40$, 4.1 ± 0.3 ng/ml) ($P=0.7656$, Mann–Whitney *U*-test). We also measured MIA levels in peritoneal wash fluid from patients with GC (Figure 3a). Of two peritoneal wash cytology-positive samples, one showed a very high MIA level.

To our knowledge, although some MMPs are good serum markers for cancer detection (Zucker *et al.*, 1999), there are no reports regarding MMP-10 levels in serum from patients with cancer including GC. MMP-10 was also detected in serum samples. In contrast to levels of the MIA, high levels of MMP-10 were detected in serum samples from most of the patients with GC (mean$\pm$s.e.; stage I, 455.8 ± 38.1 pg/ml; stage II, 526.5 ± 68.5 pg/ml; stage III, 574.1 ± 61.1 pg/ml; stage IV, 546.0 ± 51.0 pg/ml), even at stage I (Figure 3b). Levels of MMP-10 in serum samples from the patients with GC showing MMP-10-positive immunostaining ($n=34$, 553.0 ± 38.3 pg/ml) were higher than those with GC showing MMP-10-negative immunostaining ($n=35$, 451.0 ± 33.7 pg/ml), but not statistically significant ($P=0.1770$, Mann–Whitney *U*-test). High levels of MMP-10 were also detected in serum samples from some healthy individuals (81.4 ± 25.5 pg/ml) and some patients with gastritis (47.8 ± 23.0 pg/ml). When the cutoff level for MMP-10 was set at 200 pg/ml, the sensitivity and specificity for detection of GC was 94.2% (65/69) and 85.0% (51/60), respectively. Sensitivity for patients with stage II–IV GC was 100%. Levels of MMP-10 in all 27 peritoneal wash cytology-negative samples were below 50 pg/ml (Figure 3b). Two peritoneal wash cytology-positive samples showed levels of MMP-10 that were significantly higher than those in peritoneal wash cytology-negative samples.

Effect of MIA on cell growth and invasive activity of MKN-28 cells

High levels of MIA mRNA expression were correlated with T grade, N grade, and tumor stage in GC tissues. In addition, immunostaining for MIA protein was correlated with T grade, N grade, tumor stage, and poor prognosis. MIA acts as a potent tumor cell growth inhibitor for malignant melanoma cells (Blesch *et al.*, 1994) but not for pancreatic cancer cells (El Fitori *et al.*, 2005), whereas overexpression of MIA enhances the invasiveness of both melanoma cells and pancreatic cancer cells (Bosserhoff *et al.*, 2001; El Fitori *et al.*, 2005). To investigate the biologic significance of MIA in GC, the MKN-28 GC cell line was stably transfected with vector expressing MIA. MKN-28 cells were selected for low MIA expression (data not shown). Cells were transfected with plasmid vectors capable of expressing MIA constitutively. Clones were selected in G418 and examined for MIA expression by MIA ELISA (Figure 4a). Clones that expressed MIA at significantly increased levels relative to the parent are designated as MKN-28-1, MKN-28-2, and MKN-28-3. To determine the effect of MIA on cell growth, MTT assays were performed. Cell growth of MKN-28 cells expressing higher levels of MIA did not differ from that of cells transfected with empty vector up to days 2 (Figure 4b). We then performed Boyden chamber

