significantly poor prognosis than those with normal levels. Therefore, CEA and CA19-9 are not sensitive diagnostic marker but useful marker for prognosis and recurrence.

CA 72-4

CA 72-4 is a carbohydrate tumor marker recognized by two monoclonal antibodies, B72.3 and CC49 [18]. B72.3 antibody reacts with a tumor-associated glycoprotein called TAG-72. Overall positivity of CA 72-4 is 30-40% in gastric cancer patients [14, 19, 20]. However, positive rate (diagnostic sensitivity) of CA 72-4 depends on tumor staging; about 10% for early (stages I-II) and over 50% for advanced (stages III and IV) diseases. Combination of CA 72-4, CEA and CA19-9 improve the diagnostic sensitivity without impairment of the diagnostic sensitivity. CA 72-4 is more sensitive than CEA and CA19-9 in detecting recurrence of gastric cancer, indicating a useful marker for managing gastric cancer. Multivariate analysis has shown that CA 72-4 is one of the independent prognostic factors for gastric cancer [21].

Possible Serological Tumor Markers

Quantitatively measurable substances in sera that cause development and progression of cancer can be tumor marker. Specificity of tumor markers depends on specificity of the substances in tumor not in normal tissue. Mostly after H. pylori-related chronic gastritis, gastric cancer develops by accumulation of various alterations of oncogenes, tumor suppressor genes, DNA repair genes, growth factors/receptors, cell cycle regulators and cell adhesion molecules and so on [22]. Many studies to elucidate whether these molecules and gene products are tumor marker have been performed, and several are reported to be useful in clinical diagnosis for detecting cancer, monitoring recurrence and foreseeing prognosis. Those must be confirmed by multi-institutional prospective study.

Interleukin

Interleukins (ILs), inflammation-associated cytokines may serve as tumor marker because development and growth/invasion of gastric cancer are associated with inflammatory process. Interleukin-8 (IL-8) also acts as angiogenic factor for gastric cancer [23]. Serum levels of IL-1 beta, IL-6 and IL-8 are significantly increased in gastric cancer patients in comparison with those in controls, while tumor necrosis factor-alpha (TNF-alpha) serum level is greatly reduced in patient group [24, 25]. One report demonstrated that serum IL-8 levels over cut-off point (1.77 pg/mL) occurred in almost all gastric cancer patients but in only few (less than 1%) controls [26]. Preoperative serum levels of soluble IL-2 receptor (IL-2R) in gastric cancer patients are significantly higher than those of normal controls [27]. IL-2R levels are also significantly higher in patients with lymph-node metastasis than without metastasis, suggesting a value for detecting metastatic disease preoperatively. Serum IL-12

levels in gastric cancer patients are significantly higher than those of the healthy controls [28]. There is no correlation between serum IL-12 and serum CEA, CA19-9 or CA 72-4, but is significant relationship between serum IL-12 and soluble IL-2R levels. Serum IL-18 levels in gastric cancer patients are significantly higher compared with the mean level in healthy controls [29]. IL-18 levels decrease after surgical resection. Serum IL-18 level is identified as an independent preoperative prognostic factor in multivariate survival analysis.

Growth Factor and Angiogenic Factor

Growth factors and angiogenic factors participate in tumor growth, invasion and metastasis. Serum levels of vascular endothelial growth factor-A (VEGF-A) in gastric cancer patients are significantly higher than those in controls [30]. There is a significant association between serum VEGF-A levels and tumor stage, as well as invasion and metastasis. Serum VEGF-A level is an independent prognostic factor for survival by multivariate regression analysis. VEGF-C is a lymphangionenic factor and correlates with lymphatic vessel density. Serum VEGF-C levels are significantly higher in gastric cancer patients than in controls [31]. Increased serum levels of VEGF-C are associated with advanced stage and presence of metastasis. Kaplan-Meier survival analysis reveals that VEGF-C is an indicator for poor prognosis. Transforming growth factor (TGF)-beta1 is a negative regulatory factor for epithelial cell growth and deregulation of TGF-beta system participates in cancer proliferation. The mean level of serum TGF-beta1 of gastric cancer patients is significantly higher than that of healthy controls [31]. Venous invasion is significantly correlated with elevated serum TGF-beta-1 levels by logistical regression analysis. Hepatocyte growth factor (HGF), identical to scatter factor, is a secretory glycoprotein from stromal fibroblasts and macrophages that increases the motility of various types of cancer cells. The mean value of serum HGF in gastric cancer patients is significantly higher than that in healthy controls, and a significant increase in serum HGF levels is found in both early and advanced stage patients compared with control subjects [33]. The serum HGF level is significantly higher in patients with vessel invasion than in those without invasion.

Matrix Metalloproteinase

Matrix metalloproteinases (MMPs) induce extracellular matrix breakdown that is associated with normal tissue remodeling and cancer invasion and metastasis. Both serum and plasma MMP-9 levels correlate with active MMP-9 identified by zymography [34]. Serum MMP-11 levels are significantly elevated in gastric cancer patients compared with those of the control subjects [35]. The positive expression of MMP-11 is well correlated with metastasis of gastric cancer. High levels of MMP-10 are detected in serum samples from most of the gastric cancer patients even at stage I, while some healthy individuals and gastritis patients show high serum MMP-10 levels [36]. When the cutoff level for MMP-10 is set at 200pg/mL, the sensitivity and specificity for detection of gastric cancer is 94% and 85%, respectively, indicating that MMP-10 is the most useful serum marker for gastric cancer

screening. Abnormally high levels of serum tissue inhibitor of MMP (TIMP)-1 are detected in 17% of gastric cancer patients with a specificity of 98% [37]. Serum TIMP-1 is positively associated with depth of tumor invasion and metastasis. A higher serum TIMP-1 group is significantly associated with poorer survival rates than the lower serum TIMP-1 group.

