Table 1. Up-regulated and down-regulated tags and genes in gastric cancer obtained by SAGE

Commonly up-regulated and down-regulated tags and genes in gastric cancer in comparison with normal gastric epithelia	
Up-regulated	APOC1, S100A4, NDUF2, TEBP, COL1A2, SUFU, SYAP1, KIAA0930, KIAA1694, TFF3, CEACAM6, FLJ20249, FLJ22167, EIF4A1, COLPH2, G3BP, YF13H12, KRT7, SH3BP2, COL1A1, LOC284371
Down-regulated	CAGCGCTTCT (no match), CACCTCCCCA (no match), AGCCTCCCCA (no match), ACCCTCCCCA (no match), LIPF, AACCTCCCCC (no match), CHIA, TAGTGCTTCT (no match), TACAAGGTCC (no match), GTGGTCAGCT (no match), ATP4B, FLJ20410, MBD3, CAGTGCTTTT (no match), Hs.199360, Hs.353061
The 20[1] most up-regulated and down-regulated tags and genes in advanced gastric cancer in comparison with early gastric cancer	
Up-regulated	TCCCCGTAAA (no match), TCCCGTACAT (no match), CDH17, FUS, PRO1073, FLJ36926, FLJ30146, PAI-RBP1, COL1A2, TCCTATTAAG (no match), COL1A1, GRAP2, HNRPL, NUTF2, ERP70, PES1, CYP2J2, DAG1, IQGAP1, IL16, FXYD3, COQ4, LOC91966, CTBP1, TTCGGTTGGT (no match), alpha4GnT, Hs.290723, AKT3, CCT3, HMG20A
Down-regulated	Hs.216636, LOC116228, SH3MD2, NAB1, TTCCCCCAAA (no match), DDX5, VMP1, LOC51123, LZK1, CGCAGATCAG (no match), IFRD2, Hs.284464, RPS4Y, RPS4Y2, UAP1, Hs.180804, CATTAAATTA (no match), IKBKAP, ARPC3, NAGA, UBE3A, TRAG3, PNN, CTAATTCTTT (no match), TCCATCGTCC (no match)
The 20[1] most up-regulated and down-regulated tags and genes in lymph-node metastasis in comparison with primary tumor of gastric cancer	
Up-regulated	SCAND1, RGS5, S100A11, RNPC2, APOE, FLJ10815, RNASE1, H3F3B, P24B, LOC151103, CLDN3, MRPL14, PRex1, TCCCCTATTA (no match), Hs.105379, ATP5G1, NPD007, MGC3180, WDR11, ARPC1B, ABTB2, DNAJB1, HMGN2, KIAA1393, RAP1B, FLJ12150, STUB1
Down-regulated	ERdj5, RPL27A, DHRS3, E2IG5, USP7, CTSL, KRTHB1, KRTHB3, TGCACTACCC (no match), ALG12, S100A9, CTAGCTTTTA (no match), ELOVL5, LOC375463, GGGGGAGTTT (no match), ACTGCCCTCA (no match), SPC18, CTNND1, CYP20A1, FLJ11151, RPS17, ZYX, RPS16, GCTTTCTCAC (no match), BCL2L2

The gene symbol is shown, while the UniGene ID is given if the symbol is not present. No match, tag sequence is not matched to any known gene.
1) Because some genes share the same SAGE tag, gene numbers are more than 20.

compare our tags with those of other SAGE libraries using SAGEmap.

Identification of gastric cancer-specific genes by SAGE

To identify gastric cancer-specific genes, SAGE tags from six primary gastric cancers were compared with those from normal gastric epithelia, and the 20 most up-regulated and 20 most down-regulated tags were selected.[22] This produced a dataset of 128 tags because of overlapping. By complete linkage clustering among eight gastric libraries in the SAGEmap database, clusters with commonly up-regulated genes and down-regulated genes in all the gastric cancers were identified (Table 1). The up-regulated gene cluster included *APOC1, NDUF2, TEBP, COL1A1* and so on, in addition to *TFF3* and *S100A4*, which are known to be up-regulated in gastric cancer. Quantitative real-time RT-PCR on 46 cases of gastric cancer revealed that *APOC1, CEACAM6* and *YF13H12* were frequently overexpressed (more than 50% of cases showed a tumor/normal ratio >2) in gastric cancer tissues, and these should be novel genetic markers for gastric cancer. On the other hand, the down-regulated gene cluster included *LIPF* (gastric lipase), *CHIA, ATP4B, MBD3* and many unknown genes (tags whose corresponding genes have not been identified). Because they were commonly down-regulated in gastric cancer, the unknown genes are novel candidates for gastric-specific tumor suppressors. The reverse SAGE technique will help to identify these genes.[24]

Another approach to identify gastric cancer-specific genes is the selection of candidate genes by comparing SAGE libraries of gastric cancer with those of various normal tissues in the SAGEmap database. We picked up about 60 genes which were detected in our gastric cancer libraries, but not in the libraries from 15 kinds of normal tissues, especially important or crucial organs, including brain, lung, heart, liver, kidney, etc. We then examined the expression of these genes in normal human tissues by RT-PCR, and representative results are shown in Fig. 2. Many genes were expressed at various levels in normal tissues, although the comparison of SAGE data suggested them to be gastric cancer-specific. Some genes (genes H, I, J, K) were not expressed significantly in normal tissues, but were expressed in gastric cancer cells. Therefore, these may be gastric cancer-specific, or at least cancer-specific, and could be candidates for novel genetic markers.

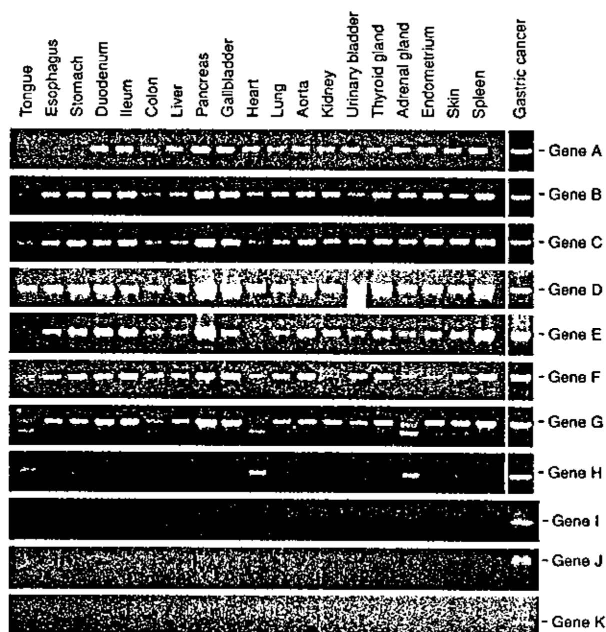

Fig. 2. Expression of genes detected in SAGE libraries of gastric cancer, but not in the libraries of 15 normal tissues. Semi-quantitative RT-PCR on various normal human tissues revealed that genes H, I, J and K are not expressed significantly in normal tissues, whereas they are detected in gastric cancer cells.

Identification of genes involved in invasion and metastasis by SAGE

The genes participating in invasion and metastasis can be identified by comparing SAGE libraries between cancers with and without invasion and metastasis. For this purpose, we first compared SAGE libraries between an early cancer (T1, N0,

M0, stage IA)[25] and an advanced cancer (T2, N2, M0, stage IIIA).[22] Both were well-differentiated-type gastric cancer (tub1) with intestinal phenotype expressing MUC2. The 20 most up-regulated and down-regulated tags and corresponding genes in the advanced cancer are shown in Table 1. Up-regulated genes include some unknown genes, *CDH17, FUS* and so on. Quantitative real-time RT-PCR demonstrated the frequent overexpression of *FUS, CDH17, COL1A1* and *COL1A2* and its positive correlation with degree of invasion, metastasis and advanced stage. *FUS* is a tumor-associated fusion gene, especially in myxoid liposarcoma, and it may have a role in regulating transcription and maintaining chromosomal stability.[26] *CDH17* (cadherin 17, liver-intestine cadherin) is known to be up-regulated in intestinal metaplasia and well-differentiated-type gastric cancer of intestinal phenotype.[27] Our immunohistochemical study confirmed that overexpression of cadherin 17 is associated with advanced stage and intestinal histology (Fig. 3, A and B), and cadherin 17-positive patients showed a poorer prognosis than the negative patients.

Using the same strategy, we tried to identify genes involved in metastasis. We compared SAGE libraries between the primary gastric cancer (por1) and its lymph-node metastatis from the same patient.[22] The 20 most up-regulated tags and corresponding genes in the metastatic tumor included *SCAND1, RGS5, S100A11, RNPC2, APOE* and so on (Table 1). Among them, *APOE* (apolipoprotein E) expression was confirmed to be associated with T grade, N grade and advanced stage. Immunohistochemically, apolipoprotein E was expressed mainly in stromal cells which are also positive for CD68, suggesting tumor-associated macrophages. Apolipoprotein E-positive cells were more prominent in the metastatic tumor than in the primary tumor (Fig. 3, C and D). Therefore, APOE may be a novel marker for metastasis, although the mechanism involved remains to be elucidated.

Candidate novel biomarkers of gastric cancer

Ideal biomarkers for cancer should have the following four characteristics.[14] First, they should be expressed at high levels in tumors and at greatly reduced levels in normal tissues. Second, the elevated expression should occur early and remain elevated during the neoplastic process. Third, the markers should be elevated in a majority of clinical samples. Fourth, the markers should be expressed on the cell surface or secreted to facilitate detection. Genes and molecules that meet all these criteria are especially useful for the development of diagnostic tools for early cancer detection. Moreover, if the function of the gene product is involved in the neoplastic process, such a gene is not just a biomarker, but can be a therapeutic target. In the course of the SAGE study, we found that *REGIV* (regenerating gene type IV) meets these criteria. A comparison of the expressed tags of scirrhous-type gastric cancer (por2) with those of normal gastric epithelia showed that *REGIV* was the second most up-regulated gene after *TFF3*.[22] Virtual northern analysis revealed that *REGIV* expression is narrowly restricted in comparison with other tissues and cancers in the SAGEmap database. Furthermore, quantitative RT-PCR showed that about a half of gastric cancers overexpressed *REGIV* mRNA, regardless of tumor stage and histological differentiation, whereas low levels of *REGIV* expression were limited to non-neoplastic gastrointestinal and pancreatic tissues. RegIV belongs to the calcium-dependent lectin superfamily, and is known to increase in inflammatory bowel diseases and a portion of colon