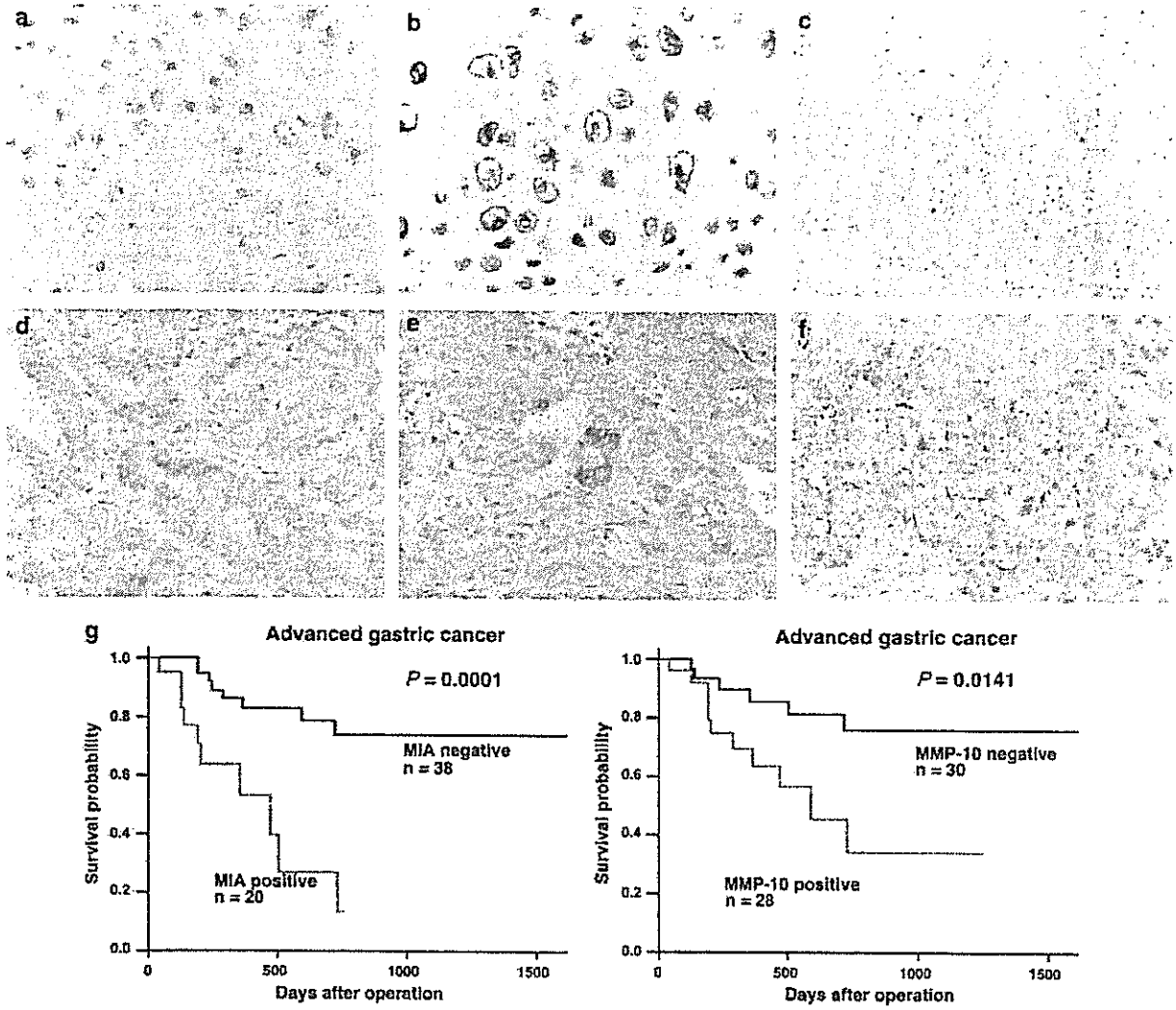


Figure 2 Immunohistochemical analysis of MIA, MMP-10, and DKK4 in noncancerous tissue and GC tissue. Staining for MIA (**a**) and MMP-10 (**b**) was found in peribronchial cartilage of the lung. DKK4 staining (**c**) was detected in a small number of epithelial cells in the duodenum. In GC samples, staining for MIA (**d**), MMP-10 (**e**), and DKK4 (**f**) was found in GC cells. Original magnifications, **a**, **b**, and **d–f**, ×400; **c**, ×200. (**g**) Prognostic value of MIA and MMP-10 staining. The prognosis of patients with MIA- or MMP-10-positive tumors was significantly worse in the group of 58 advanced GC patients ($P=0.0001$ and 0.0141, respectively, log-rank test).

invasion assays. MIA-transfected MKN-28 cells were up to three times more invasive than cells transfected with empty vector on day 2 (MKN-28-1, $P=0.014$; MKN-28-2, $P=0.046$; MKN-28-3, $P=0.025$) (Figure 4c).

