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Pathology International 2009; **59**: 121–136 doi:10.1111/j.1440-1827.2009.02329.x

Review Article

Transcriptome dissection of gastric cancer: Identification of novel diagnostic and therapeutic targets from pathology specimens

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Gastric cancer is the fourth most common malignancy in the world, and mortality due to gastric cancer is second only to that from lung cancer. 'Transcriptome dissection' is a detailed analysis of the entire expressed transcripts from a cancer, for the purpose of understanding the precise molecular mechanism of pathogenesis. Serial analysis of gene expression (SAGE) is a suitable technique for performing transcriptome dissection. Gastric cancers of different stages and histology were analyzed on SAGE, and one of the largest gastric cancer SAGE libraries in the world was created (GEO accession number GSE 545). Through SAGE, many candidate genes have been identified as potential diagnostic and therapeutic targets for the treatment of gastric cancer. Regenerating islet-derived family, member 4 (*Reg IV*) participated in 5-fluorouracil (5-FU) resistance and peritoneal metastasis, and its expression was associated with an intestinal phenotype of gastric cancer and with endocrine differentiation. *GW112* expression correlated with advanced tumor stage. Measurement of Reg IV and GW112 levels in sera indicated a sensitivity of 57% for detection of cancer. SPC18 participated in tumor growth and invasion through transforming tumor growth factor-α upregulation. Palate, lung, and nasal epithelium carcinoma-associated protein (PLUNC) was a useful marker for gastric hepatoid adenocarcinoma. Expression of *SOX9*, *HOXA10*, *CDH17*, and loss of claudin-18 expression were associated with an intestinal phenotype of gastric cancer. Information obtained from transcriptome dissection greatly contributes to diagnosis and treatment of gastric cancer.

Key words: claudin-18, gastric cancer, GW112, PLUNC, Reg IV, serial analysis of gene expression, SOX9, SPC18, transcriptome dissection

'Molecular pathology' encompasses morphopathological genomics to comprehensively understand abnormalities in morphology and function of genes and molecules. We have been studying novel diagnostic and therapeutic targets through global gene expression, epigenetics, genetic polymorphisms and their predispositional effect towards gastric cancer.[1,2] It is important to understand the pathology of cancer in order to facilitate proper medical care, with knowledge of both morphological and molecular abnormalities, and to show concretely what can be done using this knowledge of pathology, to make potential clinical applications a reality.

Although gastric cancer incidence decreases through westernization of eating habits and a decrease in *Helicobacter pylori* infection, it is the fourth most common malignancy in the world and approximately 900 000 people suffer from gastric cancer every year.[3] Mortality due to gastric cancer is second only to that from lung cancer. Although the prognosis for patients with early gastric cancer has been prolonged drastically by current methods of diagnosis and treatment, that for advanced cancer remains poor. In gastric cancer patients of all stages, the 5 year survival rate after diagnosis is around 50% in Japan, and is ≤30% in other countries.[3] Therefore, areas that need attention for better treatment of gastric cancer are: detection at an early stage; and effective medical treatments for advanced cancers. For these purposes, novel diagnostic and therapeutic targets are required to be found.

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This paper was presented at the 97th Annual Meeting of the Japanese Society of Pathology, as the Japan Pathology Award Lecture.

Received 27 September 2008. Accepted for publication 20 October 2008.

In the history of the Japanese Society of Pathology, the report of the Japan Pathology Award lectures (formerly the homework reports) on gastric cancer was initially presented by Kunio Ota (Tokyo Medical and Dental University), 'Pathogenesis of gastric cancer' in 1964; and was followed by Tateo Nagayo (Aichi Cancer Center), Setsuya Fujita (Kyoto Prefectural University of Medicine), Eiichi Tahara (Hiroshima University), and Masae Tatematsu (Aichi Cancer Center). At the 81st Annual Meeting of the Japanese Society of Pathology held in Sendai in 1992, the Japan Pathology Award lecture was presented by Tahara, who reported that through molecular pathology, different genetic pathways of stomach carcinogenesis might be discerned for poorly differentiated and well-differentiated gastric cancers.[4,5]

After this lecture the molecular basis of gastric cancer has been studied intensively and the recent understanding of the molecular events involved in stomach carcinogenesis can be summarized as follows.[1,6] A variety of genetic and epigenetic alterations occur during multistep stomach carcinogenesis. These include activation of oncogenes and growth factors/receptors, inactivation of tumor suppressor genes, DNA repair genes and cell adhesion molecules, and abnormalities in cell cycle regulators and so on. The genetic alterations that are found in gastric cancer are gene amplification, point mutation and loss of heterozygosity, while representative epigenetic changes are gene silencing by DNA methylation and histone modification. Genetic polymorphism can predispose an endogenous cause and increase susceptibility to cancer. Some of these changes occur commonly in both differentiated and undifferentiated types, and some differ depending on the histological type or mucin phenotype (gastric vs intestinal). Many of these abnormalities have been applied to molecular diagnosis[7–9] but there may be important abnormalities that have not yet been clarified, and these may become new targets for diagnosis and treatment.

In this report we describe a method for transcriptome dissection, novel cancer-specific genes identified through global analysis of gene expression, the significance of these genes in diagnosis and treatment and in stomach carcinogenesis and differentiation.

TRANSCRIPTOME DISSECTION

'Transcriptome dissection' is a term we created to describe 'a detailed analysis of entire transcripts in affected tissues, to understand the precise molecular mechanisms of pathogenesis'. Using this approach, new candidate genes for diagnosis, treatment and prevention are able to be discovered that build a bridge to clinical applications from basic research.