Cell Adhesion Molecules

E-cadherin is the most important cell adhesion molecule in gastric cancer. The mean of serum soluble E-cadherin concentration is significantly higher in gastric cancer patients than in healthy subjects. The concentrations correlate with tumor size [38]. Prospective study revealed that the sensitivity for predicting disease recurrence using the cut-off level of 10,000ng/mL at 3months and at 6 months post-surgery was 47% and 59% respectively, which was significantly better compared with the sensitivity of CEA [39]. Serum levels of soluble intercellular adhesion molecules-1 (ICAM-1) are significantly increased in gastric cancer patients. ICAM-1 concentrations are also significantly higher in patients with distant metastasis and peritoneal spread [40].

Oncogene and Tumor Suppressor Gene

c-erbB2 (also known as HER-2), a member of EGFR family, encodes transmembrane glycoprotein with tyrosine kinase activity and participates in tumor progression of gastric cancer. Preoperative serum HER-2/c-erbB2 levels in gastric cancer patients are significantly higher than those in controls [41]. Serum HER-2/c-erbB2 levels decrease significantly after radical resection of the primary tumor and are an independent prognostic factor for survival. One of the most frequent genetic alterations in human cancers is found in p53 tumor suppressor gene. Serum p53 protein is detected in 35% of gastric cancer patients [42]. The serum concentrations of the p53-positive patients are highly elevated compared with healthy individuals. Alterations in p53 protein can be immunogenic and enable the formation of p53 serum autoantibodies [43]. p53 antibodies are detected in serum of 20% of gastric cancer patients. All p53 antibody-positive patients have immunohistochemically p53-positive tumors. A significant correlation of p53 antibody is found with a higher tumor stage and also with a poor prognosis of survival.

DNA Methylation

Hypermethylation of CpG islands is associated with silencing of various tumor suppressor genes, such as hMLH1, p16 and E-cadherin, and participates in stomach carcinogenesis [44]. Aberrant DNA methylation can be readily detected in tumor-derived DNA recovered from the serum of gastric cancer patients. Many studies have been performed to determine whether aberrant DNA methylation is useful diagnostic and prognostic marker [45-48]. DNA methylation of p16 and E-cadherin is detected in serum of 30-50% of gastric

cancer patients whereas none of the control serum shows aberrant methylation. Aberrant methylation in serum DNA is accompanied with methylation in the corresponding tumor samples. It is also reported that Aberrant methylation in serum DNA from gastric cancer patients is found in APC (17%), hMLH1 (41%), TIMP3 (17%), DAP-kinase (48%), GSTP1 (15%), p15 (56%), and RARbeta (24%) [45, 47 ,48]. Some of the DNA methylation are associated with tumor stage and prognosis. The combined use of certain methylation markers will increase the significance of diagnostic value.

Histological Prognostic Factors

Genes and molecules participating in proliferation, invasion and metastasis such as growth factors and their receptors, cell cycle regulators, cell adhesion molecules and matrix-degrading enzymes are good prognostic factors [49] (Table 2). If the antibodies usable in immunohistochemistry are available, the corresponding molecules must be diagnostic and prognostic factors in histological sections.

Table 2. Histological prognostic factors of gastric cancer

Growth factor / cytokine	EGF, TGF-alpha, EGF receptor, Her2/c-erbB2, IL-8, VEGF-A, VEGF-C
Cell cycle regulator	cyclin E, CDC25B, p27
Cell adhesion molecules	E-cadherin, Dysadherin, Cadherin-17, CD44v6, CD44v9, Desmoglein-2, Claudin-18
Matrix metalloproteinase	MMP
Others	p53, HIF

Growth Factors, Cytokines and Angiogenic Factors

Gastric cancer cells express a variety of growth factors and their receptors to make an autocrine and paracrine loops, participating in not only tumor growth but also invasion and metastasis [50]. Simultaneous positive staining of EGF/TGF-alpha and EGF receptor (EGFR) correlates with deep invasion, advanced stage and poor prognosis [49]. Multivariate analysis reveals that EGFR overexpression is a possible independent unfavorable prognostic factor [51]. The overexpression associated with gene amplification of the *HER-2/c-erbB2* is frequently associated with well differentiated type gastric cancer. HER-2/c-erbB2 protein expression correlates with invasion and metastasis, and merges as an independent prognostic indicator by the Cox regression model [52]. Neovascularization enhances the growth of primary tumors and provides an avenue for hematogenous metastasis. Gastric cancer cells produce various angiogenic factors, including VEGF, IL-8, basic fibroblast growth factor (bFGF) and platelet-derived endothelial cell growth factor (PD-ECGF) [23, 53, 54]. Prognosis in gastric cancer patients with high IL-8 and VEGF expression levels is significantly poorer than that with low expression levels [55]. In curatively treated gastric

cancer patients, VEGF immunostaining correlates with worse survival in both univariate and multivariate analyses [56].

Cell Cycle Regulators

Cell cycle checkpoints are regulatory pathway that control cell cycle transitions, DNA replication and chromosome segregation. Abnormalities in cell cycle regulators are involved in stomach carcinogenesis through genomic instability and unbridled cell proliferation. The cyclin E gene is amplified in 15-20% of gastric cancer and the strong immunostaining of cyclin E is found in 27% of gastric cancer, which is associated with tumor stage, invasion and metastasis [57]. Strong immunostaining of CDC25B, a cell cycle-related phosphatase, is detected in 49% of gastric cancer, which is also associated with tumor progression [58]. The expression of $p27^{KIP1}$, a cyclin-dependent kinase (CDK) inhibitor, is lost in over 50% of gastric cancer immunohistochemically [59]. Decrease of p27-positive cells significantly correlates with advanced tumor stage, depth of invasion and lymph-node metastasis. The expression of p27 shows an inverse correlation with the expression of cyclin E [60]. Within the cyclin E positive tumors, the five-year survival rate is higher in patients with a p27 positive tumor than in those with a p27 negative tumor. Furthermore, expression of $p21^{WAF1/CIP1}$ alone or in combination with p27 is associated with favorable prognosis [61].