Discussion

Several tumor (breast cancer, lung cancer, and renal cell cancer)-specific genes have been identified by a combination of subtractive hybridization and cDNA microarray technology (Amatschek *et al.*, 2004). In this study, we searched for GC-specific genes by SAGE data analysis and quantitative RT–PCR. True cancer-specific genes were not found, but *APIN*, *TRAG3*, *CYP2W1*, *MIA*, *MMP-10*, *DKK4*, *GW112*, *REGIV*, and *HORMAD1* were expressed much more highly in GC than in 14 types of normal tissues. As these genes were identified by SAGE and quantitative RT–PCR analysis of bulk GC tissues, immunohistochemistry was required to determine which cells expressed these genes. Antibodies against MIA, MMP-10, and DKK4 were available, and staining for all three proteins was confirmed in GC cells. But, DKK4 expression was present in only two out of 151 GC cases and generally absent.

MIA was first isolated as an 11-kDa protein secreted by malignant melanoma cell lines (Blesch *et al.*, 1994). MIA is a potent inhibitor of proliferation of malignant melanoma cells and other neuroectodermal tumor cells (Blesch *et al.*, 1994). Overexpression of MIA has been reported in breast cancer (Bosserhoff *et al.*, 1999),

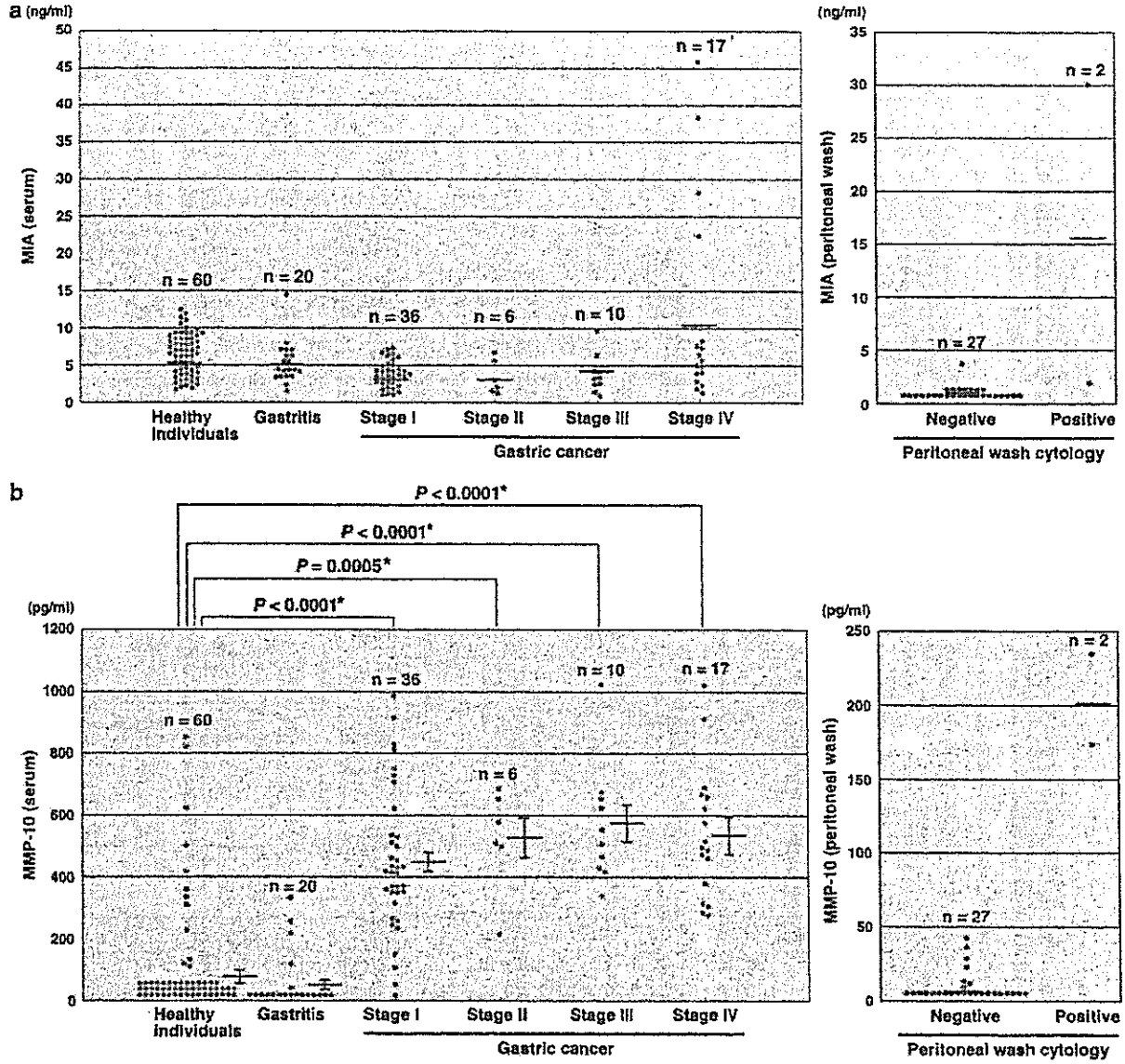
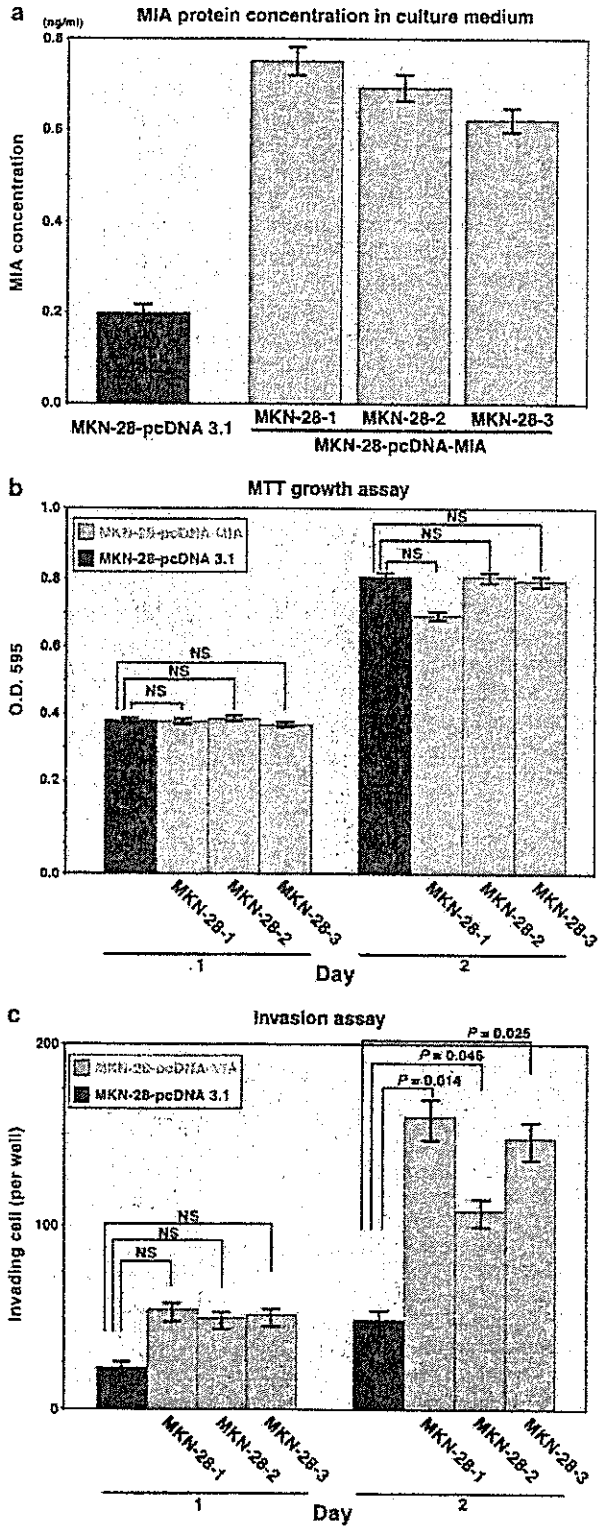