As a method of transcriptome dissection, serial analysis of gene expression (SAGE) was utilized as a powerful technique that allows global analysis of gene expression in a quantitative manner, without a prior knowledge of the exact sequence of the genes.[10] SAGE is based on the following principles. A short nucleotide sequence tag (approx. 10 bp) is sufficient to uniquely identify a transcript, provided it is isolated from a defined position within the transcript. Concentration of short sequence tags allows the efficient analysis of transcripts in a serial manner through the sequencing of multiple tags within a single clone. Because the SAGE tag numbers directly reflect the abundance of mRNA, SAGE data are highly accurate and quantitative. Completion of the human genome sequence has facilitated the mapping of specific genes to individual tags. Now, the SAGEmap database includes approximately 400 SAGE libraries online and is available to the public (http://www.ncbi.nlm.nih.gov/SAGE/). The advantage of SAGE is that we can study expression of genes of interest in other tissues registered in the database. Four SAGE studies of gastric cancer have been so far reported.[11–14] We examined five samples of gastric cancer of different stage and histology from four patients and uncovered a total of 137 706 tags including 38 903 unique tags.[14] This SAGE library is one of the largest gastric cancer libraries in the world and the sequence data are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue). Many of the genes specifically upregulated or downregulated in cancer were found by comparison of SAGE libraries from gastric cancer against normal tissue. In combination with quantitative reverse transcription–polymerase chain reaction (RT-PCR), immunohistochemistry and other methods, several candidates for novel metastasis-related genes, tumor suppressor genes and serum tumor markers were identified.[14,15]

An advantage of quantitative SAGE data is that they can be compared with other samples (not just normal tissue control). In silico analysis was performed to detect any difference in molecular bases of gastric cancer from the East and the West. Our libraries were compared with the libraries from the West that were produced by El-Rifai *et al.* as part of the Cancer Genome Anatomy Project.[11,14] A clear separation was found between the East and West tumor libraries, which had 54 differentially expressed tags.[16] These may contribute to the geographical differences in incidence and possible biological behavior of gastric cancer, and differences found in outcome for patients.

Figure 1 illustrates the strategy of clinical application from transcriptome dissection.[1,6] From detailed gene expression information, specifically upregulated or downregulated genes in a cancer can be identified. The expression of these genes is confirmed in a large number of cases on quantitative RT-PCR and immunohistochemistry. If the specific gene encodes a secretory protein, this may be detected in the blood and should be a novel serum marker of gastric cancer.

For such a molecule, a simple measuring system such as ELISA can be established for blood samples, which can then be applied for early cancer detection. After functional analysis we can then know whether the genes are novel targets that could potentially be used for molecular target therapy. Polymorphisms of genes can highly alter their expression in cancer, and may be novel risk factor candidates, and this information could possibly be used for personalized cancer prevention. A custom-made complementary DNA (cDNA) microarray with the specific genes identified on SAGE, known cancer-related genes, and known genetic markers for chemosensitivity, would be a useful tool to obtain information on biological behavior from, and sensitivity to, therapy in the clinical setting.

A practical approach is introduced here to identify cancer-specific genes, and candidate diagnostic and therapeutic targets using a SAGE database search.[17] If a gene participates in tumor progression and is specifically expressed in cancerous but not in normal tissues, the gene could be not only a cancer-biomarker, but also a possible therapeutic target, treatment of which might have minimal adverse effects. By comparing SAGE libraries of gastric cancer with those of various normal tissues in the SAGEmap database, 54 genes were identified in the gastric cancer libraries that were not present in the libraries from 14 normal tissues including brain, lung, heart, liver, kidney and so on.[17] Expression of these genes was then confirmed in tissue samples from gastric cancers and normal human organs on quantitative RT-PCR. Representative results are shown in Fig. 2. Nine genes including APin protein (*APIN*), taxol resistance-associated gene 3 (*TRGA3*), cytochrome P450, family 2, subfamily W, polypeptide 1 (*CYP2W1*), melanoma inhibitory activity (*MIA*), matrix metalloproteinase-10 (*MMP-10*), dickkopf homolog 4 (*DKK4*), *GW112*, regenerating islet-derived family, member 4 (*Reg IV*), and HORMA domain-containing 1 (*HORMAD1*) were found to have gastric cancer-specific expression.

NOVEL TARGETS FOR DIAGNOSIS AND TREATMENT OF GASTRIC CANCER

New candidate genes, *Reg IV*, *GW112*, *MMP-10* and *SPC18*, identified from transcriptome dissection will be described in terms of diagnostic and therapeutic targets.

Reg IV

Reg IV belongs to the calcium-dependent lectin superfamily, and was isolated as a gene upregulated in inflammatory bowel diseases in 2001.[18] Overexpression of *Reg IV* has been detected in colorectal carcinoma and adenoma on RT-PCR and *in situ* hybridization,[19,20] and it has been reported that high *Reg IV* expression is associated with 5-fluorouracil (5-FU) resistance in a colon cancer cell line.[19] When this analysis was begun, the expression and distribution of *Reg IV* in human tumors remained unclear, and it was assumed that it possibly participates in defense against mucosal injury, and proliferation of mucosal epithelia.

Additional study of *Reg IV* expression on quantitative RT-PCR showed that *Reg IV* was also expressed in colon cancer, but not in lung cancer and breast cancer.[21] Normal gastrointestinal tract and pancreas tissue expressed *Reg IV* at low levels, indicating that *Reg IV* was not truly cancer specific. On immunohistochemistry it was found that in non-neoplastic tissues, Reg IV was expressed in beta cells of the endocrine pancreas, goblet cells in intestinal metaplasia of the stomach, and neuroendocrine cells of the small intestine (Fig. 3a,d).[21] Twenty to thirty percent of gastric, colorectal and pancreatic cancers were positive for Reg IV expression, as were most gastrointestinal carcinoids. In accordance with quantitative RT-PCR, expression in all breast and lung cancer samples was negative. In gastric cancer, two staining patterns were noted: mucin-like staining and perinuclear staining (Fig. 3c,d). The former pattern might be associated with intestinal differentiation, and the latter might be associated with neuroendocrine differentiation. These will be discussed later.