Cell Adhesion Molecules

E-cadherin plays a major role in epithelial tissues to regulate morphogenesis and inhibit cell infiltration, and loss of E-cadherin expression participates in invasion and metastasis. Multivariate analyses reveal that reduced E-cadherin expression is an independent prognostic factor [62]. Dysadherin is a cancer-associated cell membrane glycoprotein, down-regulates E-cadherin expression and promotes metastasis [62]. The patients of gastric cancer with both increased dysadherin and reduced E-cadherin have the worst prognosis although dysadherin is not an independent factor. Cadherin-17, also called liver-intestine cadherin, is a structurally unique member of the cadherin superfamily. Positive cadherin-17 staining is detected in 67% of gastric cancer, which is associated with advanced tumor stage [63]. The prognosis of patients with positive cadherin-17 staining is significantly poorer than that of the negative cases. CD44 is an important cell adhesion molecule and its variants generated by alternative splicing modulate cell to cell interaction, movement and finally metastatic potential. There is a significant survival advantage in patients with expression of low CD44 sharing variant exon 6 (CD44v6) compared with those with high expression [64]. The expression of CD44v9 is associated with not only tumor invasion, metastasis and advanced stage but also tumor recurrence mortality of gastric cancer [65]. Desmoglein-2 is one of the components of the cell-cell adherence junction. A Significant correlation is found between a decrease in desmoglein-2 staining and loss of differentiation and peritoneal metastasis [66]. The prognosis of patients with desmoglein-2 negative tumors is significantly worse than that of those with positive tumors, while desmoglein-2 is not an independent prognostic factor by multivariate analysis. Claudins are components of tight junction strand and create a barrier to prevent paracellular transport of lipids and proteins. Claudin-18 immunostaining is reduced

in 58% of gastric cancer, which is associated with the intestinal mucin phenotype (MUC2 and CD10) [67]. In advanced cases, patients with gastric cancer showing reduced claudin-18 expression gave a significantly worse survival rate than those with preserved claudin-18 expression.

Matrix Metalloproteinases

A balance of the activities between matrix-degrading enzymes and their inhibitors is an important in determining tumor invasion and metastasis. Among various MMPs, the expression of MMP-7, also known as matrilysin, is correlated with vessel invasion and both lymphatic and hematogenous metastasis [68]. Multivariate analysis reveals that MMP-7 expression status at the invasive front is an independent prognostic factor [69]. About 30% of gastric cancer are positive for both MMP-1 and protease-activated receptor-1 (PAR-1) immunohistochemically, which is associated with invasion and metastasis [70]. Multivariate analysis indicates that combined MMP-1 and PAR-1 or PAR-1 alone is an independent prognostic factor. MMP-2 immunostaining in cancer cells is associated with advanced stage, depth of invasion, non-curative surgery, and poor survival, although MMP-2 was not an independent prognostic factor [71]. MMP-10 staining is positive in 45% of gastric cancer, which is associated with depth of tumor invasion [36]. The prognosis of patients with MMP-10 positive tumor is significantly worse than that with negative tumors among advanced gastric cancer cases.

Others

Concerning well-known tumor suppressor gene product p53, numerous reports studying p53 abnormalities in gastric cancer in relation with patients' prognosis have been published. The prognostic impact of p53 remains controversial. There are reports indicating that abnormal p53 expression significantly affects cumulative survival rate and that p53 status also influences response to chemotherapy [72, 73]. Our immunohistochemical study on more than 2500 cases of gastric cancer demonstrated no correlation between p53 expression and clinicopathological parameters such as invasion and metastasis [57]. Gastric cancers with combined expression of p53 and hypoxia-inducible factor 1alpha (HIF-1alpha) more frequently show poorly differentiation, infiltrative growth and lymph-node metastasis compared with the negative tumors [74]. Furthermore, the patients of gastric cancer with p53-positive and HIF-1apha-positive show the worst prognosis. By multivariate analysis, HIF-1alpha is found to be one of the independent prognostic factors. Cyclooxygenases (COXs) catalyze the initial, rate-limiting steps of prostaglandin synthesis from arachidonic acid. A cumulative survival is significantly worse in patients with strong COX-2 expression than in those with weak expression, and COX-2 is an independent prognostic factor by multivariate analysis [75]. CDX2, a transcription factor expressed in the intestine, is implicated in the development and maintenance of the intestinal mucosa and is associated with intestinal type gastric cancer. CDX2 immunostaining in the nucleus is detected in 60-70% of intestinal type

gastric cancer. Multivariate analysis for the overall survival rate reveals that CDX2 positive gastric cancer patients survive significantly longer than CDX2 negative patients, indicating that CDX2 is an independent prognostic factor [76, 77].