Figure 3 Analysis of serum and peritoneal wash fluid samples from patients with GC by ELISA. (a) High levels of MIA were detected in serum samples from four patients with stage IV GC. Yellow bars indicate the cutoff levels defined in this study. Red bars indicate the mean of protein levels. In peritoneal wash fluid samples, one and two cytology-positive samples showed high levels of MIA. (b) High levels of MMP-10 were detected in 65 serum samples from patients with GC, including stage I GC. Among 60 serum samples from healthy individuals, high levels of MMP-10 were detected in 15. Yellow bars indicate the cutoff levels defined in this study. Red bars indicate mean of protein levels. Error bars indicate s.e. from the mean. In peritoneal wash fluid samples, one and two cytology-positive samples showed high levels of MMP-10. *Mann–Whitney *U*-test.

colorectal cancer (Hau *et al*., 2002), glioma (Hau *et al*., 2004), and pancreatic cancer (El Fitori *et al*., 2005), and association of MIA with tumor progression has been reported. We found that expression of MIA was correlated with T grade, N grade, tumor stage, and patient prognosis, indicating that it plays an important role in GC progression and may serve as a good marker of GC progression. In contrast to malignant melanoma, high MIA levels were detected in only four of 17 serum samples from patients with stage IV GC, although overexpression of MIA was frequently found in primary GC tissues by immunohistochemistry. Therefore, MIA is not a suitable serum marker for early detection for GC, but it is a good indicator of a poor prognosis. We cannot explain the discrepancy between MIA expression level in primary GC and in serum. We did confirm that MIA was present in the culture medium of GC cells stably transfected with MIA, suggesting that MIA is secreted by GC cells. The origin of MIA in serum samples may be circulating GC cells but not primary GC cells. Levels of MIA in serum from patients with pancreatic cancer are

reported to be low, despite MIA mRNA and protein overexpression in pancreatic cancer tissues (El Fitori *et al.*, 2005).



In addition to the usefulness of MIA as an indicator of poor prognosis, it has been reported that MIA enhances migration and invasion ability and inhibits apoptosis of melanocytic cells (Bosserhoff *et al.*, 2001; Poser *et al.*, 2004). Here, we showed that transfection of MIA enhanced invasive activity of MKN-28 cells. As expression of MIA was highly specific to cancer cells, it may be a good therapeutic target with less adverse effects for various types of cancers, including GC.

Among the nine genes overexpressed in GC, MMP-10 (also known as stromelysin 2) was frequently overexpressed in GC. MMPs induce extracellular matrix breakdown associated with normal tissue remodeling and are associated with tissue destruction in arthritis, cancer invasion, and metastasis (Nelson *et al.*, 2000; Visse and Nagase, 2003). Overexpression of MMP-10 has been reported in cancers of the lung, head, and neck (Muller *et al.*, 1991), esophagus (Mathew *et al.*, 2002; Sharma *et al.*, 2004), brain (Thorns *et al.*, 2003), and liver (Bodey *et al.*, 2000). The present immunohistochemical study showed MMP-10 to be correlated with a poor prognosis in patients with GC. Importantly, high levels of MMP-10 protein were detected in serum samples from most of the patients with GC, even at stage I. An available tumor marker for GC is carcinoembryonic antigen (CEA) (Molnar *et al.*, 1976). Despite the reliability of CEA as a marker for detection of GC, the preoperative rate of serum CEA positivity in GC is 20–40% (Koga *et al.*, 1987; Shimizu *et al.*, 1987). In the present study, of 36 serum samples from patients with stage I GC, 32 (88.9%) showed high levels of MMP-10 protein, indicating that MMP-10 is a serum tumor marker with high sensitivity. In contrast, immunohistochemical staining of MMP-10 was detected in 35 (42.2%) of 83 stage I GC samples. This discrepancy between immunohistochemical and ELISA results may be due to methodological differences. MMP-10 immunohistochemical results were evaluated with reference to the percentage of stained cancer cells; the intensity of immunostaining was not evaluated because of lack of internal control in immunohistochemistry. More detailed quantitative methods for the measurement of MMP-10 protein will be necessary to determine the relation between MMP-10 protein levels in serum samples and primary GC samples. In addition, because high levels of MMP-10 protein were detected in nine (15.0%) of 60 serum samples from healthy individuals, characterization of these individuals is