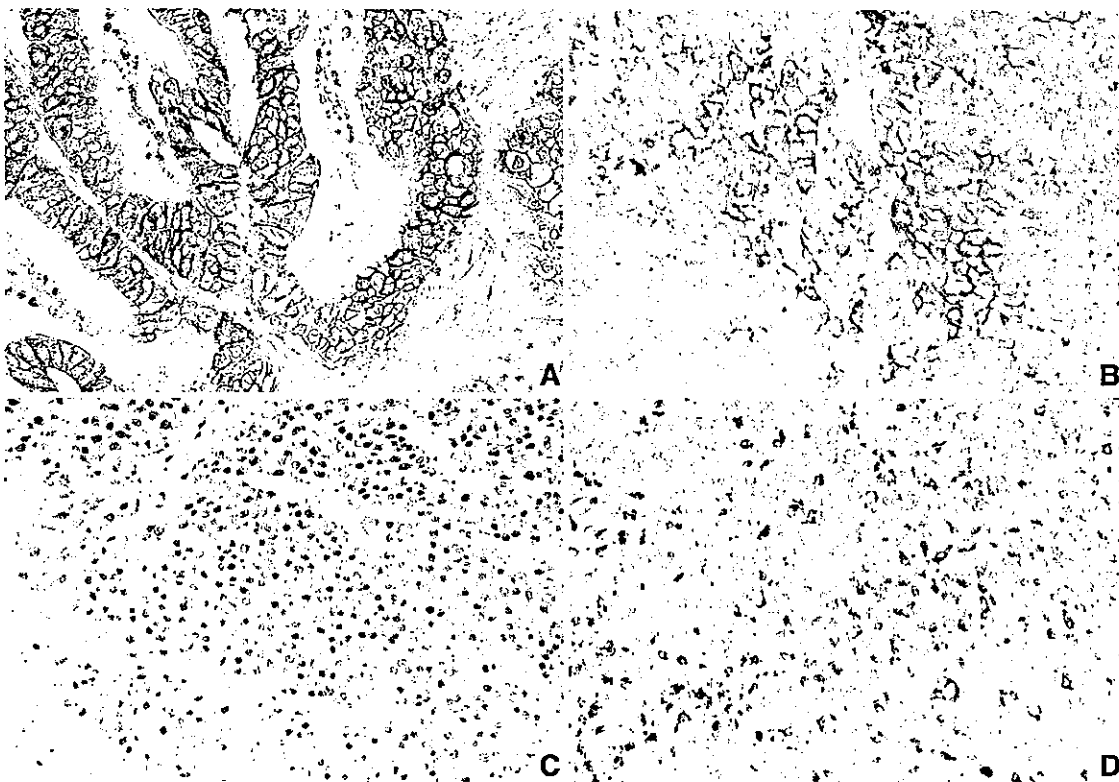

Fig. 3. Expression of cadherin 17 and apolipoprotein E in gastric carcinomas (immunohistochemical analysis). Cadherin 17 is expressed at the cell surface and cell-cell border of well-differentiated-type gastric adenocarcinoma with intestinal phenotype (A) and poorly differentiated solid-type gastric adenocarcinoma (B). Apolipoprotein E-positive cells are more prominent in metastatic tumor (D) than in primary tumor (C) of poorly differentiated solid-type adenocarcinoma.

cancers.[28,29] Transfection of a RegIV expression vector (pcDNA-RegIV-V5) into gastric cancer cell lines enhanced invasion and inhibited apoptosis. RegIV-V5 was detected in culture media of the transfected cells, indicating that RegIV is secreted by cancer cells. These findings strongly suggest that RegIV may be involved in the neoplastic process, and therefore, RegIV is not just a biomarker, but a novel therapeutic target for gastric cancer.

To obtain an anti-RegIV agent for diagnostic and therapeutic purpose, one way is the production of antibody, but another is the production of oligonucleotides that specifically bind to target molecules. Small non-coding RNA has been focused on recently as having a range of potential functions including regulation of gene expression. Oligonucleotide sequences which recognize target molecules with high affinity and specificity, called aptamers, can be isolated by systemic evolution of ligands by exponential enrichment (SELEX) process.[30,31] Recombinant RegIV protein from full-length RegIV cDNA and a random sequence oligonucleotide library are mixed and incubated. Bound oligonucleotides are selected and amplified by PCR. This cycle is repeated at least 10 times to obtain a specific aptamer. Aptamers rival antibodies because they are

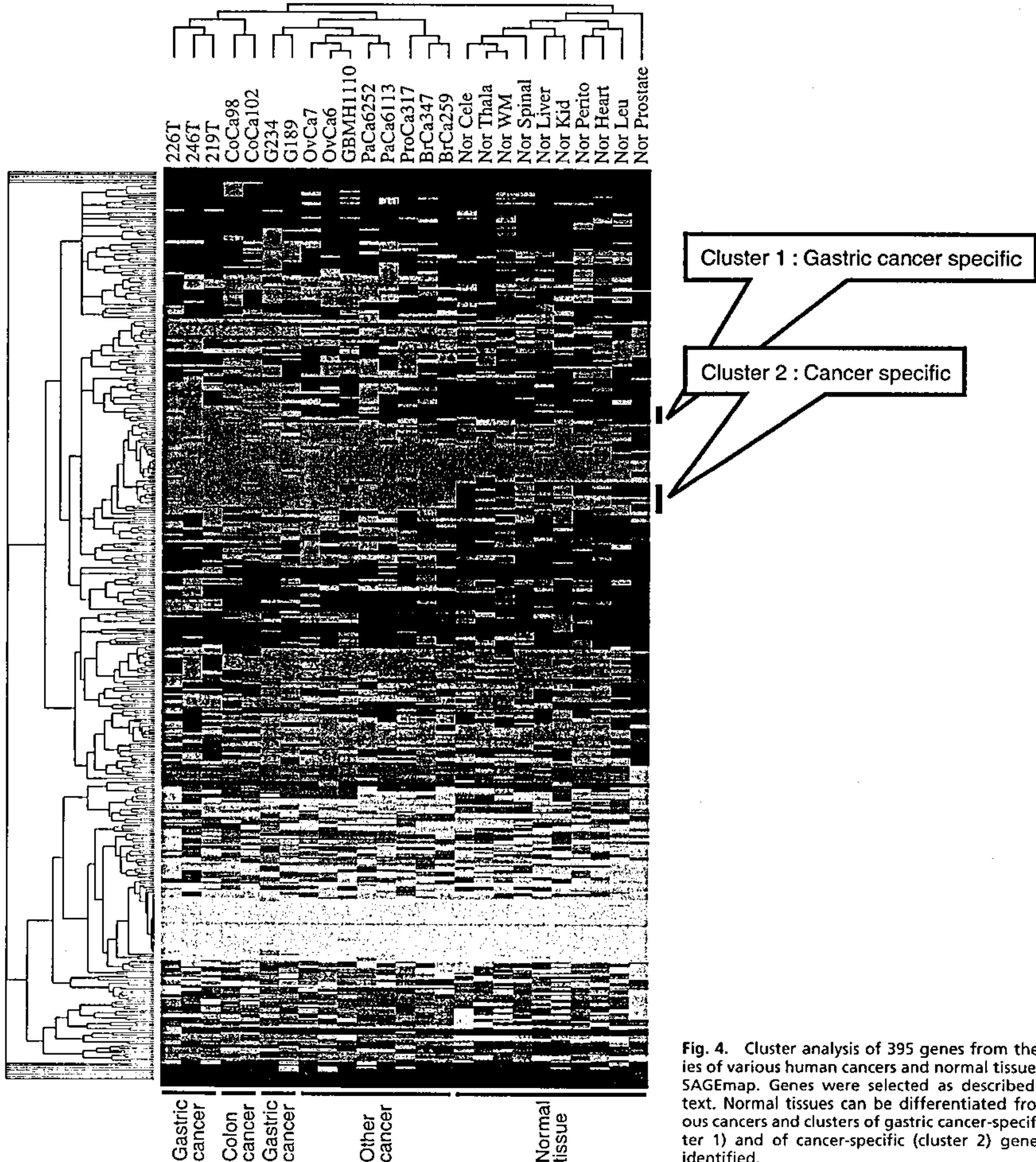

Fig. 4. Cluster analysis of 395 genes from the libraries of various human cancers and normal tissues in the SAGEmap. Genes were selected as described in the text. Normal tissues can be differentiated from various cancers and clusters of gastric cancer-specific (cluster 1) and of cancer-specific (cluster 2) genes were identified.

highly specific, able to distinguish family members, exhibit reversible equilibrium binding, and can be synthesized automatically in an *in vitro* system.

Development of specialized cDNA microarray for study and diagnosis of gastric cancer

Microarray studies have uncovered many genes related to biological behavior, such as metastasis and prognosis and sensitivity to chemotherapy.[8,9,32–34] On the other hand, SAGE has identified many differentially expressed genes and candidate novel biomarkers. However, it is hard to study large numbers of clinical samples and to apply the technique in a clinical setting. Because differential expression must be confirmed by other methods, many genes still remained unconfirmed. Thus, we decided to prepare a custom-made microarray for the study of stomach carcinogenesis and possible clinical application. A similar approach has been introduced in ovarian cancers.[35] The microarray, named Ex-STOMACHIP, consists of 395 genes selected based on the following three criteria. 1) The 164 genes which were selected as the 20 most up-regulated and down-regulated tags in the six SAGE libraries of gastric cancer studied by us, as already mentioned. 2) Known genes participating in stomach carcinogenesis, including genes of growth factors/receptors, cell cycle regulators, metalloproteinases, adhesion molecules, and so on. 3) Genes related to metastasis and chemosensitivity identified by other cDNA microarray studies. Using these sets of genes, hierarchical clustering was performed among various SAGE libraries of cancerous and non-cancerous tissues in the SAGEmap database. As shown in Fig. 4, gastric cancer tissues or other cancers including cancers of the ovary, pancreas and breast were differentiated from various normal tissues, and several gene clusters were identified. For instance, cluster 1 is gastric cancer-specific, and cluster 2 is specific for cancer in general. When results among gastric cancers with various differences in morphology and biological behavior were compared, clear gene clusters could be identified. Thus, Ex-STOMACHIP should be a useful tool not only to study mechanisms of stomach carcinogenesis, but also to obtain information about biological behavior and sensitivity to therapy in the clinical setting.

New strategy of gene diagnosis of gastric cancer

In 1993, we established a gene diagnosis system for gastrointestinal pathology specimens and performed this as a routine service until 2000, using so-called classical molecular and genetic markers, including *p53, APC, p27, EGFR*, microsatellite assay and so on.[3,36] We analyzed more than 10,000 cases, and obtained much useful information concerning differential diagnosis, grade of malignancy and susceptibility of multiple primary cancers. Now, the molecular diagnosis of pathology specimens must move into the era of genomic medicine. Here, we have outlined our search for new genetic markers of gastric