Search for Novel Tumor Markers through Global Analysis of Gene Expression

Identification of novel biomarkers for diagnosis and novel targets for treatment is a major goal in conquest of gastric cancer. Transmembrane or secretory proteins expressed specifically in cancer may be ideal markers for cancer diagnosis while genes and molecules whose function is involved in the neoplastic process may constitute a therapeutic target. Genome-wide study of gene expression is of great advantage to identify such novel targets. Serial analysis of gene expression (SAGE) is a powerful technique to allow global analysis of gene expression in a quantitative manner without a prior knowledge of the sequence of the gene [78, 79]. We have performed on 5 samples of gastric cancer with different histology and stages, and created one of the largest SAGE libraries of gastric cancer in the world, containing a total of 137,706 expressed tags including unique 38,903 tags [80]. Sequence data are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue). If a gene participates in tumor progression and specifically expressed in cancer but not in normal tissues, the gene can be not only cancer-biomarker but also a therapeutic target with minimal adverse effect [79]. By comparing SAGE libraries of gastric cancer with those of various normal tissues in the SAGEmap database, we picked up 54 genes which were detected in our gastric cancer libraries but not in the libraries from 14 normal tissues including brain, lung, heart, liver, kidney, etc [36]. The expression of these genes was validated in gastric cancers and normal human tissues by quantitative RT-PCR and 9 genes, APIN, TRAG3, CYP2W1, MIA, MMP-10, DKK4, GW112, REG4, and HORMAD1, were found to be cancer-specific.

Novel Serological Tumor Markers

REG4, regenerating islet-derived family gene member 4 (encoding Reg IV), was originally cloned as an up-regulated gene in inflammatory bowel diseases [81]. CDX2 induces the expression of Reg IV, while Reg IV enhanced the expression of SOX9. Reg IV immunostaining is detected in 30% of gastric cancer, which is associated with intestinal mucin phenotype and neuroendocrine differentiation [82]. Reg IV expression in clinical gastric cancer samples is significantly associated with resistance to the combination chemotherapy of 5-FU and cisplatin, while no association is found between Reg IV expression and tumor progression or prognosis [83]. Reg IV enhances peritoneal metastasis through induction of AKT phosphorylation and bcl-2/bcl-XL expression in animal model that is suppressed by Reg IV-siRNA treatment [84]. Serum Reg IV concentration in presurgical gastric cancer patients is significantly elevated even at stage I [83]. The diagnostic sensitivity of serum Reg IV (36%) is much superior to that of serum CEA or CA19-9.

GW112, also called OLFM4 or hGC-1, was originally cloned from human myeloid cells and encodes a secretory glycoprotein of 510 amino acids [85]. GW112 is normally expressed in bone marrow, intestine and prostate and the expression is confirmed in inflamed mucosa of ulcerative colitis and certain cancers [86, 87]. GW112 protein binds to GRIM-19, participating in inhibition of apoptosis [88]. Because GW112 also interacts with cadherin and lectins, GW112 may facilitate cell adhesion and invasion [89]. GW112 immunostaining is found in epithelial cells at the bottom of crypt in small intestine but not detected in normal colon. Strong GW112 expression is detected in 60% of gastric cancer. An inverse correlation is noticed between Reg IV positive and GW112 positive cases. High serum levels of GW112 are detected in 31% of gastric cancer patients regardless of tumor stage. Importantly, there is no correlation between serum Reg IV and GW112 levels. Combination of Reg IV and GW112 reveals a sensitivity of 57% in detecting gastric cancer. Therefore, combined measurement of serum Reg IV and GW112 is novel and highly sensitive diagnostic marker.

Melanoma inhibitory activity (MIA) was first isolated as a secreted protein from malignant melanoma cell lines [90]. MIA enhances invasion ability in gastric cancer cells [36]. Immunostaining of MIA significantly correlates with depth of tumor invasion, lymph-node metastasis and tumor stage. The patients with MIA-positive cancer show significantly poorer prognosis than those with MIA-negative cancer. High level of MIA in serum is detected only in stage IV patients.

MMP-10 is one of the cancer-specific genes identified by SAGE data analysis [36]. As aforementioned above, the diagnostic sensitivity and specificity of serum MMP-10 is 94% and 85%, respectively, indicating that MMP-10 is suitable for gastric cancer screening.

Novel Histological Tumor Markers

Signet-ring cell carcinoma is a unique subtype of adenocarcinoma that is characterized by abundant intracellular mucin accumulation. Signet-ring cell carcinoma typically develops in the stomach but also occurs in various organs such as the lung, breast and so on. In some cases, the primary site of origin is difficult to determine. All signet-ring cell carcinomas of the stomach and colorectum are immunohistochemically positive for Reg IV (figure 1), whereas all of those of the lung and breast are negative for Reg IV [91]. Reg IV is a specific histological marker for signet-ring cell carcinoma of gastrointestinal tract.

Gastric hepatoid adenocarcinoma is an extrahepatic tumor characterized by morphologic similarities to hepatocellular carcinoma, and is known to have an aggressive clinical course and poor survival. Screening for genes up-regulated in gastric cancer by comparing gene expression profiles from SAGE and microarray identified the palate, lung, and nasal epithelium carcinoma-associated protein (PLUNC) gene as one of the most up-regulated genes by microarray [92]. Immunostaining for PLUNC reveals strong and extensive staining of PLUNC in most of gastric hepatoid adenocarcinoma (figure 2), whereas only 7% of conventional gastric cancers show focal immunostaining of PLUNC. The patients with PLUNC positive gastric cancer show a significantly worse survival rate than those with PLUNC negative tumor. PLUNC staining is also detected in liver metastases, whereas it not observed in primary hepatocellular carcinoma or in normal adult or fetal liver. Therefore,

PLUNC is a novel histological marker for gastric hepatoid adenocarcinoma, and PLUNC immunostaining serves as a specific indicator to distinguish metastatic hepatoid adenocarcinoma of the stomach in the liver from primary hepatocellular carcinoma.