Figure 4 Expression of MIA in MKN-28 cells. (a) MIA protein levels in the culture media of MKN-28 cells transfected with pcDNA-MIA or pcDNA 3.1 constructs were measured by ELISA. In MKN-28 cells stably transfected with pcDNA-MIA, increased MIA levels were detected. (b) Effect of MIA expression on cell growth of MKN28 cells. Cell growth was assessed by MTT assay at 1 and 2 days after seeding on 96-well plates. (c) Effect of MIA on cell invasion. MKN-28 cells transfected with pcDNA-MIA or pcDNA 3.1 constructs were incubated in Boyden chambers. After 1 and 2 days, invading cells were counted. Bars and error bars, mean and s.d. of three different experiments. On day 2, the clones were up to three-fold more invasive than the empty vector-transfected cells. NS = not significant.

necessary to determine if MMP-10 is a valid serum tumor marker. At least, gastritis may not be a reason for high levels of MMP-10 protein because it was not detected in 15 of the 20 serum samples of patients with gastritis, and only three samples showed low levels (200–400 pg/ml). Overexpression of MMP-10 was reported in human diabetic corneas (Saghizadeh *et al.*, 2001), but no expression was observed in synovial samples from patients with rheumatoid arthritis (Hembry *et al.*, 1995).

Clinical trials using MMP inhibitors as cancer therapeutics have been reported (Rudek *et al.*, 2001; Rizvi *et al.*, 2004). MMP inhibitors inhibit a broad spectrum of MMPs, and several adverse effects have been reported. MMP-10 activates proMMP-7 and proMMP-9 (Nakamura *et al.*, 1998), which are thought to be particularly important for the malignant behavior of GC cells (Nomura *et al.*, 1996; Yamashita *et al.*, 1998). As expression of MMP-10 was narrowly restricted in cancer, MMP-10-specific inhibitors may provide antitumor drugs with less adverse effects for the treatment of various types of cancers, including GC.

Of two peritoneal wash cytology-positive samples, one showed a high MIA level. The levels of MMP-10 in peritoneal wash fluid were higher in two cytology-positive samples than those in cytology-negative samples. Peritoneal wash cytology is a frequently performed and important technique in the diagnosis of peritoneal dissemination. However, it often fails to detect malignant cells. It has been reported that peritoneal wash cytological examination is the most significant factor predicting peritoneal recurrence, with a sensitivity of 56% (Bando *et al.*, 1999). As pellets from peritoneal wash fluid are used for cytology, it may be useful to investigate the levels of MIA or MMP-10 in the supernatants of cytology-negative peritoneal wash fluid to detect micrometastases of GC cells in the peritoneal cavity. As we studied here a small number of peritoneal wash fluids, additional investigation with more samples will clarify whether measurement of the levels of MIA or MMP-10 is useful to detect occult cancer cells in the supernatants of cytology-negative peritoneal wash fluid.

DKK4 immunostaining was identified in only 1.3% of GC samples, whereas overexpression of *DKK4* mRNA was observed in 25.0% of GC samples by quantitative RT–PCR. As bulk GC tissues were used for quantitative RT–PCR analysis, the resulting data may not reflect the expression levels of *DKK4* in cancer cells alone. DKK4-positive cells were identified in the intestinal metaplasia of the stomach by immunostaining. Expression of *DKK4* mRNA observed in quantitative RT–PCR may be due to non-neoplastic tissue contamination, such as intestinal metaplasia. *DKK4* suppresses the Wnt signaling pathway (Mao and Niehrs, 2003), which is thought to participate in carcinogenesis (Beachy *et al.*, 2004). Thus, DKK4 may not be a good therapeutic target, at least for GC.

In addition to MIA, MMP-10, and DKK4, *APin protein*, *GW112*, and *Reg IV* are reported to be secretory proteins (Clark *et al.*, 2003; Solomon *et al.*, 2003). These secreted molecules may constitute good serum tumor markers. Creation of antibodies and immunohistochemical and functional analyses of *APin protein*, *GW112*, and *Reg IV* should be performed. In addition, recent data have shown the antiapoptotic activity of GW112 (Zhang *et al.*, 2004).