Concerning the biological role of Reg IV, forced expression of *Reg IV* in TMK-1 gastric cancer cells inhibited 5-FU-induced apoptosis through activation of epidermal growth factor receptor (EGFR), induction of Bcl-2 and cytochrome c, and inhibition of caspase-9 and -3.[22] Therefore, Reg IV may serve as an indicator of the resistance of a cancer to 5-FU-based chemotherapy. The expression of Reg IV was then examined on immunostaining in samples from recurrent gastric cancer patients who had been treated with a combination chemotherapy of low-dose 5-FU and cisplatin. As predicted, all Reg IV-positive patients had 'no change' or 'progressive disease'.[22] Furthermore, it was found that Reg IV promoted peritoneal dissemination metastasis in a mouse model.[23] *Reg IV*-transfected MKN-28 gastric cancer cells were inoculated into the peritoneal cavity of nude mice. Number and size of metastatic tumors were higher in *Reg IV* transfectants than in controls. In this system the expression of the phosphorylated form of the epidermal growth factor (EGF) receptor, Bcl-2, Bcl-XL, survivin, and the phosphorylated form of v-akt murine thymoma viral oncogene homolog (AKT) was upregulated. This peritoneal metastasis was inhibited by treatment with *Reg IV*-small interfering RNA (siRNA). In the clinical specimens it was confirmed that the *Reg IV* levels were high in gastric cancer patients with peritoneal metastasis, and *Reg IV*-positive gastric cancer patients had poorer prognosis than *Reg IV*-negative patients.[24] Overall these findings indicate that Reg IV is a novel diagnostic tool, and a potential therapeutic target for the treatment of gastric cancer.

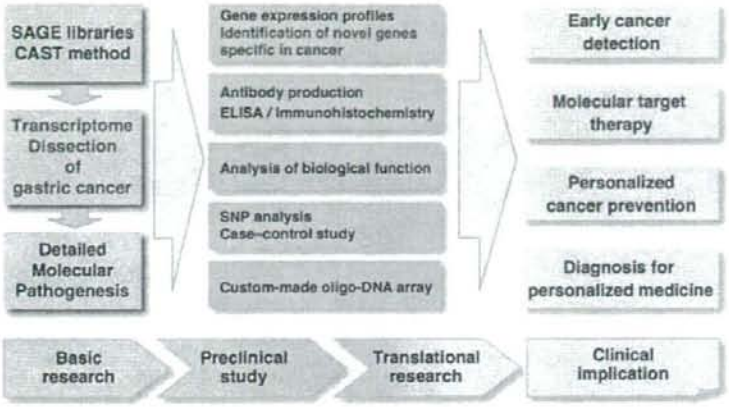

Figure 1 Strategy to search for novel genes of gastric cancer through transcriptome dissection and its clinical implication. CAST, *Escherichia coli* ampicillin trap; SAGE, serial analysis of gene expression; SNP, single nucleotide polymorphism.

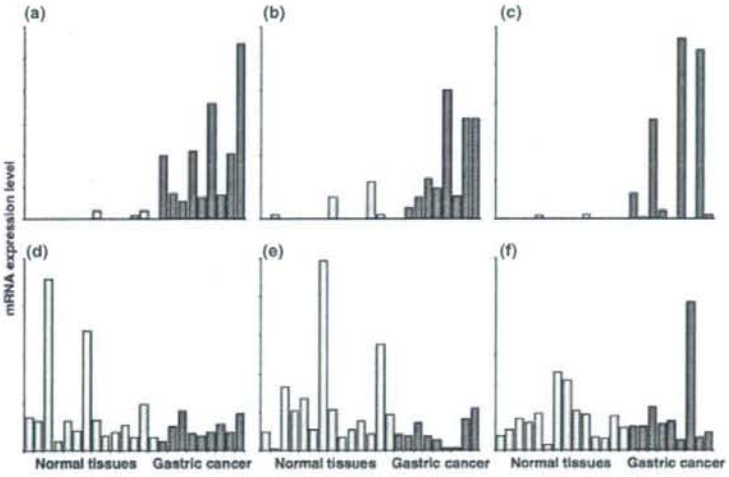

Figure 2 Representative results of quantitative RT-PCR of various normal tissues (brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, spleen, leukocytes) and gastric cancers. mRNA expression level of (**a**) matrix metalloproteinase-10 (*MMP-10*), (**b**) *GW112* and (**c**) taxol resistance-associated gene 3 (*TRAG3*) were much higher in gastric cancers than in normal tissues. These three are indicative of expression of genes detected in the SAGE libraries of gastric cancer-specific gene. (**d**) arginyltransferase 1 (*ATE1*); (**e**) bromodomain containing 4 (*BRD4*); (**f**) v-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (*ETS2*).

Analysis of the amino acid sequence of the Reg IV protein suggested that it should be secreted.[14] It was confirmed on western blotting that native Reg IV was present in the culture media of gastric cancer cells that expressed high levels of *Reg IV* mRNA (Fig. 4) and that V5-tagged Reg IV protein was detected in not only cell extracts, but also culture media from a Reg IV-V5-expressing gastric cancer cell line, but not control cells. Although early detection is especially important for the treatment of gastric cancer, no good serological marker exists for detection of early cancer.[25] Tests for known tumor markers such as CEA and carbohydrate antigen (CA) 72-4 do not have satisfactory sensitivity for early detection, although they may have prognostic impact. Reg IV levels in the sera of gastric cancer patients and healthy controls were then measured on ELISA to determine whether Reg IV is a serum tumor marker or not. Serum levels of Reg IV protein were similar between the healthy controls and patients with chronic active gastritis, and serum Reg IV levels were significantly elevated in gastric cancer patients, even at stage I.[22] If the cut-off level was set at 2 ng/ml, specificity was 99%, and diagnostic sensitivity was 36%, which is much superior to that of serum CEA (14%) or CA19-9 (15%).