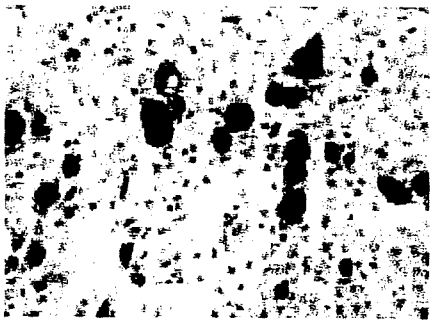

Figure 1. Immunostaining of Reg IV in a signet-ring cell carcinoma of the stomach.

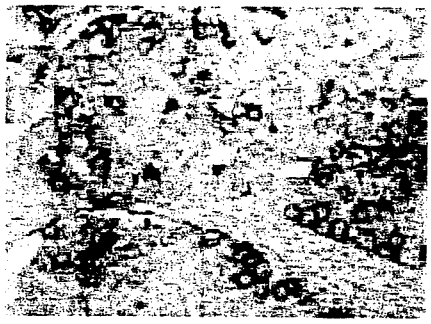

Figure 2. Immunostaining of PLUNC in a hepatoid adenocarcinoma of the stomach.

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Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis

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Summary

Background Analyses of microRNA expression profiles have shown that many microRNAs are expressed aberrantly and correlate with tumorigenesis, progression, and prognosis of various haematological and solid tumours. We aimed to assess the relation between microRNA expression and progression and prognosis of gastric cancer.

Methods 353 gastric samples from two independent subsets of patients from Japan were analysed by microRNA microarray. MicroRNA expression patterns were compared between non-tumour mucosa and cancer samples, graded by diffuse and intestinal histological types and by progression-related factors (eg, depth of invasion, metastasis, and stage). Disease outcome was calculated by multivariable regression analysis to establish whether microRNAs are independent prognostic factors.

Findings In 160 paired samples of non-tumour mucosa and cancer, 22 microRNAs were upregulated and 13 were downregulated in gastric cancer; 292 (83%) samples were distinguished correctly by this signature. The two histological subtypes of gastric cancer showed different microRNA signatures: eight microRNAs were upregulated in diffuse-type and four in intestinal-type cancer. In the progression-related signature, miR-125b, miR-199a, and miR-100 were the most important microRNAs involved. Low expression of let-7g (hazard ratio 2·6 [95% CI 1·3–4·9]) and miR-433 (2·1 [1·1–3·9]) and high expression of miR-214 (2·4 [1·2–4·5]) were associated with unfavourable outcome in overall survival independent of clinical covariates, including depth of invasion, lymph-node metastasis, and stage.

Interpretation MicroRNAs are expressed differentially in gastric cancers, and histological subtypes are characterised by specific microRNA signatures. Unique microRNAs are associated with progression and prognosis of gastric cancer.

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Introduction

Gastric cancer is the fourth most common human malignant disease and the second most frequent cause of cancer-related death worldwide.[1] Improvement of diagnosis and treatment has resulted in good long-term survival for patients with early gastric cancer, whereas the outlook for individuals with advanced disease remains poor.[2] Advanced gastric cancer frequently recurs as nodal and haematogenous metastases and peritoneal dissemination. Although several types of non-surgical treatment have been assessed, surgical resection is still the primary curative treatment for localised gastric cancer.

Data from several studies show that various genetic alterations cause tumorigenesis and progression of gastric cancer.[3,4] Inactivation of runt-related transcription factor 3 (*RUNX3*) by methylation has also been reported.[5] Several groups have undertaken high-throughput analyses of gastric cancer expression profiles by DNA microarrays[4] and microdissection.[6] However, markers for tumorigenesis and progression of gastric cancer have not yet been discovered and specific therapeutic targets have not been identified.

A new class of small non-coding RNAs—microRNAs—has been discovered.[7] Mature microRNAs are composed of 19–25 nucleotides and are cleaved from 60–110-nucleotide hairpin microRNA precursors in the cytoplasm by the RNase III enzyme Dicer.[8] Single-stranded microRNAs bind mRNAs of potentially hundreds of genes at the 3′ untranslated region with imperfect complementarity, resulting in degradation of target mRNAs and inhibition of translation.[8] Several target-prediction programs have been developed, but very few targets have been proved experimentally.[9] MicroRNAs play a part in crucial cellular processes, including development, differentiation, stress response, apoptosis, and proliferation.[8,10] 475 human microRNAs have been reported to date (miRBase version 9.2; University of Manchester, Manchester, UK);[11] this number could reach 800–1000 through experimental confirmation of predicted microRNA genes.[12]

Microarray platforms have been developed for analysis of microRNA expression, and data show that several microRNAs are expressed aberrantly in various haematological and solid malignant diseases.[13–16] MicroRNAs act as novel oncogenes or tumour-suppressor genes.[17,18] We and others have noted that alterations in microRNA expression correlate highly with progression and prognosis of human tumours.[19–24] Thus, focusing on microRNAs in gastric cancer could yield new insights

into the biological behaviour of this disease. For oncogenic microRNAs, antagomirs are a type of antisense oligonucleotide that inhibit microRNA function in vivo effectively;[25–27] for tumour-suppressive microRNAs, reconstitution with microRNA precursor sequences has an antitumour effect. Therefore, microRNAs are possible therapeutic targets for cancer.[22,28]

To ascertain whether microRNA expression signatures can differ between gastric cancer and non-tumour mucosa, we undertook genome-wide microRNA expression profiling in two sets of gastric tissues. With expression-profile results for these samples and associated clinical variables, we investigated the association between microRNAs and histological types, tumour progression, and prognosis of gastric cancer.