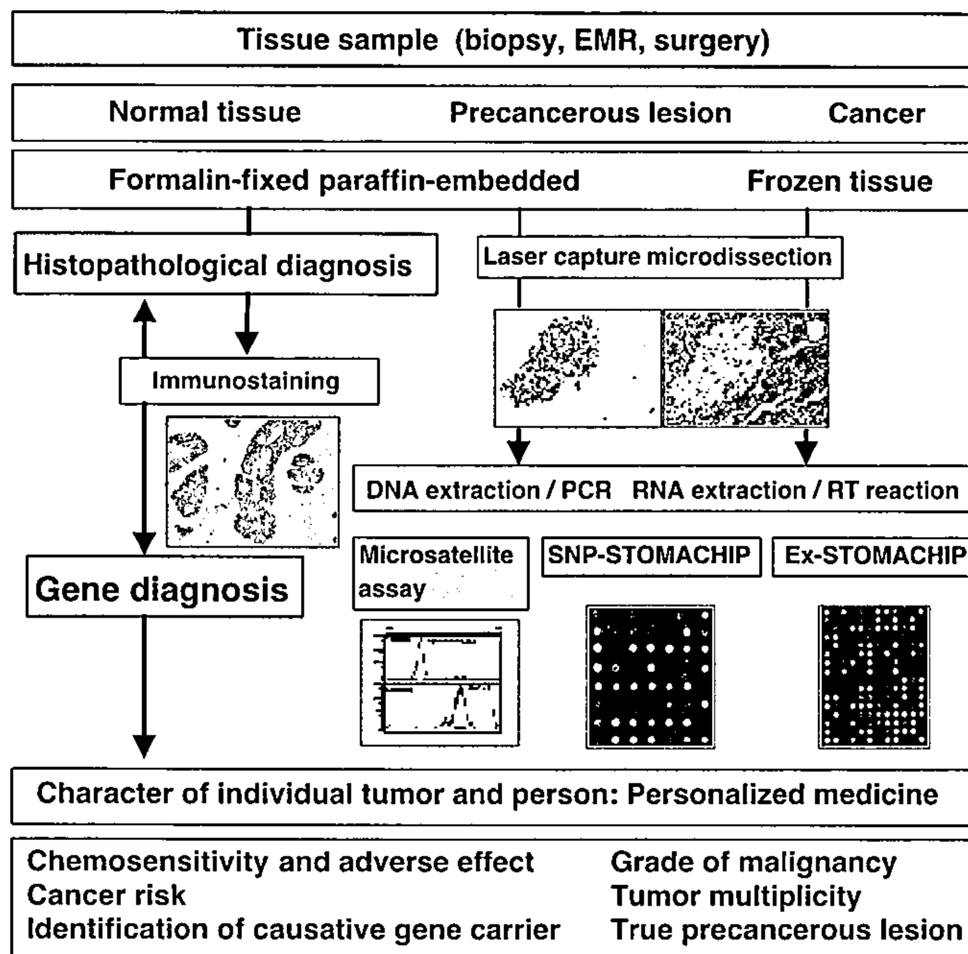

Fig. 5. New strategy of gene diagnosis of gastric cancer using pathology samples.

cancer and their clinical implications. Many new candidate biomarkers of gastric cancer were identified by SAGE, and can be introduced into clinical diagnosis through two approaches. One is the use of custom-made arrays for analysis of tissue samples, and the other is detection systems for blood samples using RNA aptamer or antibody. Information about SNPs in highly differentially expressed genes will be directly connected with cancer prevention. Fig. 5 illustrates the new strategy of gene diagnosis of gastric cancer on pathology samples obtained by biopsy, endoscopic mucosal resection or surgery. Tissues are collected from samples freshly frozen or fixed with formalin and embedded in paraffin, and DNA and RNA are recovered using laser capture microdissection, if necessary. The gene expression profile is examined by the use of Ex-STOMACHIP to obtain information about grade of malignancy and chemosensitivity, as well as possible side effects. Microsatellite analysis predicts tumor multiplicity. If these analyses are extended to mucosa without overt morphological aberration, super-early diagnosis of gastric cancer should be possible. Analysis of genetic polymorphism will give information about cancer risk and sensitivity to chemotherapy. Combinations of these approaches can not only achieve cancer detection, but also clarify the character of the individual tumor and patient, thereby leading to personalized medicine and cancer prevention.

Conclusion

SAGE is a powerful technique to identify novel genes associated with gastric cancer, and to search for new biomarkers of gastric cancer. Our SAGE libraries are the largest gastric cancer libraries in the world. By comparing the tags expressed in gastric cancers with those in normal gastric epithelia or each other, many differentially expressed genes were identified. *CEACAM6*, *APOC1* and *YF13H12* are commonly up-regulated in gastric cancer, while *FUS*, *CDH17*, *COL1A1*, *COL1A2* and *APOE* are associated with invasion and metastasis. RegIV, secreted by cancer cells, inhibits apoptosis, suggesting that RegIV may serve as a novel biomarker and therapeutic target. Production of RNA aptamer should be effective to establish a detection system for blood samples. A custom-made array, named Ex-STOMACHIP, consisting of 395 genes, including highly differentially expressed genes identified by SAGE, is useful to study molecular stomach carcinogenesis and to obtain information about biological behavior and sensitivity to therapy in the clinical setting. The combination of gene expression profiling and determination of genetic polymorphism will allow characterization of individual cancers and patients, leading directly to personalized medicine and cancer prevention.

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ADAM8 as a Novel Serological and Histochemical Marker for Lung Cancer

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ABSTRACT

Purpose and Experimental Design: **We have been investigating genes involved in pulmonary carcinogenesis by examining gene expression profiles of non–small-cell lung cancers to identify molecules that might serve as diagnostic markers or targets for development of new molecular therapies. A gene encoding ADAM8, a disintegrin and metalloproteinase domain-8, was selected as a candidate for such molecule. Tumor tissue microarray was applied to examine expression of ADAM8 protein in archival lung cancer samples from 363 patients. Serum ADAM8 levels of 105 lung cancer patients and 72 controls were also measured by ELISA. A role of ADAM8 in cellular motility was examined by Matrigel assays.**

Results: **ADAM8 was abundantly expressed in the great majority of lung cancers examined. A high level of ADAM8 expression was significantly more common in advanced-stage IIIB/IV adenocarcinomas than in adenocarcinomas at stages I–IIIA. Serum levels of ADAM8 were significantly higher in lung cancer patients than in healthy controls. The proportion of the serum ADAM8-positive cases defined by our criteria was 63% and that for carcinoembryonic antigen was 57%, indicating equivalent diagnostic power of these two markers. A combined assay using both ADAM8 and carcinoembryonic antigen increased sensitivity because 80% of the lung cancer patients were then diagnosed as positive, whereas only 11% of 72 healthy volunteers were falsely diagnosed as positive. In addition, exogenous expression of ADAM8 increased the migratory activity of mammalian cells, an indication that ADAM8 may play a significant role in progression of lung cancer.**

Conclusions: **Our data suggest that ADAM8 should be useful as a diagnostic marker and probably as a therapeutic target.**

INTRODUCTION

Lung cancer is one of the most common cancers in the world, and non–small-cell lung cancer (NSCLC) accounts for ~80% of those cases (1). Because the prognosis of advanced lung cancer remains poor, development of novel therapeutic and diagnostic strategies is an urgent goal (2). Tumor markers that are currently available for lung cancer, such as carcinoembryonic antigen (CEA), serum cytokeratin 19 fragment (CYFRA 21-1), and progastrin-releasing peptide (pro-GRP), are not satisfactory for diagnosis at an early stage or for monitoring the disease because of their relatively low sensitivity and specificity in detecting the presence of cancer cells (3–5). Although the precise pathways involved in lung tumorigenesis remain unclear, some evidence indicates that tumor cells express cell surface markers unique to each histologic type at particular stages of differentiation. Because cell surface proteins are considered more accessible to immune mechanisms and drug delivery systems, identification of cancer-specific cell surface and secretory proteins is likely to be an effective approach to development of novel diagnostic markers and therapeutic strategies.

We have been screening genes encoding transmembrane/secretory proteins that are up-regulated in lung cancers, with cDNA microarrays and tumor cells purified by laser-capture microdissection (6, 7). To verify the biological and clinicopathological significance of the respective gene products, we have been performing tumor tissue microarray analysis of clinical lung cancer materials. This systematic approach revealed that a disintegrin and metalloproteinase domain-8 (ADAM8), a cell surface disintegrin and metalloproteinase domain-8 molecule, was frequently transactivated in primary lung cancers.

ADAM family members are implicated to be involved in the proteolytic processing of membrane-bound precursors, and they modulate cell-cell and cell-matrix interactions. ADAM8 encodes a protein of 824 amino acids with a COOH-terminal transmembrane domain and potential extracellular adhesion and protease domains (8, 9). This molecule, localized to the plasma membrane, is processed by autocatalysis into two forms; one is derived by removal of a prodomain and the other is a remnant protein composed of the extracellular region with a disintegrin domain at the NH_2 terminus (10). ADAM8 behaves as an active metalloprotease *in vitro*, hydrolyzing myelin basic protein and a variety of peptide substrates based on the cleavage sites of membrane-bound cytokines, growth factors, and receptors (11–

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14). Other studies have demonstrated overexpression of some ADAM family proteins in a variety of human tumors (15, 16), but involvement of ADAM8 in human cancer was not indicated previously.

We report here the identification of ADAM8 as a novel diagnostic marker and a potential target for therapeutic agents/antibodies and also provide evidence for its possible role in human pulmonary carcinogenesis.

MATERIALS AND METHODS

Cell Lines and Clinical Samples. The 23 human lung cancer cell lines used in this study included nine adenocarcinomas (A427, A549, LC319, NCI-H1373, PC-3, PC-9, PC-14, NCI-H1666, and NCI-H1781), nine squamous cell carcinomas (EBC-1, LU61, NCI-H520, NCI-H1703, NCI-H2170, RERF-LC-AI, SK-MES-1, NCI-H226, and NCI-H647), one large-cell carcinoma (LX1), and four small-cell lung cancers (SCLCs; DMS114, DMS273, SBC-3, and SBC-5). All cells were grown in monolayers in appropriate media supplemented with 10% 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 (6). A total of 363 formalin-fixed primary tumors (206 adenocarcinomas, 118 squamous cell carcinomas, 25 large-cell carcinomas, and 14 SCLCs) and adjacent normal lung tissue samples from 336 patients undergoing surgery at Saitama Cancer Center (Saitama, Japan) and from postmortem materials (27 individuals) obtained from Hiroshima University (Hiroshima, Japan) were also used in this study.

Serum samples were obtained with informed consent from 72 healthy individuals as controls (40 males and 32 females; median age, 61.5 ± 9.1 SD; range, 42–89 years). The healthy individuals showed no abnormalities in complete blood cell counts, C-reactive proteins, erythrocyte sedimentation rates, liver function tests, renal function tests, urinalyses, fecal examinations, chest X-rays, or electrocardiograms. Serum samples were also obtained with informed consent from 105 lung cancer patients admitted to Hiroshima University Hospital (78 males and 27 females; median age, 68.0 ± 10.8 SD; range, 30–84 years). Samples were selected for the study on the basis of the following criteria: (*a*) patients were newly diagnosed and previously untreated and (*b*) their tumors were pathologically diagnosed as lung cancers (stages I–IV). These 105 cases included 62 adenocarcinomas, 25 squamous cell carcinomas, and 18 SCLCs. Clinicopathological records were fully documented. Serum was obtained at the time of diagnosis and stored at −80°C. Disease staging in all 105 cases was supported by a computed tomography scan of the chest and abdomen, bone scintigraphy, and magnetic resonance imaging of the head.

Semiquantitative Reverse Transcription-PCR Analysis. Total RNA was extracted from cultured cells and clinical tissues with Trizol reagent (Life Technologies, Inc., Gaithersburg, MD), 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 reverse transcribed with oligo$(dT)_{12-18}$ primer and SuperScript II reverse transcriptase (Life Technologies, Inc.). Semiquantitative reverse transcription-PCR experiments were carried out with synthesized *ADAM8* gene-specific primers (5′-GTGTGTGTACGTGTCTCCAGGT-3′ and 5′-CAGACAAGATAGCTGACTCTCCC-3′), or with β-actin (*ACTB*)-specific primers (5′-ATCAAGATCATTGCTCCTCCT-3′ and 5′-CTGCGCAAGTTAGGTTTTGT-3′) as an internal control. All PCR reactions involved initial denaturation at 94°C for 2 minutes followed by 22 (for *ACTB*) or 28 cycles (for *ADAM8*) of 94°C 30 s, 54°C to 60°C for 30 seconds, and 72°C for 60 seconds on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

Northern Blot Analysis. Human multiple tissue blots (BD Biosciences, Palo Alto, CA) were hybridized with ^{32}P-labeled PCR products. Prehybridization, hybridization, and washing were done according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at −80°C for 1 week.