GW112

GW112, also called olfactomedin 4 (*OLFM4*) or human G-CSF clone-1 (*hGC-1*), was originally cloned from human myeloid cells and encodes a secreted glycoprotein of 510 amino acids.[26] GW112 is normally expressed in the bone marrow, intestine and prostate, and altered expression is observed in various cancers including those of the colon,

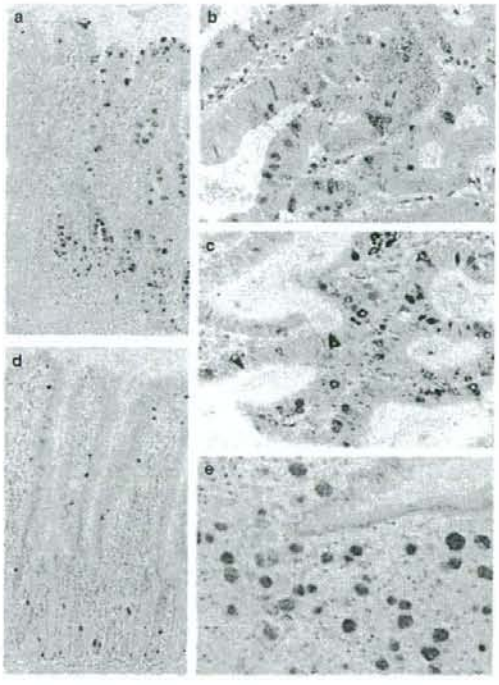

Figure 3 Immunostaining of regenerating islet-derived family, member 4 (Reg IV) in non-neoplastic tissues and gastric cancers. Reg IV is expressed (**a**) in goblet cells of intestinal metaplasia of the stomach and (**d**) neuroendocrine cells of the small intestine. (**b**,**c**,**e**) Strong expression is observed in gastric cancers. There are two staining patterns: (**b**) mucin-like staining and (**c**) perinuclear staining. (**e**) Gastric signet-ring cell carcinoma is also positive for Reg IV.

breast, and lung cancers.[27,28] Because GW112 interacts with GRIM19, cadherin and lectins, GW112 may facilitate apoptosis, tumor growth and invasion.[29,30]

On quantitative RT-PCR low levels of *GW112* expression were detected in some normal tissues, while strong expression was detected in gastric cancers.[17,31] Fifty-eight percent of gastric cancers had overexpression of *GW112* associated with stage III and IV cancers. A monoclonal antibody was then produced for GW112, and immunohistochemistry undertaken. GW112 was expressed in epithelial cells at the bottom of the intestinal crypt and intestinal metaplasia of the stomach, and strong GW112 expression was detected in 60% of gastric cancers (Fig. 5). Significant association was found between GW112 expression and intestinal histology. Furthermore, a significant inverse correlation was detected between Reg IV protein level and that of GW112 in gastric cancer.

An ELISA system was established to measure serum levels of these proteins. Approximately 30% of gastric cancer

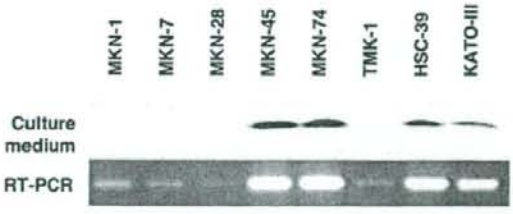

Figure 4 Detection of regenerating islet-derived family, member 4 (Reg IV) in culture media of gastric cancer cell lines. Western blot analysis of culture media of eight gastric cancer cell lines and northern blot analysis of cell extracts are shown. Reg IV protein was detected in culture media of cell lines that express *Reg IV* mRNA at high levels. RT-PCR, reverse transcription–polymerase chain reaction.

patients displayed high levels of GW112 in their sera regardless of tumor stage, even at stage I. Specificity of the test was 95%, indicating that GW112 is also a good serum tumor marker for gastric cancer. Importantly, no correlation was detected between Reg IV and GW112 levels. Measurement of Reg IV and GW112 indicated a sensitivity of 57% for detection. A combination of Reg IV and GW112 may serve as a highly sensitive biomarker for gastric cancer. Clinical application is expected.

MMP-10

MMP-10, also known as stromelysin 2, is one of the cancer-specific genes identified using the same method as that for *Reg IV* and *GW112* (Fig. 2).[17] Among the nine cancer-specific genes identified, *MMP-10* was most frequently overexpressed in gastric cancer. MMP induce extracellular matrix breakdown, associated with tissue destruction during cancer invasion and metastasis.[32] Overexpression of MMP-10 has been reported in various cancers such as cancers of the lung, esophagus and liver. Our immunohistochemical study demonstrated MMP-10 to be correlated with tumor progression and a poor prognosis for gastric cancer patients. Importantly, high levels of MMP-10 protein were detected in serum samples from >90% of gastric cancer patients, regardless of tumor stage, while diagnostic specificity was 85%.[17] In samples from patients with stage I gastric cancer, 89% showed high serum levels of MMP-10. Therefore, MMP-10 is extremely useful for screening and early detection of gastric cancer.

SPC18

Signal peptidase complex 18KDa (*SPC18*) is another new therapeutic candidate gene identified through transcriptome

dissection. As already mentioned, SAGE is a powerful technique for performing a global analysis of gene expression in a quantitative manner. It is difficult, however, to apply SAGE in the clinical setting to study large numbers of clinical samples because mass sequencing is required. In contrast, microarrays are a conventional technique for examining the expression of large numbers of genes at the same time. A custom-made microarray for the study of stomach carcinogenesis and possible future clinical application was prepared. The microarray, named Ex-STOMACHIP, consisted of 395 genes (478 cDNAs) and contained: genes selected as the 20 most upregulated and downregulated tags in SAGE libraries of gastric cancer; genes known to participate in carcinogenesis; and genes related to metastasis and chemosensitivity identified from other microarray studies.[15] Using Ex-STOMACHIP in combination with quantitative RT-PCR on 42 samples of gastric cancer, *SPC18* was identified as one of the significantly upregulated genes in stage III/IV gastric cancer compared with stage I/II.