Methods

Tissue samples

For microRNA expression profiling, we obtained gastric tissue samples (cancer lesions and adjacent non-tumour mucosae) from patients who underwent gastrectomy between 2002 and 2005 at the University of Tokyo (group 1) and between 1998 and 2005 at Hiroshima University (group 2). We gathered all samples in the same manner, and they were snap-frozen immediately in liquid nitrogen and stored at −80°C until RNA and protein extraction could be done. Since microdissection is difficult to do in diffuse-type gastric cancer, for technical uniformity we used bulk tissue for all cases.

We obtained study approval from the ethics committee at the University of Tokyo and every patient from the University of Tokyo gave written informed consent for samples to be used. Because we did not obtain written informed consent for samples from Hiroshima University, for strict privacy protection, identification information was removed before analysis; this procedure is in accordance with ethical guidelines for human genome or gene research enacted by the Japanese Government and was approved by the ethics review committee of the Hiroshima University School of Medicine.

Panel: Patient cohorts and of analyses undertaken

STEP 1: MicroRNA expression patterns in gastric cancer (non-tumour mucosa *vs* cancer)

Samples

61 pairs in group 1 and 99 in group 2 were analysed independently

Statistical methods

1 Class comparison by BRB-ArrayTools; paired *t* test ($p<0.01$)
2 Class prediction by BRB-ArrayTools; paired class prediction by the leave-one-out cross-validation method

Samples

169 non-tumour mucosae (64 samples from group 1 and 105 from group 2) and 184 cancers (81 samples from group 1 and 103 from group 2) (unpaired condition)

Statistical methods

Average linkage clustering with centred Pearson correlation with 35 microRNAs

STEP 2: MicroRNA expression patterns and histological types (diffuse-type *vs* intestinal-type gastric cancer)

Samples

103 diffuse-type and 81 intestinal-type gastric cancer samples

Statistical methods

1 Class comparison by BRB-ArrayTools; two-sample *t* test ($p<0.001$)
2 Average linkage clustering with centred Pearson correlation with the 19 most significant microRNAs ($p \leq 2\times10^{-4}$)

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(Continued from previous column)

STEP 3: MicroRNA expression and tumour progression correlation

Samples

- T3 and T4 *vs* T1 (101 *vs* 15 samples)
- Lymph-node metastasis (N) positive *vs* negative (126 *vs* 54 samples)
- Stage IV *vs* I (51 *vs* 37 samples)
- Peritoneal dissemination (P, CY) positive *vs* negative (33 *vs* 76 samples)
- Haematogenous metastasis (H, M) positive *vs* negative (12 *vs* 169 samples)

Statistical methods

1 Class comparison by BRB-ArrayTools; two-sample *t* test ($p<0.01$, for haematogenous metastasis, $p<0.05$)
2 Venn diagram of T, N, and stage
3 Significance analysis of microarrays (SAM) with rank-regression option for T and stage

STEP 4: MicroRNA expression and prognosis correlation

Samples

101 cases have information for disease outcome and underwent curative surgery. All 182 cases had surgery (curative or non-curative)

Overall survival

- Statistical methods
  1 Univariate Cox proportional hazards regression in BRB-ArrayTools
  2 Kaplan-Meier survival curves
  3 Multivariable Cox proportional hazards regression analysis

Disease-free survival

- Statistical methods
  1 Univariate Cox proportional hazards regression in BRB-ArrayTools
  2 Kaplan-Meier survival curves
  3 Multivariable Cox proportional hazards regression analysis

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For miRBase see http://www.mirbase.org

Procedures

We did RNA labelling and hybridisation on microRNA microarray chips and undertook postprocessing, as described previously.[13,15,19–21] Briefly, 5 μg of total RNA from every sample was reverse transcribed with biotin end-labelled random-octamer oligonucleotide primers. Hybridisation of biotin-labelled complementary DNA was done on the Ohio State University custom microRNA microarray chip (OSU_CCC version 3.0; ArrayExpress [European Bioinformatics Institute, Cambridge, UK], array design A-MEXP-620), which contains nearly 1100 microRNA probes, for 326 human and 249 mouse microRNA genes, spotted in duplicates. We washed and processed the hybridised chips to detect biotin-containing transcripts with streptavidin Alexa Fluor 647 conjugate (Invitrogen, Carlsbad, CA, USA) and scanned them on a microarray scanner (4000B; Axon Instruments, Sunnyvale, CA, USA).

We analysed microarray images with GenePix Pro 6.0 (Axon Instruments). Average values of the replicate spots for every microRNA sample were background subtracted, normalised, and subjected to further analysis. Only probes for human mature microRNAs were used for analysis. We implemented quantile normalisation with the Bioconductor 1.8 package affy 1.1.2.

MicroRNAs were retained when they were present in at least 20% of samples and when they had changes of more than 1·5-fold from the gene median in at least 20% of samples. Absent calls (background-level signals on the microarray) were removed at a threshold of 4·5 (log_2 scale) before statistical analysis. After the filtration, we included 237 microRNAs in further statistical analyses.

MicroRNA nomenclature is according to miRBase version 9.2.[11] The microarray dataset is deposited in ArrayExpress (experiment number E-TABM-341) according to MIAME (minimum information about a microarray experiment) guidelines.

For ArrayExpress see http://www.ebi.ac.uk/arrayexpress/

For the Bioconductor 1.8 package affy 1.1.2 see http://www.bioconductor.org

See Online for webappendix

Statistical analysis

The panel summarises the analyses. We identified differentially expressed microRNAs with BRB-ArrayTools version 3.5.0 (Biometric Research Branch, National Cancer Institute, Bethesda, MD, USA),[29] and significance analysis of microarrays (SAM) version 3.0. The webappendix contains further descriptions of the methods used.