Immunohistochemistry and Tissue Microarray. Tumor tissue microarrays were constructed with 363 formalin-fixed primary lung cancers, as published previously (17–19). The tissue area for sampling was selected based on visual alignment with the corresponding H&E-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 with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case, and 5-μm sections of the resulting microarray block were used for immunohistochemical analysis.

Three independent investigators assessed ADAM8 positivity semiquantitatively, recording staining intensity as absent (scored as 0), weak (scored as 1+), or strongly positive (scored as 2+), without prior knowledge of clinicopathological data. Cases were accepted as strongly positive only if reviewers independently defined them as such. Contingency tables were used to analyze the relationship of ADAM8 expression in NSCLCs to clinicopathological data.

To investigate the presence of ADAM8 protein in clinical samples that had been embedded in paraffin blocks, we stained the sections in the following manner. Briefly, 50 μg/mL goat polyclonal antihuman ADAM8 antibody (R&D Systems, Inc., Minneapolis, MN) were added after blocking of endogenous peroxidase and proteins, and the sections were incubated with horseradish peroxidase-labeled antigoat IgG (Histofine Simple Stain MAX PO (G), Nichirei, Tokyo, Japan) as the secondary antibody. Substrate-chromogen was added, and the specimens were counterstained with hematoxylin.

Flow Cytometric Analysis. Lung cancer cells (1×10^6 cells) were incubated with a mouse monoclonal antihuman ADAM8 antibody for detecting the ectodomain of the protein (0.34 mg/mL; R&D Systems, Inc.) or control mouse IgG (0.34 mg/mL; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 hour. The cells were washed in PBS and then incubated with AlexaFluor 488-conjugated antimouse IgG (Molecular Probes, Eugene, OR) at 4°C for 30 minutes. The cells were washed in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson Labware, Bedford, MA) and analyzed by ModFit software (Verity Software House, Inc., Topsham, ME). Mean fluorescence intensity was calculated as a relative signal-intensity value, *i.e.*, of cells treated with antihuman ADAM8 antibody/cells treated with control mouse IgG.

ELISA. Serum levels of ADAM8 were measured by ELISA using a commercially available enzyme test kit (R&D Systems, Inc.). In brief, 3-fold diluted sera were added to a 96-well microplate precoated with monoclonal antibody specific for ADAM8 and incubated for 2 hours at room temperature. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for ADAM8 was added to the wells and incubated for 2 hours at room temperature. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution (R&D Systems, Inc.) was added to the wells and allowed to react for 30 minutes. The reaction was stopped by adding 100 μL of 2 N sulfuric acid. Color intensity was determined by a photometer at a wavelength of 492 nm, with a reference wavelength of 630 nm. Levels of CEA in serum were measured by ELISA with a commercially available enzyme test kit (HOPE Laboratories, Belmont, CA), according to the same protocol as above. Differences in the levels of ADAM8 and CEA between tumor groups and a healthy control group were analyzed by Mann-Whitney *U* tests. The levels of ADAM8 and CEA were additionally evaluated by receiver-operating characteristic curve analysis to determine cutoff levels with optimal diagnostic accuracy and likelihood ratios. The correlation coefficients for these two markers were calculated with Pearson's correlation coefficient. Significance was defined as $P < 0.05$.

Matrigel Invasion Assay. NIH3T3 and COS-7 cells transfected either with plasmids expressing ADAM8 or with mock plasmids were grown to near confluence in DMEM containing 10% FCS. The cells were harvested by trypsinization, washed in DMEM without addition of serum or proteinase inhibitor, and suspended in DMEM at 1×10^5 cells/mL. Before preparing the cell suspension, the dried layer of Matrigel matrix (Becton Dickinson Labware) was rehydrated with DMEM for 2 hours at room temperature. DMEM (0.75 mL) containing 10% FCS was added to each lower chamber in 24-well Matrigel invasion chambers, and 0.5 mL (5×10^4 cells) of cell suspension were added to each insert of the upper chamber. The plates of inserts were incubated for 22 hours at 37°C. After incubation, the chambers were processed; cells invading through the Matrigel were fixed and stained by Giemsa as directed by the supplier (Becton Dickinson Labware).

RESULTS

ADAM8 Expression in Lung Tumors, Cell Lines, and Normal Tissues. To search for novel molecules to serve as diagnostic markers and/or targets for development of therapeutic agents for lung cancer, we had applied cDNA microarray analysis to identify genes that were transactivated in the majority of NSCLCs examined. Among 23,040 genes screened, we identified the *ADAM8* transcript, indicating 2-fold or higher expression (mean fold expression, 5.6 ± 4.5 SD; range, 2.2–16.4) in cancer cells than in normal lung cells (control) in 63% of the NSCLC samples examined, and confirmed its transactivation by semiquantitative reverse transcription-PCR experiments in 8 of 10 additional NSCLC tissues and in 11 of 23 lung cancer cell lines (Fig. 1*A*). Northern blot analysis with human cDNA as a probe detected a 3.5-kb transcript but at a very low level and only in leukocytes, lymph node, and bone marrow among 16 normal tissues examined (data not shown).

We also examined expression of ADAM8 protein in clin-

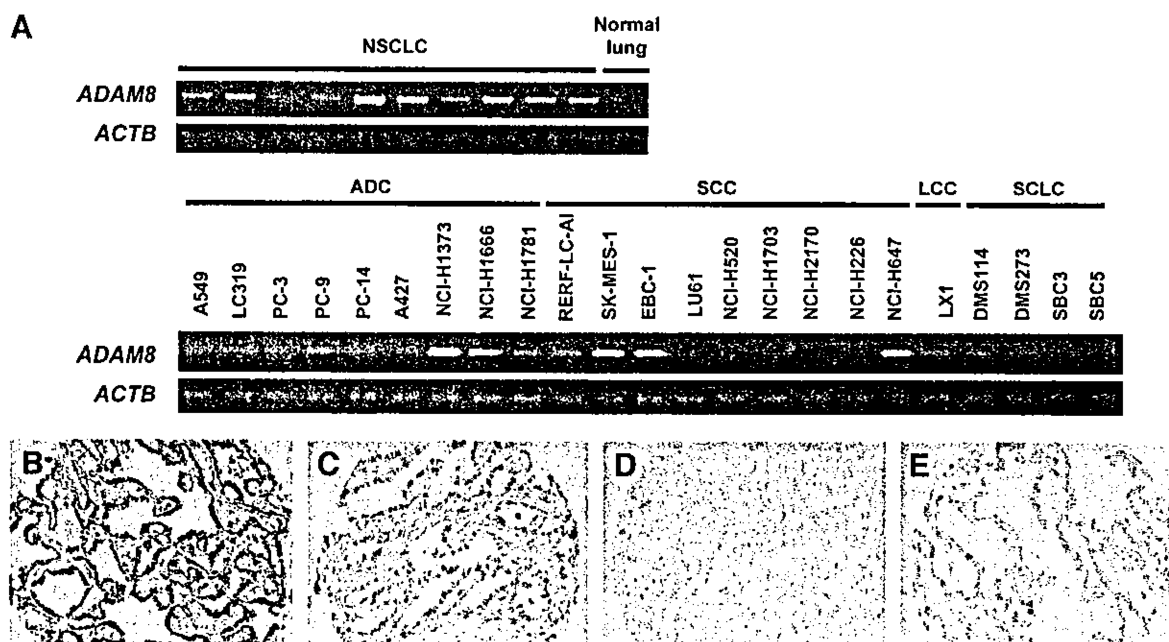

Fig. 1 Validation of ADAM8 expression in lung tumors and cell lines. *A*, expression of *ADAM8* in a normal lung tissue and 10 clinical NSCLC samples (*top panel*) and 23 lung cancer cell lines (*bottom panel*) detected by semiquantitative reverse transcription-PCR analysis. ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma; SCLC, small-cell lung cancer. *B–E*, representative images after immunohistochemical staining of lung tissues, with anti-ADAM8 antibody on tumor tissue microarrays (×100). Examples are shown of strong (*B*), weak (*C*), and absent (*D*) ADAM8 expression in ADCs and of no expression in normal lung (*E*).

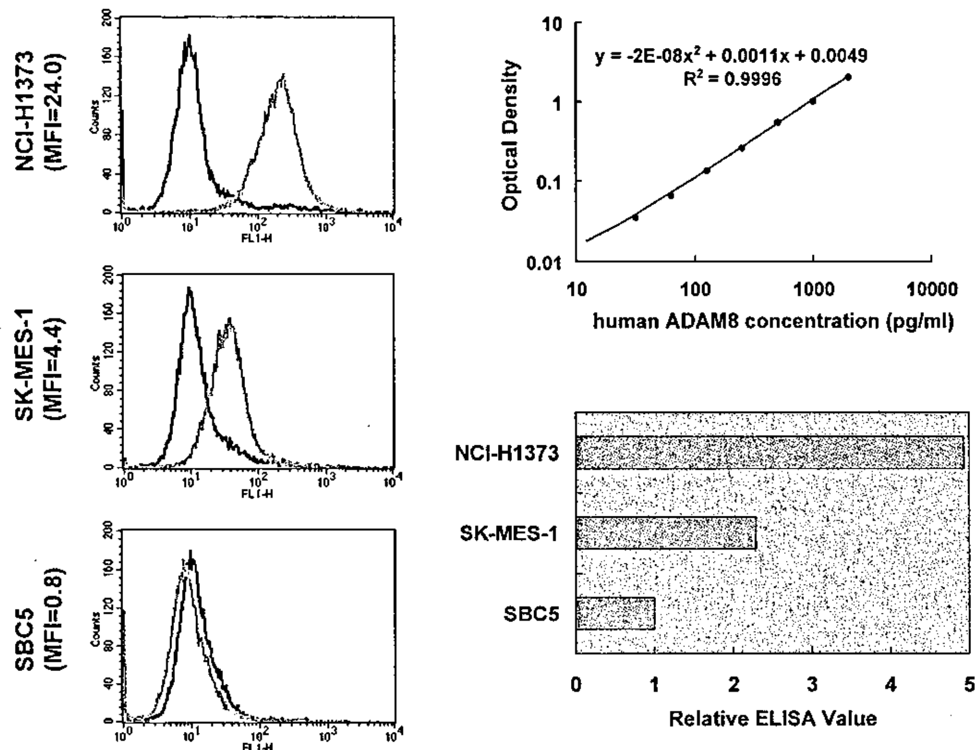

Fig. 2 Cellular localization and secretion of ADAM8 in lung cancer cells. *A*, expression of ADAM8 on cell surfaces in lung cancer lines NCI-H1373, SK-MES-1, and SBC-5 evaluated by flow cytometric analysis. Mean fluorescence intensity (MFI) was calculated as relative signal intensity values of cells treated with antihuman ADAM8 antibody (*green*)/cells treated with mouse IgG (control; *black*). *B*, standard curve of sandwich ELISA with anti-ADAM8 antibody (*X* axis, ADAM8 concentration; *Y* axis, absorbance). *C*, specific detection of ADAM8 with ELISA in conditioned medium from ADAM8-expressing NCI-H1373 and SK-MES-1 cells and nonexpressing SBC-5 cells. The *X* axis indicates the relative ELISA values (concentration of secreted ADAM8 from NCI-H1373 or SK-MES-1/concentration of secreted ADAM8 from SBC-5 cells).

ical lung cancers by tissue arrays with anti-ADAM8 antibody. ADAM8 localized at the plasma membrane, as well as in the cytoplasm of tumor cells, but was hardly detectable in surrounding normal tissues (Fig. 1*B–E*). Positive staining was observed in 158 (77%) of 206 adenocarcinomas cases examined, 55 (47%) of 118 squamous cell carcinomas, 16 (64%) of 25 large-cell carcinomas, and 11 (79%) of 14 SCLCs, whereas no staining was observed in any of the normal portions of the same tissues. We classified a pattern of ADAM8 expression on the tissue array ranging from absent/weak (scored as 0 ~ 1+) to strong (scored as 2+). Expression levels of ADAM8 were not associated with any of the clinicopathological factors in squamous cell carcinomas. However in adenocarcinomas, strong ADAM8 staining was significantly more common in stages IIIB–IV tumors (22 of 60; 37%) than in stages I–IIIA tumors (34 of 146, 23%; $P = 0.049$; χ^2 test). The sample sizes of large-cell carcinomas and SCLCs were too small to be evaluated additionally.