Secretory proteins are usually synthesized as precursors with signal peptides that are cleaved by a family of signal peptidases following membrane translocation.[33] Signal peptidase complex (SPC) has five distinct subunits, and SPC18 and SPC21 are presumed to have catalytic activity.[34,35] Substrate specificity, however, remains unknown and no study has been reported on their expression and role in cancer. Quantitative RT-PCR demonstrated that *SPC18* was overexpressed in 40% of gastric cancer samples, and the overexpression was significantly associated with advanced tumor stage and presence of lymph node metastasis.

An expression vector containing *SPC18* was constructed and introduced into the MKN-1 gastric cancer cell line, which has a low level of *SPC18* expression. Under a condition of 1% serum, *SPC18* significantly stimulated cell proliferation, measured on 3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *SPC18* was also found to stimulate cell migration and invasion, monitored by wound healing assay and matrigel invasion assay. The important question is what the target secretory proteins of the signal peptidase SPC18 are, in relation to tumor growth and invasion. The level of transforming growth factor (TGF)-α in the culture media was measured and found to be increased 10–20-fold in *SPC18*-transfected MKN-1 cells in comparison with control MKN-1 cells. The same pattern was observed for EGF levels in the culture media. Furthermore, *SCP18*-siRNA treatment specifically reduced the levels of TGF-α and EGF in SPC18-producing KATO-III gastric cancer cells. Therefore, TGF-α and EGF might be important targets for SPC18. It was also confirmed that *SPC18* stimulated tumor growth in SCID mice. EGFR was activated in the formed tumor with forced expression of *SPC18*. Thus, SPC18 promoted TGF-α/EGF secretion possibly by processing these precursors, resulting in EGFR activation and stimulation of tumor growth and invasion. Therefore, SPC18 participates in the growth and invasion of tumors, partly through TGF-α or EGF upregulation, and may serve as a marker of high-grade malignancy, as well as a novel therapeutic target.

APPLICATION TO HISTOPATHOLOGICAL DIAGNOSIS

Here Reg IV and palate, lung, and nasal epithelium carcinoma-associated protein (PLUNC) are provided as an example of candidates identified from transcriptome dissection used for histopathological diagnosis.

Reg IV

Signet-ring cell carcinoma is a unique subtype of adenocarcinoma that is characterized by abundant intracellular mucin accumulation, and a crescent-shaped nucleus displaced toward one end of the cell. Signet-ring cell carcinoma typically occurs in the stomach, but can arise in almost every organ, including the large intestine, lung and breast, and can present as distant metastases. A typical example is the Krukenberg tumor, which is a metastatic signet-ring cell carcinoma that occurs in the ovary. Because this cancer has a morphological identity irrespective of the primary site of origin or metastatic status, it is difficult to determine the primary site. It was earlier confirmed that expression of Reg IV is limited to cancers of the stomach, colon and pancreas, whereas lung cancers and breast cancers do not express Reg IV. The immunohistochemical characteristics of signet-ring cell carcinoma were then analyzed from various organs including the stomach, colorectum, breast and lung, using antibodies against Reg IV and known tissue markers such as MUC2, MUC5AC, cytokeratin (CK) 7, CK20, caudal-related homeobox gene 2 (CDX2), thyroid transcription factor-1 (TTF-1), mammaglobin, gross cystic disease fluid protein-15 (GCDFP15), and estrogen receptor (ER).[36]

All gastric and colorectal signet-ring cell carcinomas were positive for Reg IV expression (Fig. 3e), whereas none of them expressed TTF-1, mammaglobin, GCDFP15 or ER. MUC2, MUC5AC, CK7, CK20 and CDX2 were expressed in 50–80% of gastric signet-ring cell carcinomas, while MUC2, CK20 and CDX2 were expressed in >80%, and MUC5AC and CK7 in 38% and 12% of colorectal signet-ring cell carcinoma, respectively. In contrast, none of the pulmonary and breast signet-ring cell carcinomas expressed Reg IV. While most of the pulmonary signet-ring cell carcinomas expressed TTF-1, most of the breast signet-ring cell carcinomas showed cytoplasmic staining for GCDFP15 and nuclear staining of ER. As expected, TTF-1 was a good tissue marker for signet-ring cell carcinomas originating from the lung, and GCDFP15 and ER were good markers for signet-ring cell carcinomas of

the breast. In summary, Reg IV is a strong immunohistochemical marker for gastrointestinal signet-ring cell carcinoma, with a specificity and sensitivity of 100%.

PLUNC

Another example of a histopathological diagnostic marker identified through transcriptome dissection is PLUNC.[37] Of the technologies for high-throughput analysis of gene expression, the principles of microarray analysis and SAGE are totally different. Microarray analysis is based on hybridization, while SAGE is based on PCR and sequencing. Therefore, a list of upregulated or downregulated genes extracted using each method may be different. The lists of the 20 most upregulated genes detected on microarray analysis using GeneChip Human Genome U133Plus 2.0 array (Affimetrix, Santa Clara, CA, USA) and SAGE (GSM9103)[14] in the same sample of poorly differentiated adenocarcinoma of the stomach were compared. There were no genes that were the same among the top 20 differentially expressed genes that were extracted using the two methods. *PLUNC* was the gene showing the greatest increase in expression in gastric cancer on microarray analysis, but only three tags of *PLUNC* were detected on SAGE.