Although we identified several genes that were overexpressed in GC SAGE libraries, there were many genes that were overexpressed according to SAGE but not quantitative RT–PCR. The inconsistent results between SAGE and quantitative RT–PCR may be due to the small number of SAGE libraries. Among 54 candidates, the expression of many genes was highest in the normal pancreas or duodenum by quantitative RT–PCR. To further identify cancer-specific genes, detailed SAGE libraries of normal tissues, such as pancreas and duodenum, are needed.

In conclusion, our present study yielded a list of genes that are potential tumor markers of GC. We identified MMP-10 as a serum tumor marker for diagnosis of GC and identified MIA and MMP-10 as prognostic indicators of GC. We identified several genes by quantitative RT–PCR that have not previously been implicated in GC. Although the functions of these genes, *APin*, *GW112*, and *Reg IV*, are not well understood in cancer, they may provide novel therapeutic targets for GC. Our current data also provide information with respect to the expression of these genes throughout the body. As both MIA and MMP-10 play important roles in tumor cell invasion, specific inhibitors against MIA or MMP-10 may constitute good anticancer drugs with less adverse effect. As the number of samples from normal organs studied here was small, additional examination will certify the specificity of nine GC-specific genes identified in this study.

Materials and methods

Tissue samples

In all, 195 primary tumors, 69 serum samples, and 29 peritoneal wash samples were collected from patients diagnosed with GC. Patients were treated at the Hiroshima University Hospital or an affiliated hospital.

For quantitative RT–PCR, 44 GC samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained during surgery at the Hiroshima University Hospital or an affiliated hospital. We confirmed microscopically that the tumor specimens were predominantly (>80%) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. Noncancerous samples of heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain, and spinal cord were purchased from Clontech (Palo Alto, CA, USA).

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 151 patients who had undergone surgical excision for GC. Of the 151 patients, 59 had early GC and 92 had advanced GC. Early GC is limited to the mucosa, or the mucosa and submucosa, regardless of nodal status. Advanced GC is a tumor whose invasion is beyond muscularis propria (Hohenberger and Gretschel, 2003). Information on patient prognosis was available for 58 of the 92 advanced GC cases.

Among 151 GC cases used for immunohistochemical analysis, serum samples were available for ELISA from 69 GC cases (44 men and 25 women; age range, 35–88 years; mean, 68.7 years). Serum samples were collected presurgically, before initiation of therapy, and stored at −80°C until analysis. Serum samples from 20 patients with chronic-active gastritis with *Helicobacter pylori* infection (13 men and seven women; age range, 57–85 years; mean, 68.8 years) were also collected. The presence of *H. pylori* was determined with the following tests: the H&E staining and Giemsa staining of biopsied tissues, rapid urease test, and urea breath test. Control serum samples were obtained from 60 healthy individuals (32 men and 28 women; age range, 30–83 years; mean, 63.4 years). Peritoneal wash samples from 29 patients with GC were obtained at the time of surgery at the Hiroshima University Hospital or an affiliated hospital.

Histologic classification (intestinal-type or diffuse-type) was according to the Lauren classification system (Lauren, 1965). Tumor staging was according to the TNM staging system (Sobin and Wittekind, 2002). As written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis; this procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Identification of candidate GC-specific genes

To identify GC-specific genes, we used four GC SAGE libraries (W226T, GSM8867; W246T, GSM8505; S219T, GSM7800; P208T, GSM9103) published by us and 14 normal SAGE libraries (white matter, GSM676; cerebellum, GSM695; thalamus, GSM713; heart, GSM1499; lung, GSM762; stomach, GSM784; colon, GSM728; liver, GSM785; kidney, GSM708; leukocyte, GSM709; peritoneum, GSM738; skeletal muscle, GSM819; spinal cord, GSM2386; lymph node, GSM14785) available from the SAGEmap online database (http://www.ncbi.nlm.nih.gov/SAGE/; Lal *et al*., 1999). We compared tags from each GC SAGE library with those of normal SAGE libraries. To exclude tags generated by sequencing errors, we selected only tags that occurred at least three times in each GC SAGE library. In addition, we selected tags that were not found in any of the 14 normal SAGE libraries.