Secretion of ADAM8 in Lung Cancer Cells. We then validated ADAM8 expression on the surfaces of lung cancer cells with flow cytometry and anti-ADAM8 monoclonal antibody. This analysis indicated that the antibody bound to NCI-H1373 and SK-MES-1 cells in which ADAM8 transcript had been detected at a high level but not to SBC-5 cells, which did not express ADAM8 (Fig. 2*A*).

Because the extracellular domain of ADAM8 protein is thought to be secreted (10), we applied ELISA method to examine its presence in the culture media of these cell lines. High levels of ADAM8 protein were detected in media of NCI-H1373 and SK-MES-1 cultures but not in the medium of SBC-5 cells (Fig. 2, *B* and *C*). The amounts of detectable ADAM8 in the culture media accorded well with the expression levels of ADAM8 on the cell surfaces detected with flow cytometric analysis.

Serum Levels of ADAM8 in Lung Cancer Patients. Because the *in vitro* findings had suggested a possibility for development of a novel tumor maker for lung cancer, we investigated whether the extracellular domain of ADAM8 is secreted into sera of patients with lung cancer. ELISA experiments detected ADAM8 in serologic samples from lung cancer patients and also from normal individuals; serum levels of ADAM8 in lung cancer patients were 431 ± 249 pg/mL (mean ± SD) and those in healthy individuals were 267 ± 56 pg/mL. The difference was significant with *P* of <0.001 (Mann-Whitney *U* test). When classified according to histologic type, the serum levels of ADAM8 were 427 ± 286 pg/mL in adenocarcinomas patients, 467 ± 210 pg/mL in squamous cell carcinomas patients, and 400 ± 112 pg/mL in SCLC patients (Fig. 3*A*); the differences among the three histologic types were not significant. High levels of serum

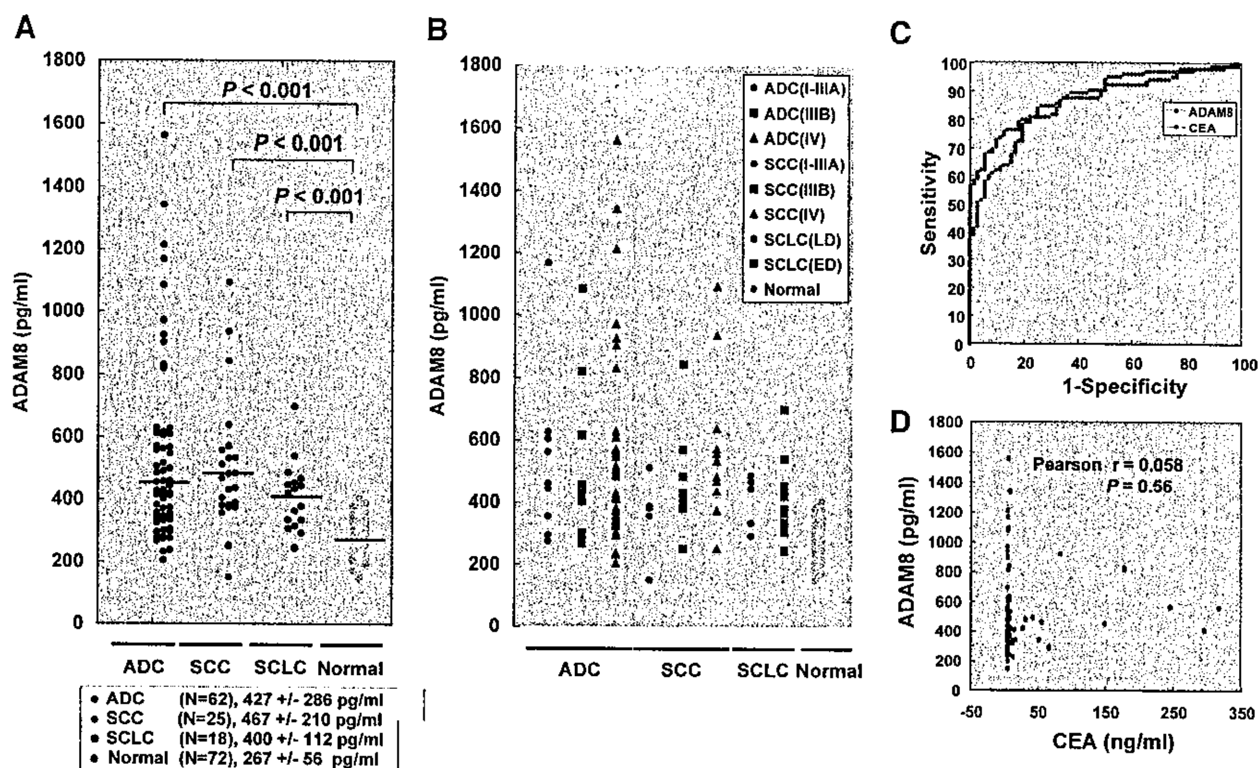

Fig. 3 Serologic concentration of ADAM8 determined by ELISA in patients with lung cancers and in healthy controls. *A*, distribution of ADAM8 in sera from patients with lung adenocarcinoma (ADC), lung squamous cell carcinoma (SCC), or SCLC. Averaged serum levels are shown as *black lines*. Differences were significant between ADC patients and healthy individuals ($P < 0.001$, Mann-Whitney *U* test), between SCC patients and healthy individuals ($P < 0.001$) and between SCLC patients and healthy individuals ($P < 0.001$). *B*, distribution of ADAM8 in sera from patients at various clinical stages of lung cancers. LD indicates limited disease; ED, extensive disease. *C*, receiver-operating characteristic curve analysis of ADAM8 (*blue*) and CEA (*red*) as serum markers for lung cancer (*X* axis, 1-specificity; *Y* axis, sensitivity). *D*, relationship between serum levels of ADAM8 and CEA (*X* axis, CEA concentration; *Y* axis, ADAM8 concentration).

ADAM8 were detected even in patients with earlier-stage tumors (stages I–IIIA; Fig. 3*B*). We also found no significant association between the serum ADAM8 level and age or gender (Table 1).

Comparison of ADAM8 and CEA as Tumor Markers. To evaluate the feasibility of using serum ADAM8 level as a tumor detection marker, we also measured by ELISA serum levels of CEA, a conventional tumor marker, in the same patients and controls. ADAM8 and CEA were additionally analyzed by drawing receiver-operating characteristic curves to determine their cutoff levels (Fig. 3*C*). The sum of the area under the receiver-operating characteristic curve for serum ADAM8 value was slightly larger than that for serum CEA, suggesting slightly better specificity and likelihood for ADAM8 as diagnostic marker for lung cancer. Cutoff levels in this assay were set to result in optimal diagnostic accuracy and likelihood ratios for ADAM8 and CEA, *i.e.*, 379 pg/mL for ADAM8 and 5.3 ng/mL for CEA. As shown in Fig. 3*D*, the correlation coefficient between serum ADAM8 and CEA values was not significant (Pearson's correlation: r = 0.058, $P = 0.56$), indicating that measuring both markers in serum can improve overall sensitivity for detection of NSCLC to 80% (for diagnosing NSCLC, the sensitivity of CEA alone is 57% and that of ADAM8 is 63%.). False-positive results for either of the two tumor markers among 72 normal volunteers (control group) amounted to 11% (8 of 72), whereas the false-positive rates for CEA and ADAM8 in the same control group were 5.5% (4 of 72) each.

Table 1 Correlation of serum ADAM8 level with age or gender

	Serum ADAM8			
Factors	Percentage (%)	No. of patients	Mean ± SD	*P*
Lung cancer patients				
Gender				
Male	74	78	442 ± 232	0.54*
Female	26	27	418 ± 299	
Age (y)				
≤65	43	45	413 ± 288	0.26*
>65	57	60	445 ± 218	
Normal individuals				
Gender				
Male	56	40	267 ± 52	0.65*
Female	44	32	273 ± 62	
Age (y)				
≤65	71	51	266 ± 53	0.08*
>65	29	21	308 ± 60	

* Mann-Whitney *U* test.

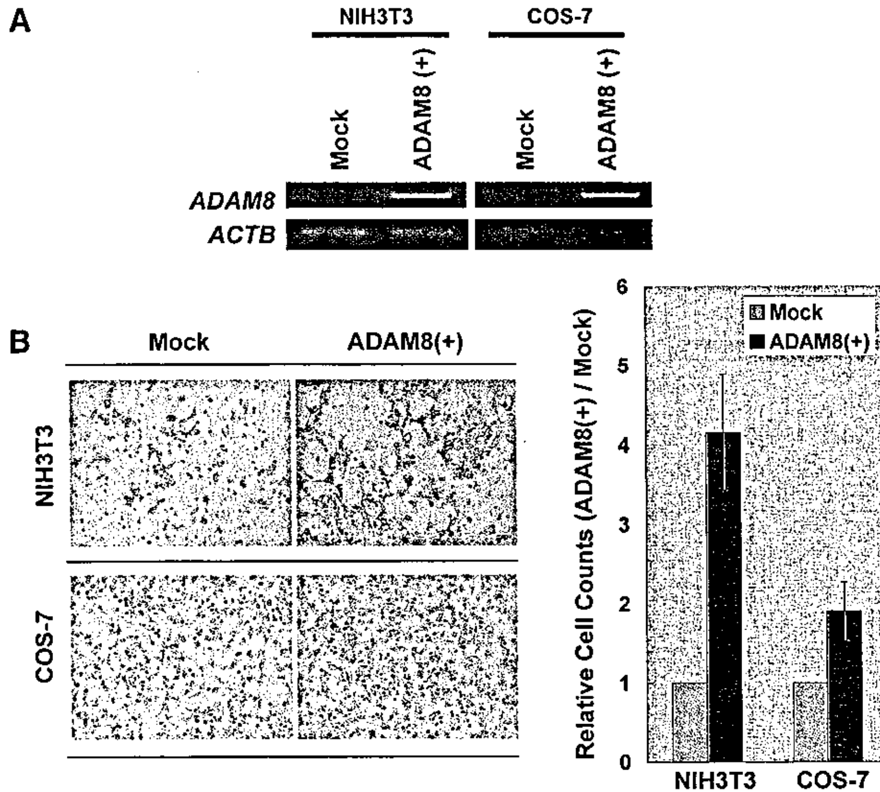

Fig. 4 Promotion of invasiveness of mammalian cells transfected with ADAM8-expressing plasmids. *A*, transient expression of *ADAM8* in NIH3T3 and COS-7 cells, detected by semiquantitative reverse transcription-PCR analysis. *B*, assay demonstrating the invasive nature of NIH3T3 and COS-7 cells in Matrigel matrix after transfection with expression plasmids for human *ADAM8*. *Left panel* shows Giemsa staining (×200), and *right panel* represents the relative number of cells migrating through the Matrigel-coated filters (cells with ADAM8-expressing plasmids/cells with mock plasmids). Assays were performed three times and in triplicate wells.