PLUNC was first cloned as a gene expressed in embryonic palate, nasal epithelium, and adult lung of the mouse by the differential display method.[38] Human *PLUNC* shows a similar expression pattern.[39] It is known that *PLUNC* encodes a secretion protein, and may participate in the early immune response against bacterial and viral infection in the upper respiratory tract, but the biological function of PLUNC remains poorly understood.[40] PLUNC is reported to be commonly expressed in adenocarcinoma, but not in small cell carcinoma and squamous cell carcinoma of the lung.[40,41] No information for PLUNC expression is available for other cancers. Quantitative RT-PCR showed that *PLUNC* expression was detected in adult trachea and lung, and one of the gastric cancers, while all other normal organs examined did not express *PLUNC*. Immunohistochemically, only 9% of gastric cancers were positive for PLUNC. Among those, cases with >30% of tumor cells stained were found to be hepatoid adenocarcinoma of the stomach (Fig. 6).

Gastric hepatoid adenocarcinoma is the primary gastric cancer producing AFP, and represents approximately 5% of all stomach cancers.[42] Liver metastasis is frequent and the prognosis is poor in comparison with ordinary gastric cancer.[43] Gastric hepatoid adenocarcinoma has histological features that mimic hepatocellular carcinoma, and discrimination is extremely difficult from hepatocellular carcinoma. It is challenging to differentiate hepatoid adenocarcinoma from hepatocellular carcinoma on the basis of morphology, especially if it metastasizes in the liver. So far there has been no marker that can completely distinguish both. It was reported that immunohistochemical detection of HepPar1, CK19 or CK20 is useful for differentiating between the two.[44] HepPar1 staining is detected more frequently in hepatocellular carcinoma than in gastric hepatoid adenocarcinoma, whereas staining for CK19 and CK20 is detected more frequently in gastric hepatoid adenocarcinoma than in hepatocellular carcinoma. But many exceptions to this exist. Most of the gastric hepatoid adenocarcinomas in both primary and liver metastatic tumors were positive for PLUNC, whereas no HCC was PLUNC positive. Therefore, PLUNC is a novel and useful marker for differentiating gastric hepatoid adenocarcinoma from hepatocellular carcinoma.

ROLE OF THE NEWLY IDENTIFIED GENES IN DEVELOPMENT, DIFFERENTIATION AND PROGRESSION OF GASTRIC CANCER

Using transcriptome dissection based on SAGE, part of the molecular mechanism of development, differentiation and progression of gastric cancer has been clarified. Here, the connection with tumor differentiation is introduced. Gastric cancers are histologically classified into two major groups: differentiated and undifferentiated types, or intestinal and diffuse types based on glandular structure.[45,46] In addition, gastric cancers are also classified into intestinal (I) and gastric (G) types on the basis of the mucin expression profile such as expression of MUC2, CD10, MUC5AC, and MUC6.[47,48] CDX1 and CDX2 are intestine-specific transcription factors, and SOX2 is known to be stomach specific.[49,50] Some differences in molecular events have been noted: for instance, hypermethylation of the hMLH1 gene and microsatellite instability are frequent in the G type, and p53 mutation is frequent in the I type.[1,51,52] Also, with regard to biological difference, it has been suggested that the G type behaves more aggressively than the I type.[53]

Reg IV

As aforementioned, there are two staining patterns of Reg IV in gastric cancer: mucin-like staining and perinuclear staining. Mucin-like staining of Reg IV was significantly associated with MUC2-positive I type, and double staining demonstrated co-expression in the same tumor cells (Fig. 7a; Table 1).[21] In HT-29 colon cancer cells with forced expression of *CDX2-ER*, 4-hydroxytamoxifen (4-OHT) induces nuclear translocation and activates *CDX2*.[54] In this system *CDX2* induced the expression of *Reg IV*. On immunofluorescent staining, all of the Reg IV-positive cells in both intestinal metaplasia and gastric cancer were also positive for CDX2, but CDX2-positive cells were not always Reg IV positive (Fig. 7b;

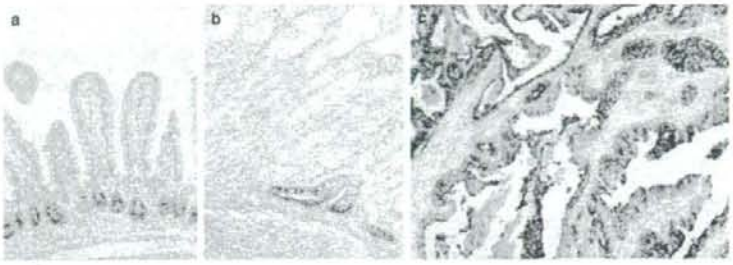

Figure 5 Immunostaining of GW112 in non-neoplastic tissues and gastric cancer. (**a**) GW112 is expressed in the cytoplasm of epithelial cells in the bottom area of the small intestine and (**b**) intestinal metaplasia of the stomach. (**c**) GW112 is strongly expressed in well-differentiated gastric cancer.

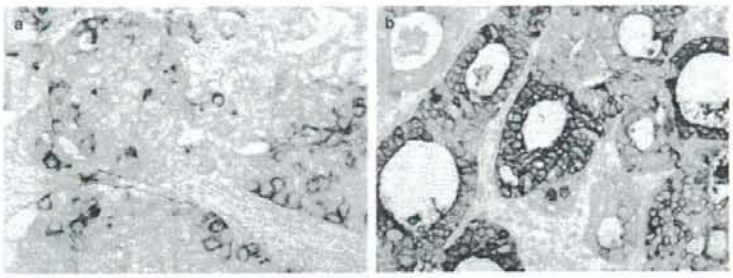

Figure 6 Immunostaining of palate, lung, and nasal epithelium carcinoma-associated protein (PLUNC) in hepatoid adenocarcinoma of the stomach. (**a**) Many tumor cells with a solid structure resembling hepatocellular carcinoma are positive for PLUNC. (**b**) Tumor cells in the tubular component of hepatoid adenocarcinoma are also positive for PLUNC.