After filtration of microRNAs, we used the paired *t* test (level of significance, $p<0·01$) to independently analyse pairs of non-tumour mucosa and cancer samples from groups 1 and 2. We undertook class prediction with the leave-one-out cross-validation method, taking into account that samples were paired (eg, pairs of non-tumour mucosae and cancer lesions from the same patient).

We used hierarchical cluster analysis to generate a tree cluster showing the separation of every class. For hierarchical clustering, we used average linkage metrics and centred Pearson correlation of microRNAs identified between non-tumour mucosa and gastric cancer and between diffuse-type and intestinal-type gastric cancer (Cluster 3.0). For tree visualisation, we used Java Treeview version 1.1.1.

We identified microRNAs whose expression was related significantly to overall survival and disease-free survival of patients (endpoint of cancer-specific death and recurrence, respectively). We undertook univariate Cox proportional hazards regression in BRB-ArrayTools, and we judged microRNAs significant if $p<0·05$.

We used SPSS version 17.0.1 for Kaplan-Meier survival analysis and Cox proportional hazards regression. To generate survival curves, we converted continuous microRNA expression levels measured on microRNA array chips to a dichotomous variable, using the respective mean levels of expression as a threshold.[21] This procedure enabled division of samples into classes with high and low expression of microRNA. We compared survival curves by log-rank test and judged $p<0·05$ significant.

	Group 1 (n=79)	Group 2 (n=103)	p*	Total (n=182)
Age (years; mean [SD])	65·2 (9·8)	67·1 (11·6)	0·24	66·3 (10·9)
Sex			0·87	
Men	52/79 (66%)	66/102† (65%)		118/181 (65%)
Women	27/79 (34%)	36/102† (35%)		63/181 (35%)
Histological type‡			0·022	
Diffuse	53/81§ (65%)	50/103 (49%)		103/184 (56%)
Intestinal	28/81§ (35%)	53/103 (51%)		81/184 (44%)
Depth of invasion (T)‖			0·50	
T1	4/81§ (5%)	11/102† (11%)		15/183 (8%)
T2	29/81§ (36%)	38/102† (37%)		67/183 (37%)
T3	41/81§ (50%)	45/102† (44%)		86/183 (47%)
T4	7/81§ (9%)	8/102† (8%)		15/183 (8%)
Lymph-node metastasis (N)			0·028	
Negative (N0)	17/79 (22%)	37/101¶ (37%)		54/180 (30%)
Positive (N1–N3)	62/79 (78%)	64/101¶ (63%)		126/180 (70%)
Haematogenous metastasis (H, M)			0·69	
Negative	75/79 (95%)	94/102† (92%)		169/181 (93%)
Positive	4/79 (5%)	8/102† (8%)		12/181 (7%)
Peritoneal dissemination (P, CY)			<0·0001	
Negative	64/79 (81%)	12/30** (40%)		76/109 (70%)
Positive	15/79 (19%)	18/30** (60%)		33/109 (30%)
Stage††			0·13	
I	11/79 (14%)	26/102† (25%)		37/181 (21%)
II	14/79 (18%)	23/102† (23%)		37/181 (21%)
III	29/79 (37%)	27/102† (27%)		56/181 (30%)
IV	25/79 (31%)	26/102† (25%)		51/181 (28%)

Data are n (%) unless stated otherwise. *Differences between groups calculated by *t* test for age and χ^2 test for all others. †No information available for one patient. ‡Lauren's classification used for histological typing. Intestinal-type gastric cancer is almost the same as differentiated-type gastric cancer, and diffuse-type gastric cancer is almost the same as undifferentiated-type gastric cancer. §One patient had cancer in three regions. ‖Graded according to the International Union Against Cancer's TNM classification, 5th edn. ¶No information available for two patients. **No information on intraoperative cytology available for 73 patients. ††Graded according to the Japanese Classification of Gastric Cancer, 2nd English edn. Clinical stage is decided by the factors T, N, H, M, P, and CY. Stages IA and IB are regarded as stage I, and stages IIIA and IIIB as stage III.

Table 1: **Characteristics of patients and tissues**

We examined the joint effect of covariates with Cox proportional hazards regression to ascertain whether microRNAs are independent prognostic factors. We censored data for three patients who died of other diseases; data for one patient were censored before the first event (death) in overall survival and were included in the Kaplan-Meier analysis, but were removed for Cox regression analysis in overall survival.

We regarded age as a continuous covariate. T was dichotomised on the basis of absence (T1, T2) versus presence (T3, T4) of serosal invasion of tumour. Stage was dichotomised on the basis of a more than 65% 5-year survival (stages I and II) versus a less than 50% 5-year survival (stages III and IV). For all microRNAs, patients were categorised into groups with high and low expression, with respective mean levels of microRNA expression as a threshold.

We undertook univariate Cox regression to examine the effect of every clinical covariate on patient's survival. We did multivariable analysis by stepwise addition and removal of covariates found to be associated with survival in univariate models ($p<0 \cdot 10$). Conditions of the stepwise selection method were Score statistic ($p<0 \cdot 05$ for addition) and Wald statistic ($p<0 \cdot 05$ for removal). All stepwise addition models gave the same final models as did stepwise removal, and final models included only those