Activation of Cellular Migration/Invasive Activity by ADAM8. ADAM8 protein contains conserved ectodomains that are considered important for cell adhesion and protease activities; other ADAM family members are known to be associated with cellular motility and invasion (9). Hence, we examined a possible role of ADAM8 in cellular motility and invasion in Matrigel assays, with NIH3T3 and COS-7 cells. As shown in Fig. 4, transfection of ADAM8 cDNA into either cell line significantly enhanced its invasive activity through Matrigel, compared with cells transfected with mock vector.

DISCUSSION

Despite many advances in diagnostic imaging of tumors, combination chemotherapy, and radiation therapy, little improvement has been achieved within the last decade in terms of prognosis and quality of life for patients with lung cancer. Given the frequent failure of conventional treatment strategies, many cancer-related molecules have been characterized toward the goal of developing novel anticancer therapies such as molecular-targeted drugs and antibodies or cancer vaccines (20, 21). Molecular-targeted therapies are expected to be highly specific to malignant cells, with minimal adverse effects due to their well-defined mechanisms of action. Equally desirable in prospect are minimally invasive, highly sensitive, and specific new diagnostic methods that would adapt readily to clinical settings. From these points of view, tumor-specific transmembrane/secretory proteins should have significant advantages because they are presented either on the cell surface or within the extracellular space and/or in serum, making them easily accessible as molecular markers and therapeutic targets. Some tumor-specific markers already available, such as CYFRA or Pro-GRP, are transmembrane/secretory proteins (4, 5); the example of rituximab (Rituxan), a humanized monoclonal antibody against CD20-positive lymphomas, provides proof that targeting specific cell surface proteins can result in significant clinical benefits (22). As an approach to identifying novel cancer-specific cell surface or secretory proteins, we have been exploiting the power of genome-wide expression analysis to select genes that are overexpressed in cancer cells. Analysis of candidate molecules revealed ADAM8 as a potential target for development of novel tools for diagnosis and treatment of lung cancer.

ADAM8 protein is homologous to a snake disintegrin, Reprolysin (M12B), a zinc metalloprotease (9). Members of the ADAM family are cell surface proteins with a unique structure combining potential adhesion and protease domains. A published report has suggested that the ADAM8 ectodomain is cleaved by ADAM8 itself (10). Because various matrix metalloproteinases and ADAM family proteins had been described as being overexpressed in human cancers (23), ADAM8 seemed likely to have a potential role in tumor development or progression. In this study, we demonstrated that induction of exogenous expression of ADAM8 enhanced the cellular migration/invasive activity of mammalian cells. Concordantly, the strong ADAM8 staining in primary NSCLC tissues detected by tissue microarray analyses correlated with disease progression; overexpression of ADAM8 protein was significantly more common in tumors from patients with locally advanced lung cancer (stage IIIB) or distant organ metastases (stage IV) than in those with earlier

stage diseases (stages I–IIIA). The former patients are generally not candidates for curative resection (24). Although the precise mechanism of ADAM8 in lung carcinogenesis is unknown and the processes of cancer-cell invasion to adjacent tissues and distant metastasis consist of a complex series of sequential step, these results indicate that ADAM8 expression and its cleavage could promote dissemination of tumors by stimulating cell migration. ADAM8 has been described as a shedding enzyme of the low-affinity IgE receptor CD23 (13); it also appears to play an important role in physiologic and pathological cell interactions by specifically releasing a functional form of a neural-cell adhesion molecule, a homologue of L1 (CHL1), from cell surfaces (14). However, when we analyzed mRNA expression of *ADAM8*, *CD23*, and *CHL1* in lung cancer cell lines and cancer tissues by semiquantitative reverse transcription-PCR, no expression of *CD23* was detectable in most of the lung cancer samples examined, and the expression pattern of *CHL1* was not concordant with that of *ADAM8* (data not shown). Additional studies to identify unknown substrates of ADAM8 in lung cancers may contribute not only to identification of novel tumor markers and therapeutic targets but also should yield new understanding of the signaling pathway mediated by ADAM8 expression.

We also found high levels of ADAM8 protein in serologic samples from lung cancer patients. Because most of the serum samples used for were derived from patients with advanced cancers (stages IIIB–IV), we evaluated ADAM8 as a tumor marker for early diagnosis of this disease. Tissue microarray analyses of NSCLCs at stages I–IV documented ADAM8 positivity even in early-stage tumors; ADAM8 staining was observed in 176 (64%) of 274 cases at stages I–IIIA, including 111 (76%) of 146 adenocarcinomas cases, whereas as mentioned above, strong ADAM8 staining was observed more frequently in adenocarcinomas cases at advanced stages IIIB–IV than in cases at stages I–IIIA. These results indicated that ADAM8 should be useful for diagnosis of even early-stage lung cancers and that the high level of ADAM8 expression could indicate increased malignant potential of ADAM8-positive tumors.

To validate the feasibility of applying ADMA8 as the diagnostic tool, we compared serum levels of ADAM8 with those of CEA, a conventional diagnostic marker for NSCLCs, in terms of sensitivity and specificity for diagnosis. The proportions of positive cases among the same serum samples were 57% for CEA and 63% for ADAM8, whereas the false-positive rate for ADAM8 (5.5%) was the same as that of CEA, indicating equivalent or better diagnostic power of ADAM8. Furthermore, an assay combining both markers increased the sensitivity such that 80% of the patients with lung cancer were diagnosed as positive, whereas 11% of 72 healthy volunteers were falsely diagnosed as positive. Although additional validation with a larger set of serum samples covering various clinical stages will be necessary, the data presented here sufficiently demonstrate a potential clinical application of ADAM8 itself as a serologic/histochemical marker for lung cancers. We should mention also that we observed activation of *ADAM8* in nearly half of a group of pancreatic cancers, which have a significantly invasive phenotype (data not shown). This suggests that overexpression of *ADAM8* might play a significant role in progression of pancreatic cancer and could warrant investigation of serum levels of ADAM8 in patients with other types of invasive cancer as well.

In conclusion, we have identified ADAM8 as a potential marker for diagnosis of lung cancers. This molecule is also a likely candidate for development of therapeutic approaches such as antibody therapy. ADAM8 was specifically overexpressed in most lung cancer tissues we examined and was elevated in the sera of a large proportion of patients with lung cancer. ADAM8, combined with other tumor markers, could significantly improve the sensitivity of lung cancer diagnosis.

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ORIGINAL PAPER

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A single nucleotide polymorphism in the *MMP-1* promoter is correlated with histological differentiation of gastric cancer

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Abstract *Purpose*: Matrix metalloproteinase-1 (MMP-1) plays a key role in cancer invasion and metastasis by degradation of extracellular matrix (ECM) and basement membrane barriers. The 1G/2G single nucleotide polymorphism (SNP) in the *MMP-1* promoter at position −1607 bp has been reported to affect the transcriptional activity. In the light of these findings, we investigated whether this SNP in the *MMP-1* promoter is associated with the development, differentiation, and progression of gastric cancer. *Methods*: The 215 gastric cancer patients and 166 controls were used in this study. The SNP of the *MMP-1* promoter was analyzed by PCR-RFLP and sequencing. The genotype frequency was compared between cases and controls, and the association with clinicopathological parameters among cases was studied. *Results*: The frequency of 1G/2G genotypes in gastric cancer patients was similar to those in controls ($p = 0.57$). The degree of tumor invasion, the presence of lymph node metastasis, and clinical stage showed no significant association with the SNP. On the other hand, we found a significant association with histological differentiation and gender among gastric cancer patients ($p < 0.05$, respectively). *Conclusions*: The presence of 2G allele in the *MMP-1* promoter did not enhance the risk of gastric cancer; however, it may be involved in differentiation of gastric cancer.

Keywords SNP · MMP-1 · Gastric cancer

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Introduction

Gastric cancer is one of the most common cancers in many Asian countries including Japan and Korea. The poor prognosis depends on the degree of stomach wall invasion and on metastatic spread to regional lymph nodes. Degradation of extracellular matrix (ECM) and basement membrane barriers by MMPs plays an important role in tumor invasion and metastasis (Forget et al. 1999; Kohn and Liotta 1995; Liotta et al. 1991). A prognostic value of MMPs expression in tumor tissue has been reported (McDonnel and Matrisian 1991). Overexpression of MMP-1 has been demonstrated in a variety of cancers (Hewitt et al. 1991; Murray et al. 1998a; Templeton et al. 1990), and the expression of MMP-1 is associated with poor prognosis of esophageal cancer patients (Murray et al. 1998b). In colorectal cancer, the expression of MMP-1 correlated with pathological factors such as Dukes' stage, differentiation, lymphatic or vascular invasion, and tumor depth (Baker and Leaper 2003).

Genetic polymorphism of insertion of a guanine (G) nucleotide at −1607 bp in the *MMP-1* gene promoter sequence, which generates the sequence 5'-GGA-3', has been identified. This sequence generates a new binding site for ETS transcription factor, influencing its transcriptional activity (Rutter et al. 1998). Moreover, the presence of 2G allele in the *MMP-1* promoter has been reported to associate with the development and

progression of carcinomas of the ovary, endometrium, and colorectum (Ghilardi et al. 2001; Kanamori et al. 1999; Nishioka et al. 2000). The frequency of ovarian cancer patients carrying 2G alleles was significantly higher than that in non-cancer individuals (Kanamori et al. 1999); hence, the presence of 2G allele is thought to be a risk factor of endometrial cancer (Nishioka et al. 2000). Similarly, the frequency of 2G allele was higher in colorectal patients than that in controls (Ghilardi et al. 2001). The levels of *MMP-1* expression in ovarian cancer tissues among the patients carrying 2G alleles were significantly elevated, compared with those homozygously carrying 1G alleles (Kanamori et al. 1999; Nishioka et al. 2000).

In gastric cancer, *MMP-1*expression has been associated with both peritoneal and lymph node metastasis (Inoue et al. 1999); however, there is no report on the association between the *MMP-1* promoter polymorphism and the development of gastric cancer. In this study we investigated whether the 1G/2G polymorphism in the *MMP-1* promoter is associated with the development of gastric cancer. Moreover, we examined the relationship between the 1G/2G polymorphism and the clinicopathological factors among gastric cancer patients.