Figure 7 Relationship between regenerating islet-derived family, member 4 (Reg IV) and differentiation of gastric cancer. Double staining with immunofluorescent dyes shows co-expression of (**a**) Reg IV (red) and MUC2 (green) and (**b**) Reg IV (red), CDX2 (green) and 4′6′-diamidino-2-phenylindole (DAPI) (blue), and (**c**) co-localization of Reg IVs (red) and serotonin (green) in gastric cancer, respectively. CDX2, caudal-related homeobox gene 2; MUC, mucin; Reg IV, regenerating islet-derived family, member 4.

Table 1 Reg IV, MUC2, CDX2 and chromogranin A in gastric cancer on immunostaining

	Reg IV expression Positive	Fisher's exact test *P* value
MUC2		
Positive	40/52 (77%)	$P < 0.0001$
Negative	2/91 (2%)	
CDX2		
Positive	36/53 (68%)	$P < 0.0001$
Negative	6/90 (7%)	
Chromogranin A		
Positive	18/27 (67%)	$P < 0.0001$
Negative	24/116 (21%)	

CDX2, caudal-related homeobox gene 2; MUC, mucin; Reg IV, regenerating islet-derived family, member 4.

Table 1). These findings indicate that CDX2 is an upstream regulator for Reg IV expression.

The downstream targets of Reg IV are currently being searched for on GeneChip analysis, in combination with quantitative RT-PCR in *Reg IV*-transfected gastric cancer cell lines. SOX9 was found to be one of the downstream targets of Reg IV. Clear induction of SOX9 by Reg IV was detected in MKN-28 cells with an intestinal mucin phenotype. SOX9 is a member of the *sry*-related high-mobility group (HMG) box (SOX) family of HMG DNA-binding domain transcription factors, and is required for the development and differentiation of multiple cell lineages.[55] SOX9 is expressed in progenitor/stem cells and Paneth cells in the small intestine and is known to be regulated by the Wnt pathway.[56,57] Immu-

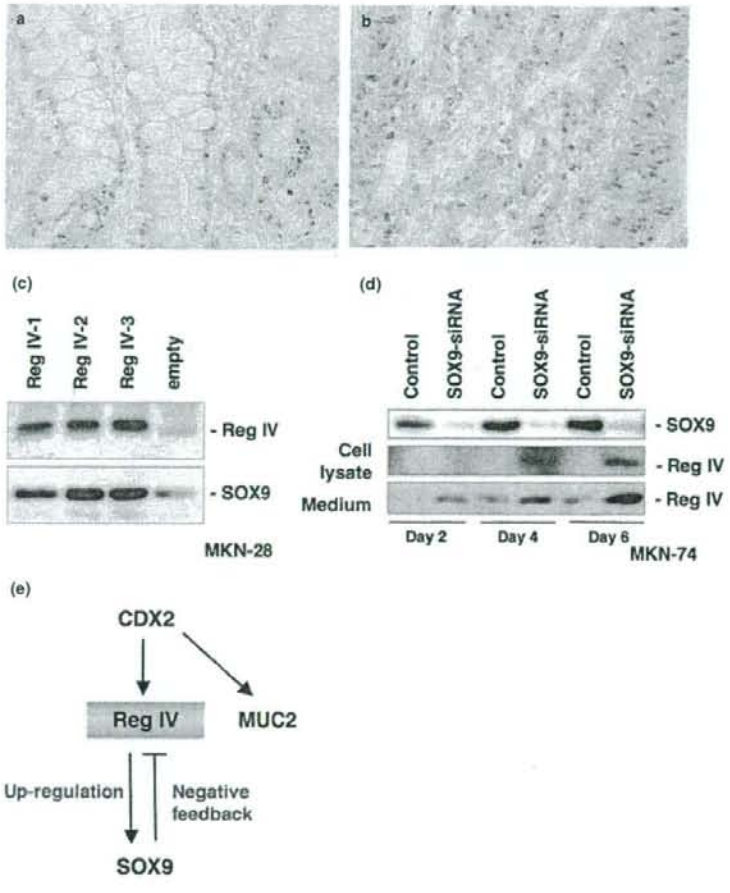

Figure 8 Expression of SOX9 and its regulation by regenerating islet-derived family, member 4 (Reg IV) in gastric cancer. (**a**) Immunostaining of SOX 9 in intestinal metaplasia of the stomach and (**b**) I type gastric cancer. (**c**) Forced expression of *Reg IV* in MKN-28 gastric cancer cells (Reg IV-1, -2, -3) induces SOX9, while (**d**) treatment of *SOX9*-siRNA to MKN-74 gastric cancer cells induces Reg IV in both cell lysate and culture medium on western blot. (**e**) Schematic illustration of the relationship between Reg IV and SOX9.

nohistochemically, SOX9 was expressed in epithelial cells at the proliferative zone of the normal gastric mucosa and bottom area of the intestinal metaplasia of the stomach (Fig. 8a). Many tumor cells of I type gastric cancer were positive for SOX9, and SOX9-positive tumor cells co-localized with *Reg IV*-positive cells. Furthermore, forced expression of Reg IV induced SOX9 in MKN-28 cells at the protein level, while addition of *SOX9*-siRNA to MKN-74 displaying a good level of SOX9 expression induced Reg IV expression (Fig. 8b). Therefore, a feedback mechanism might exist between Reg IV and SOX9 (Fig. 8c).