Evaluation of the specificity of gene expression

To evaluate the specificity of expression of each gene, a specificity index was calculated as follows: first, we identified the normal tissue in which the target gene expression was the highest of the 14 normal tissues analysed by quantitative RT–PCR (the mRNA expression level in this tissue was denoted as A). We then identified the GC among the nine GC samples in which the target gene expression was highest by quantitative RT–PCR (the mRNA expression level in this tissue was denoted as B). The ratio B/A was defined as the specificity index. When the specificity index of the target gene was >10, the gene was considered to show high specificity for GC. When the specificity index of the target gene was <10 and >2, the gene was considered to show low specificity for GC. When the specificity index of the target gene was <2, the gene was considered to show no specificity for GC.

Quantitative RT–PCR analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with an First Strand cDNA Synthesis Kit (Amersham Biosciences Corp., Piscataway, NJ, USA). PCR was performed with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously (Kondo *et al*., 2004). *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls (Kondo *et al*., 2004). Primer sequences and additional PCR conditions are available upon request.

Immunohistochemistry

A Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis. In brief, microwave pretreatment in citrate buffer was performed for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2–methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block nonspecific antibody-binding sites. Sections were incubated with the following antibody dilutions: mouse monoclonal anti-MIA (2F7), 1:50; mouse monoclonal anti-MMP-10, 1:100 (Novocastra, Newcastle, UK), and mouse monoclonal anti-DKK4, 1:100 (R&D Systems, Abingdon, UK). The specificity of the MIA antibody has been characterized in detail (Bosserhoff *et al*., 1999). Specificity of DKK4 staining was confirmed by preabsorption of the anti-DKK4 antibody with excess DKK4 protein (R&D Systems). Sections were incubated with primary antibody for 8 h at 4 C, followed by incubations with biotinylated anti-mouse IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The percentage of stained cancer cells was evaluated for each antibody. A result was considered positive if at least 50% of the cells were stained. When fewer than 50% of cancer cells were stained, the immunostaining was considered negative.

ELISA

Serum and peritoneal wash fluid levels of MIA and MMP-10 were measured with an MIA ELISA Kit (Roche Diagnostics Co., Indianapolis, IN, USA) and a Quantikine Human MMP-10 Immunoassay Kit (R&D Systems) according to the manufacturer's instructions.

Cell lines, expression vector, and stable transfection

A human GC-derived cell line, MKN-28, was kindly provided by Dr Toshimitsu Suzuki. MKN-28 cells were maintained in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MA, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

For constitutive expression of the *MIA* gene, cDNA was PCR amplified and subcloned into pcDNA 3.1 (Invitrogen Corp., Carlsbad, CA, USA). The pcDNA-MIA expression vector was transfected into MKN-28 cells with FuGENE6 (Roche Diagnostics) according to the manufacturer's instructions. Stable transfectants were selected after 2 weeks of culture with 80 μg/ml G418 (Invitrogen). The amount of secreted MIA protein in cell culture supernatants was determined by MIA ELISA.

Cell growth and in vitro *invasion assays*

Cultured cells were harvested from 80% confluent monolayer cultures by brief treatment with 0.1% trypsin and 0.1% EDTA.

The cells were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after 1 and 2 days by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Alley *et al*., 1988).

Modified Boyden chamber assays were performed to examine invasiveness. Stably transfected cells were plated at 10 000 cells per well in RPMI 1640 medium plus 1% serum in the upper chamber of a Transwell insert (8 μm pore diameter; Chemicon, Temecula, CA, USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After 1 and 2 days, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye to assess the number of cells.

Statistical methods

Statistical analyses were carried out with Fisher's exact test and the Mann–Whitney *U*-test. Kaplan–Meier survival curves were constructed for MIA or MMP-10-positive and MIA or MMP-10-negative patients. Survival rates were compared between MIA or MMP-10-positive and MIA or MMP-10-negative groups. Differences between survival curves were tested for statistical significance by log-rank test (Mantel, 1966). *P*-value less than 0.05 was considered statistically significant.

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