Materials and methods

Samples

A total of 381 peripheral blood samples from 166 healthy control subjects and 215 gastric cancer patients were employed in this study. Controls were randomly selected from those visited Hiroshima University Hospital for regular healthy check or symptoms such as appetite loss or epigastralgia. They were proved to be free from malignancy by medical examination with gastrointestinal scope and biopsy. Gastric cancer patients underwent surgical operation or endoscopic mucosal resection (EMR) at Hiroshima University Hospital. All patients and controls gave informed consent prior to enrollment in the study. The human genome research ethics screening committee of Hiroshima University School of Medicine approved this study. Gastric cancer patients were 153 males and 62 females (median age 67.7 ± 11.4 years). Histology of gastric cancer was classified according to the criteria of Lauren (1965). There were 122 patients who had an intestinal type of gastric cancer, and 93 patients who had a diffuse type. Intestinal type and diffuse type correspond to well-differentiated type and poorly differentiated type, respectively, in the histological classification of the Japanese Gastric Cancer Association (Japanese Gastric Cancer Association 1998). Alternative histological classification of the 215 gastric carcinomas was those with either intestinal or diffuse type components (pure type) or with coexistence of both types of components (mixed type; Stelzner and Emmrich 1997). Gastric cancer patients were grouped according to TNM classification (Sobin and Wittekind 2002), on the basis of the postoperative histopathological evaluation. Moreover, they were assigned to two subgroups according to the presence (N+) or absence (N–) of detectable lymph node metastasis at the time of diagnosis.

Eight human gastric carcinoma cell lines (MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, TMK-1, HSC-39, and KATO-III) were used. The TMK-1 cell lines was established in our laboratory from poorly differentiated adenocarcinoma (Ochiai et al. 1985). Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28 and MKN-74, well-differentiated adenocarcinomas; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by T. Suzuki (Fukushima Medical University, Fukushima, Japan). KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinoma, were kindly provided by M Sekiguchi (University of Tokyo, Tokyo, Japan) and by K. Yanagihara (National Cancer Center, Tokyo, Japan), respectively, (Yokozaki 2000). All of these cell lines were routinely maintained in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Whittaker, Walkersville, Md.), penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml) under conditions of 5% CO_2 in air at 37°C.

DNA extraction and PCR-RFLP analysis

The genomic DNA purification kit (Promega, Madison, Wis.) and QIAamp 96 DNA Blood kit (QIAGEN, Valencia, USA) were used for DNA extraction. The PCR-restriction fragment length polymorphism (RFLP) assay was used to determine the *MMP-1* genotypes. The PCR primers used for amplifying MMP-1 polymorphism were: forward primer 5'-TGACTTTTAAAACA TAGTCTATGTTCA-3'; reverse primer 5'-TCTTGGATTGATT TGAGATAAGTCATAGC-3'. The reverse primer was specially designed to introduce a recognition site of restriction enzyme *Alu*I (AGCT) by replacing a T with a G at the second position close to the 3' end of the primer (Zhu et al. 2001). The 1G alleles have this recognition site, whereas the 2G alleles destroy the recognition site by inserting a guanine. The target sequence was amplified in a 25-µl reaction volume containing 10–20 ng of genomic DNA, 0.2 µM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.3 µM of each primer, and 0.75 units of Ampli Taq Gold (Perkin-Elmer, Norwalk, Conn.). The PCR amplification was carried out with 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C (depending on the primer) for 30 s, and extension at 72°C for 30 s after the initial activation step of 94°C for 10 min. The 269-bp fragment was then digested with *Alu*I (TaKaRa Biomedicals, Shiga, Japan) overnight at 37°C. After overnight digestion, 269-bp (2G allele), 241-bp, and 28-bp (1G allele) fragments were loaded on an ethidium bromide stained 2.5% NuSieve 3:1 agarose (FMC Bioproducts, Rockland, Md.) gel for 60 min at 100 V. Heterozygotes displayed a combination of both alleles (269, 241, and 28 bp).

Sequencing analysis of PCR products

The PCR products were purified and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, Calif.). Plasmid DNA was extracted from individual clones by alkaline lysis plasmid minipreparation. The inserted PCR fragments obtained from each sample were sequenced with both M13 reverse and M13 forward primer using the PRISM AmpliTaq DNA polymerase FS Ready Reaction Dye Terminator Sequencing kit (Perkin-Elmer ABI, Foster City, Calif.). Reamplified DNA fragments were purified with Centri-Sep Columns (Princeton, Adelphia, N.J.) and were sequenced by ABI PRISM 310 genetic analyzer (Perkin-Elmer ABI, Foster City, Calif.).

Statistical analysis

Statistical analysis was performed with the use of Fisher's exact test. A value of $p < 0.05$ was considered significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were used for estimating the risk of association with genotypes. Odds ratios for the genotypes were calculated by the logistic regression model, adjusting for age and gender. The logistic regression analysis was performed for the association between the genotypes and clinicopathological factors (SPSS software, ver 11.0).

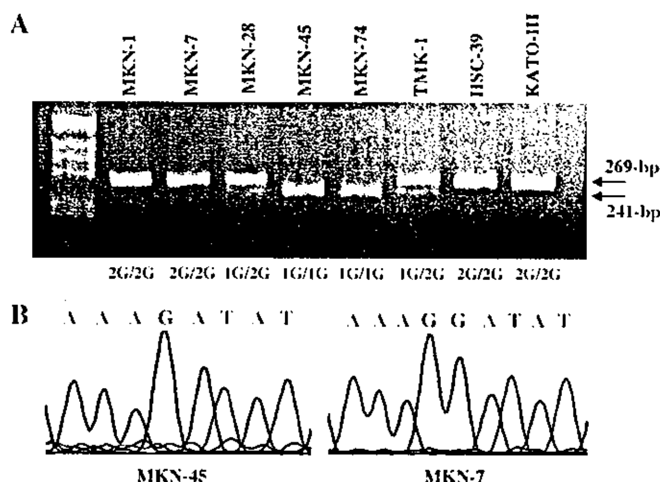

Fig. 1 A Representative PCR-RFLP analysis to confirm the variants in eight human gastric cancer cell lines. Ethidium bromide-stained 2.5% NuSieve 3:1 agarose gel used for genotyping. The target products (269-bp) in the *MMP-1* gene promoter was PCR amplified and digested with *AluI*, which cleaved the 1G allele at the polymorphic site, generating two fragments (241-bp and 28-bp, respectively), but did not cut the 2G allele. B Representative electropherogram of each type of polymorphism. The genotype of the SNP was proved to be 1G/1G in MKN-45 and 2G/2G in MKN-7

Results

We first examined the 1G/2G polymorphism in the *MMP-1* gene promoter by PCR-RFLP in eight gastric cancer cell lines, followed by sequencing: three patterns of PCR-RFLP were detected (Fig. 1A). MKN-1, MKN-7, HSC-39, and KATO-III cells had a single 269-bp DNA band; MKN-45 and MKN-74 cells showed a single 241-bp DNA band. On the other hand, MKN-28 and TMK-1 cells had heterozygous bands. Subsequent sequencing confirmed the 1G/2G genotyping of MKN-45 and MKN-7 cells (Fig. 1B). MKN-45 and MKN-7 cells were identified with 1G/1G and 2G/2G genotypes, respectively; MKN-45 and MKN-74 cells carried 1G/1G genotype; MKN-1, MKN-7, HSC-39, and KATO-III cells carried 2G/2G genotype; MKN28 and TMK-1 cells carried heterozygous 1G/2G genotype. Eight human gastric cancer cell lines used in this study had been derived from Japanese gastric cancer patients and established in Japan. There were no particularly prominent genotypes among these cell lines.

We next determined the 1G/2G genotyping among gastric cancer patients and controls; typical PCR-RFLP patterns are shown in Fig. 2 (i.e., 1G/1G or 2G/2G genotype with a single 241-bp or 269-bp band, respectively; 1G/2G genotype with 241-bp and 269-bp bands). The 1G/2G genotype distribution in gastric cancer patients and controls is shown in Table 1. The genotype distribution among controls was in good agreement with Hardy-Weinberg equilibrium. The allelic frequency in controls in our study was similar to the allele frequency reported in healthy Japanese subjects (Kanamori et al. 1999). The frequency of those carrying at least one 2G allele (1G/2G and 2G/2G) was almost equal between patients with gastric cancers (87.9%) and controls (89.7%; $p=0.57$, OR = 0.83, 95% CI = 0.43–1.59). In addition, we found that the frequency of genotypes (1G/1G vs 1G/2G + 2G/2G) did not differ by gender, age, and status of *H. pylori* infection.

Finally, we analyzed the association between the 1G/2G genotyping and clinicopathological factors among gastric cancer patients (Table 2). We found a significant difference in genotype distribution (1G/1G vs 1G/2G + 2G/2G) by histological classification (intestinal type vs diffuse type, $p=0.03$, OR = 2.84, 95% CI = 1.09–7.39). The patients carrying at least one 2G allele were more frequent in diffuse type than those in intestinal type. Furthermore, the patients carrying at least one 2G allele were more frequent in mixed type than those in pure type, with an OR of 3.81, although this was not statistically significant due to a small number of the mixed type. We also found a significant difference by gender (male vs female, $p=0.04$, OR = 3.48, 95% CI = 1.00–12.04). On the other hand, depth of tumor invasion, the presence of lymph node metastasis, and TNM classification showed no significant correlation with genotyping. In general, intestinal and diffuse types of gastric carcinomas are more frequent in males and females, respectively, implying a possible interaction between histological typing and gender. The logistic

Fig. 2 Representative 8 cases of gastric cancer patients and 5 cases of controls are shown. The target products (*269-bp*) in the *MMP-1* gene promoter were PCR amplified and digested with *AluI*. MKN-45 cells were used as positive controls for digestion with *AluI*. *Numbers above the panel* are case numbers. Genotypes are shown below each case. Case numbers 1, 3, 6, and 7 are intestinal type, and the others are diffuse type

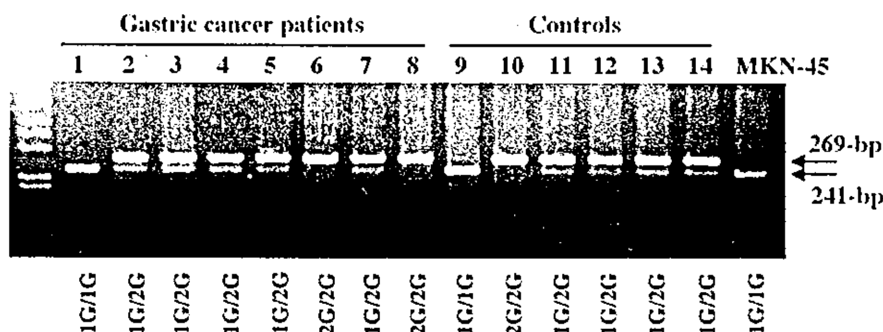

Table 1 *MMP-1*genotype distribution of the study subjects. .*CI* confidence interval