	p†	FDR (%)‡	Fold change	Chromosomal location	Gastric signature§	Proved targets‖	Cancer involvement¶
MicroRNAs upregulated in cancer							
miR-181d	$<1\times10^{-7}$	<0·01	2·3	19p13.12	Progression	*CDX2, GATA6, NLK*	Pancreas
miR-181a-1, miR-181a-2	$<1\times10^{-7}$	<0·01	2·2	1q31.3, 9q33.3	Progression	*HOXA11, BCL2, CD69, TRAα, PTPN11 (SHP2), PTPN22, DUSP5, DUSP6, KAT2B (PCAF), CDKN1B, CDX2, GATA6, NLK*	Breast, pancreas, liver, thyroid, uterus, brain
miR-181c	$<1\times10^{-7}$	<0·01	2·1	19p13.12	Progression	*CDX2, GATA6, NLK*	Lung, pancreas, liver, thyroid, uterus, brain
miR-181b-1, miR-181b-2	$<1\times10^{-7}$	<0·01	2·0	1q31.3, 9q33.3	Progression	*TCL1A, VSNL1, GRIA2, KAT2B (PCAF), AICDA (AID), CDX2, GATA6, NLK*	Breast, colon, pancreas, prostate, stomach, thyroid, uterus, brain, CLL
miR-21	$<1\times10^{-7}$	<0·01	2·0	17q23.2	Histotype, progression	*PTEN, TPM1, PDCD4, SERPINB5, BMPR2, BTG2, CDK6, IL6R, SOCS5, NFIB, SPRY2, RECK, TIMP3, TP63 (TP73L), DAXX, HNRNPK, TOPORS, TP53BP2, JMY, TGFBR2, TGFBR3, APAF1, PPIF, SPRY1, MTAP, SOX5, TGFB1, NCAPG, RTN4, DERL1, PLOD3, BASP1, MARCKS, IL12A, JAG1, LRRFIP1*	Breast, colon, lung, pancreas, prostate, stomach, liver, thyroid, uterus, ovary, brain, CLL, lymphoma
miR-25	$<1\times10^{-7}$	<0·01	1·7	7q22.1	Progression	*BCL2L11, KAT2B (PCAF), CDKN1C*	Pancreas, prostate, stomach, liver, thyroid, uterus, oesophagus, brain, AML
miR-92-1, miR-92-2	$<1\times10^{-7}$	<0·01	1·7	13q31.3, Xq26.2	..	*MYLIP, HIPK3, BCL2L11, VHL, ITGA5, TP63 (TP73L)*	Colon, pancreas, prostate, stomach, thyroid, CLL, AML
miR-93	$<1\times10^{-7}$	<0·01	1·6	7q22.1	Progression	*E2F1, CDKN1A, VEGFA, KAT2B (PCAF), STAT3, TP53INP1, TUSC2*	Colon, pancreas, prostate, stomach, ovary, AML
miR-17-5p	2×10^{-7}	<0·01	1·7	13q31.3	..	*E2F1, NCOA3 (AIB1), RUNX1 (AML1), RBL2, CDKN1A, PTEN, BCL2L11, TIMP1, VEGFA, HIF1A, CCND1, MAPK9, MAP3K8, PKD1, PKD2, PPARA, RBL1, STAT3, TSG101, KAT2B (PCAF), CRK, GAB1, MYCN, IRF1, NR4A3, RNF111, TP53INP1, APBB2, BRCA1, APP, RASSF2, TNFSF12, MAPK14, FN1, FNDC3A, BCL2, MEF2D, MAP3K12*	Breast, colon, lung, pancreas, prostate, stomach, bladder
miR-106a	3×10^{-7}	<0·01	1·7	Xq26.2	Progression	*RB1, RUNX1 (AML1), ARID4B (RBP1L1), MYLIP, HIPK3, CDKN1A, VEGFA, APP, IL10*	Colon, lung, pancreas, prostate, stomach, liver, AML
miR-20b	4×10^{-7}	<0·01	1·9	Xq26.2	Progression	*ARID4B (RBP1L1), MYLIP, HIPK3, CDKN1A, VEGFA*	..
miR-135a-1, miR-135a-2	7×10^{-7}	<0·01	2·1	3p21.1, 12q23.1	Progression	*APC, SMAD5, JAK2*	Colon, prostate, thyroid, uterus, AML, lymphoma
miR-425-5p	1×10^{-6}	<0·01	2·2	3p21.31	..	..	..
miR-106b	1×10^{-6}	<0·01	1·6	7q22.1	..	*E2F1, CDKN1A, VEGFA, KAT2B (PCAF), ITCH, APP, STAT3, MAPK14*	Colon, stomach, AML
miR-20a	3×10^{-6}	<0·01	1·8	13q31.3	..	*E2F1, E2F2, E2F3, TGFBR2, RUNX1 (AML1), CDKN1A, ZBTB7A (LRF), VEGFA, HIF1A, CCND1, STAT3, MYF5, APP, MAPK14, BCL2, MEF2D, MAP3K12*	Colon, pancreas, prostate, uterus, ovary, AML
miR-19b-1, miR-19b-2	5×10^{-6}	<0·01	1·7	13q31.3, Xq26.2	Histotype	*THBS1 (TSP1), MYLIP, HIPK3, SOCS1*	Prostate
miR-224	2×10^{-5}	0·02	2·2	Xq28	..	*API5*	Pancreas, liver, thyroid, ovary, AML
miR-18a	5×10^{-5}	0·04	1·7	13q31.3	..	*CTGF, CDKN1A, NR3C1 (GR), THBS1 (TSP1), ESR1, RUNX1 (AML1)*	Pancreas, liver, AML
miR-135b	5×10^{-5}	0·04	1·6	1q32.1	..	*APC*	Uterus
miR-19a	0·0008	0·5	1·5	13q31.3	Histotype, progression, prognostic	*PTEN, THBS1 (TSP1), SOCS1*	Uterus, CLL
miR-345	0·001	0·5	1·5	14q32.2	Progression	..	Prostate, thyroid
miR-191	0·002	1·0	1·3	3p21.31	..	..	Breast, colon, lung, pancreas, prostate, stomach

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