Genotype	Controls (%)[a] ($n = 166$)	Patients (%) ($n = 215$)	*P* value[b]	Crude OR[c] (95% CI)
Overall				
1G/1G	17 (10.3)	26 (12.1)		
1G/2G	61 (36.7)	88 (40.9)		
2G/2G	88 (53.0)	101 (47.0)	0.57	0.83 (0.43–1.59)
Gender				
Male				
1G/1G	9 (9.5)	23 (15.0)		
1G/2G	41 (43.2)	60 (39.2)		
2G/2G	45 (47.3)	70 (45.8)	0.20	0.59 (0.26–1.34)
Female				
1G/1G	8 (11.3)	3 (4.8)		
1G/2G	20 (28.1)	28 (45.2)		
2G/2G	43 (60.6)	31 (50.0)	0.18	2.49 (0.63–9.86)
Age (years)				
<65				
1G/1G	14 (11.1)	11 (11.7)		
1G/2G	46 (36.5)	39 (41.5)		
2G/2G	66 (52.4)	44 (46.8)	0.89	0.94 (0.41–2.18)
≥65				
1G/1G	3 (7.5)	15 (12.4)		
1G/2G	15 (37.5)	49 (40.5)		
2G/2G	22 (55.0)	57 (47.1)	0.39	0.57 (0.16–2.09)
H. pylori infection				
Negative	$n = 47$	$n = 44$		
1G/1G	4 (8.5)	7 (15.9)		
1G/2G	16 (34.0)	14 (31.8)		
2G/2G	27 (57.5)	23 (52.3)	0.28	0.49 (0.13–1.81)
Positive	$n = 90$	$n = 49$		
1G/1G	12 (13.4)	6 (12.2)		
1G/2G	31 (34.4)	20 (40.8)		
2G/2G	47 (52.2)	23 (47.0)	0.86	1.10 (0.39–3.15)

[a]The observed genotype distribution of controls was in agreement with Hardy-Weinberg equilibrium
[b]Correlation was analyzed by Fisher's test. The *p* values <0.05 were regarded as statistically significant. The *p* values of 1G/2G + 2G/2G genotypes relative to 1G/1G genotype
[c]Odds ratio of 2G/2G + 1G/2G genotypes relative to 1G/1G genotype

Table 2 Correlation between the genotype of SNP in the *MMP-1* promoter and clinicopathological characteristics of gastric cancer patients

	Genotype (%)			OR (95%CI)[a]	
	1G/1G	1G/2G	2G/2G	Crude	Adjusted[b]
Age (years)					
<65 ($n = 94$)	11 (11.7)	39 (41.5)	44 (46.8)	0.94	
≥65 ($n = 121$)	15 (12.4)	49 (40.5)	57 (47.1)	(0.41–2.15)	
Gender					
Male ($n = 153$)	23 (15.0)	60 (39.2)	70 (45.8)	3.48	
Female ($n = 62$)	3 (4.8)	28 (45.2)	31 (50.0)	(1.00–12.04)	
Histological classification 1[c]					
Intestinal ($n = 121$)	20 (16.5)	49 (40.5)	52 (43.0)	2.84	3.56
Diffuse ($n = 94$)	6 (6.4)	39 (41.5)	49 (52.1)	(1.09–7.39)	(1.15–11.11)
Histological classification 2[d]					
Mixed type ($n = 26$)	1 (3.8)	12 (46.4)	13 (50.0)	3.81	4.73
Pure type ($n = 189$)	25 (13.2)	76 (40.2)	88 (46.6)	(0.49–29.38)	(0.58–38.53)
Depth					
m, sm ($n = 85$)	10 (11.8)	29 (34.1)	46 (54.1)	0.97	1.02
mp ~ ($n = 130$)	16 (12.3)	59 (45.4)	55 (42.3)	(0.42–2.25)	(0.24–4.35)
Lymph node metastasis					
N (−; $n = 126$)	15 (11.9)	46 (36.5)	65 (51.6)	0.98	1.14
N (+; $n = 89$)	11 (12.4)	42 (47.2)	36 (40.4)	(0.43–2.25)	(0.16–8.13)
TNM classification[e]					
Stages I, II ($n = 145$)	20 (13.8)	55 (37.9)	70 (48.3)	1.71	3.04
Stages III, IV ($n = 70$)	6 (8.6)	33 (47.1)	31 (44.3)	(0.65–4.46)	(0.83–11.16)

Correlation was analyzed by Fisher's test. The *P* values <0.05 were regarded as statistically significant
[a]Odds ratio of 2G/2G + 1G/2G genotypes relative to 1G/1G genotype
[b]The ORs were adjusted for age and gender
[c]Histology of gastric cancer was classified according to the criteria of the Lauren
[d]Pure-type gastric cancer with either intestinal or diffuse type components; mixed-type gastric cancer with coexistence of both types of components
[e]Tumor staging was classified according to the criterion of the UICC TNM stage grouping, 6th edition, 2002, stomach

regression analysis then revealed that histological classification remained significant ($p = 0.03$, OR = 3.56, 95% CI = 1.15–11.11) even after adjustment for age and gender.

Discussion

Studies of ovarian cancer and colorectal cancer have shown that the frequency of patients carrying at least one 2G allele in the *MMP-1* promoter was significantly higher than in control subjects (Ghilardi et al. 2001; Kanamori et al. 1999); thus, the presence of 2G allele is considered to be one of the risk factors for the development of these cancers. Furthermore, pancreatic cancers frequently showed a positive staining for *MMP-1* protein in immuno-histochemical analysis, whereas the *MMP-1* expression in fetal and normal pancreatic tissues was very faint (Ito et al. 1999). In this study, we examined whether the risk of gastric cancer is associated with the 1G/2G polymorphism in the *MMP-1* promoter region. The allelic frequency in the patients with gastric cancer was similar to that in controls. It seems that the presence of 2G allele did not enhance the susceptibility for the development of gastric cancer.

However, we found a significant association between the 1G/2G polymorphism and the histological classification. The frequency of those carrying at least one 2G allele was significantly higher in the diffuse type (poorly differentiated type) of gastric cancer than that in the intestinal type (well-differentiated type) with an OR of 3.56. The *MMP-1* promoter with 2G allele has displayed significantly increased transcriptional activity than that with 1G allele in melanoma cell lines and normal fibroblasts through the ETS binding site (Rutter et al. 1998). Diffuse type of gastric cancer is usually characterized by an abundant deposition of collagen fibers, possibly requiring higher levels of *MMP-1*expression for degradation of ECM. It has beensuggested that carcinogenesis was a multicellular and multistage process in which the destruction of the microenvironment was required for conversion of normal tissue to tumor (Park et al. 2000). Although MMPs are not oncogenic or mutagenic, they alter the microenvironment and may affect the process of carcinogenesis and its histology. This polymorphism may have a more profound impact on histology and differentiation of gastric cancer.

Recent studies showed the significance of a modified histological classification, pure type or mixed type. Patients with mixed-type gastric cancer revealed poorer prognosis than those with pure type did (Stelzner and Emmrich 1997). In this study, the patients carrying at least one 2G allele were more frequent in mixed type than those in pure type, with an OR of 3.81, although this was not statistically significant due to a small number of the mixed type. Take together; these findings suggest that the presence of 2G allele in the *MMP-1* promoter may contribute to the morphogenesis of gastric carcinomas.

Several MMPs, including MMP-1, have been reported to play an important role in cancer invasion through their overexpression, which is associated with metastasis and unfavorable prognosis in esophageal cancer, ovarian cancer, cutaneous malignant melanoma, and colorectal cancer (Ghilardi et al. 2001; Kanamori et al. 1999; Murray et al. 1998b; Ye et al. 2001).Kanamori et al. (1999) observed a high expression level of the *MMP-1* in tumors carrying 2G allele than 1G homozygotes in ovarian cancers; however, in this study, 1G/2G polymorphism in the *MMP-1* promoter was not associated with invasion, lymph node metastasis, and TNM classification in gastric cancer patients. Possible explanations may be the following:

1. The degree of tumor invasion in gastric carcinoma might be determined not only by the presence of 2G allele in the *MMP-1* promoter but also the response to growth factors and cytokines. In fact, cytokines, such as interleukin-1 (IL-1), influence the expression levels of *MMP-1*(Singer et al. 1997). Especially, IL-1A acts as a growth stimulator for gastric carcinoma (Ito et al. 1993), and it correlates with liver metastasis of gastric carcinoma (Furuya et al. 1999; Tomimatsu et al. 2001). Although the presence of 2G allele in the *MMP-1* promoter displays a significantly higher transcriptional activity than the *MMP-1* promoter with 1G allele (Rutter et al. 1998), the polymorphism may not significantly affect mean expression levels of *MMP-1* in normal stromal cells. The polymorphism may increase the *MMP-1* expression in response to growth factors and cytokines (Wyatt et al. 2002).
2. Some reports revealed that overexpression of *MMP-1* was observed in stromal cells of gastric carcinoma but not in carcinoma cells (Migita et al. 1999; Otani et al. 1999). So, the presence of 2G allele of *MMP-1* promoter may not necessarily contribute to the degree of tumor invasion in gastric carcinoma.
3. In stage-I cases in our study, a large portion of them (99 of 112 cases) have 2G allele, and 37 of 54 cases carrying 2G homozygotes were treated by means of EMR. So, they are in the status of before invading to the submucosa, they have no significant correlation with tumor invasion and metastasis. In fact, our results revealed a tendency of increasing risk of development of gastric cancer with an 8.33 exceeding risk for the patients with the 2G allele (stage II vs stage III + IV;$p = 0.05$). It has been reported that MMPs can alter the microenvironment and may influence tumor formation. So, MMPs may contribute to the initial stages of cancer development, and overexpression of MMPs may be associated with elevated risk of tumorigenesis. Moreover, the polymorphism in the *MMP-1* promoter is associated with early tumor stages in lung cancer (Zhu et al. 2001). The polymorphism in the *MMP-1* promoter may be one of the pathways of the increased neoplastic risk

in the stomach. However, we do not have any data concerning pre-cancerous lesions (i.e., patients with gastric adenoma, severe atrophic gastritis with intestinal metaplasia) at present; therefore, we could not verify a relationship between the polymorphism in the *MMP-1* promoter and a pre-cancerous lesion. However, since this is a very important point to elucidate the pathway of increasing risk of tumorigenesis and progression in gastric cancer, we should clarify this issue in the near future.

On the other hand, we found a significant association with gender among gastric cancer patients ($p=0.037$, OR = 3.48, 95% CI = 1.00–12.04). This finding suggested a gender-specific effect of the *MMP-1* polymorphism. The presence of 2G allele in *MMP-1* promoter enhances the transcriptional activity. Moreover, it has been reported that the activity of MMP-1 might be regulated by sex hormones (Marbaix et al. 1992; Schneikert et al. 1996). The expression of *MMP-1* was negatively regulated by androgen (Schneikert et al. 1996), whereas the secretion and activation of *MMP-1* was inhibited by physiological concentrations of progesterone (Marbaix et al. 1992). Furthermore, being consistent with the above-mentioned information, IL-1A is a key inducer of *MMP-1* in the human endometrium. Ovarian steroids inhibited the release of IL-1A and repress MMP-1 production (Singer et al. 1997); therefore, the presence of 2G allele in the *MMP-1* promoter may affect the risk of gastric cancer in women. However, in our study, almost all women with gastric cancer are elderly and in menopausal status; the mean levels of ovarian steroids may be low among them. So, we did not find a significant association among gastric cancer patients and controls in women.

Conclusion

In conclusion, our studies suggest that the presence of the 2G allele in the *MMP-1* promoter might be associate with histological differentiation of gastric cancer; however, we could not consider lifestyle factors such as cigarette smoking, alcohol consumption, and dietary habits of our patients. These factors may contribute to the development of gastric cancer (Chen et al. 2000). Further investigations are necessary to clarify a role of this *MMP-1* polymorphism with increased number of study subjects and epidemiological data.

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