In contrast, perinuclear staining of Reg IV in gastric cancer indicated significant association with neuroendocrine differentiation monitored by the expression of chromogranin A and synaptophysin.[21] Using a tissue microarray with 630 cases of gastric cancer, connection of Reg IV with a variety of neuroendocrine hormones was examined, and it was found that Reg IV-positive cancers were significantly associated with serotonin- and gastrin-positive cancers. Furthermore, double immunofluorescent staining demonstrated that Reg IV was co-localized with serotonin (Fig. 7c; Table 1), gastrin, and pancreatic polypeptide in both cancer cells and non-neoplastic epithelial cells of the stomach.

Figure 9 summarizes the role of Reg IV and its pathway in gastric cancer. Reg IV may play a certain role in intestinal differentiation of gastric cancer through CDX2 and SOX9. Reg IV activates the EGFR and upregulates the anti-apoptotic signal, allows cells to escape from apoptosis, and participates in tumor growth and 5-FU resistance. The consequences of this association with endocrine differentiation, such as production of serotonin, and gastrin and its pathway, need further investigation.

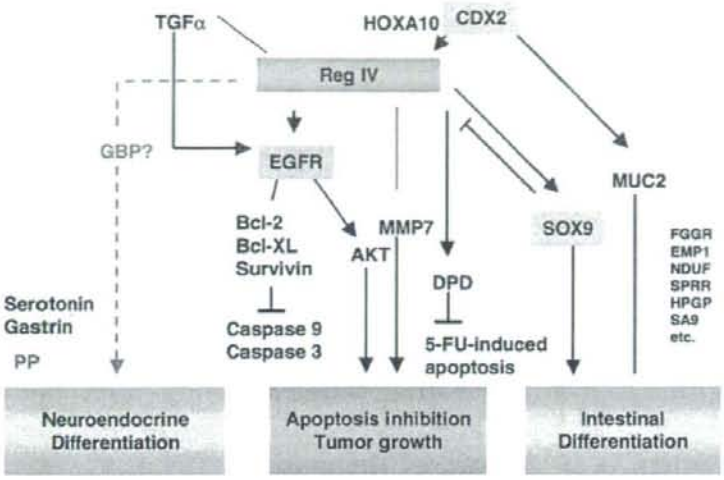

Figure 9 Role of regenerating islet-derived family, member 4 (Reg IV) and its pathway in gastric cancer.

HOXA10

Next several genes and molecules identified on transcriptome dissection are described, which relate to gastric cancer with intestinal differentiation.

Using the same method as that for identification of *PLUNC*, the lists of the 20 most upregulated genes detected on microarray analysis and SAGE (GSM8867)[14] were compared in a sample of well-differentiated gastric adenocarcinoma with an intestinal phenotype (I-type gastric cancer). Although only three genes were commonly detected as being upregulated on both methods, most of the upregulated genes extracted for each method were different. Among them, *HOXA10* had the greatest increase in expression in I-type gastric cancer on microarray analysis, whereas the tag for *HOXA10* was totally negative in SAGE.

HOXA10 is a member of the homeobox gene superfamily of transcription factors that contain a helix-turn-helix DNA-binding motif, and which participates in myeloid cell differentiation and proliferation.[58,59] HOXA10 and HOXA9 are associated with acute myeloid leukemia (*AML*) and mixed lineage leukemia (*MLL*) fusion genes.[60] HOXA10 controls uterine organogenesis during embryonic development and endometrial differentiation in adults.[61] Deregulation of HOXA10 correlates with progression of endometrial carcinoma.[62] Recently it was reported that CDX2 is an upstream regulator for *HOXA10* in myeloid cells and participates in leukemogenesis.[63] Quantitative RT-PCR indicated that 70% of gastric cancers overexpressed *HOXA10* regardless of tumor stage. On immunostaining HOXA10 was preferentially expressed in I-type gastric cancers and some of the intestinal metaplasias. The relation between HOXA10 and MUC2 was statistically significant. Because knockdown of *HOXA10* by siRNA treatment in MKN-45 gastric cancer cells reduced expression of Reg IV, HOXA10 may be an upstream regulator for Reg IV. Although the role of HOXA10 in stomach carcinogenesis remains unknown, HOXA10 is a novel marker for I-type gastric cancer.

Claudin-18

During the course of the search for novel tumor suppressor genes on SAGE data analysis, another gene related to gastric cancer with an intestinal phenotype was identified. To identify genes showing decreased expression in gastric cancer, the gastric cancer SAGE libraries (GSM8505 and GSM8867)[14] were obtained from well-differentiated gastric cancers with an intestinal phenotype, and normal stomach SAGE library (GSM784). Tags from each gastric cancer library were compared with those of the normal stomach library, and with selected tags that were downregulated in gastric cancer libraries.[64] Among 25 genes thus selected, expression of *CLDN18* (encoding claudin-18) was found to be lost in six of the eight gastric cancer cell lines on conventional RT-PCR. Quantitative RT-PCR showed that expression of *CLDN18* was downregulated in approximately 60% of gastric cancer tissues.

Claudins are components of tight junction strands.[65] Tight junctions are localized at the most apical region of polarized epithelial cells and create a barrier to prevent paracellular transport and restrict the lateral diffusion of membrane lipids and proteins. The claudin protein family consists of 24 members (claudins 1–24) and are expressed in an organ-

Table 2 Results of case–control study for G/A genotype in the promotor of CLDN-18 variant 1

Genotype	Gastric cancer (n = 154)	Control (n = 304)	P-value	Crude OR (95% CI)
Age (maen ± SD)	63.4 ± 11.1	48.7 ± 20.8	0.00005	5.17
A/A	5 (3.2%)	45 (14.8%)		(2.01–13.3)
A/G	88 (57.2%)	133 (43.8%)		
G/G	61 (39.6%)	126 (41.4)		

The observed genotype distribution of controls was in agreement with Hardy–Weinberg equilibrium. Association was analyzed on Fisher's exact test. P are values for G/G+A/G genotypes relative to A/A genotype. OR are for G/G+A/G genotypes relative to A/A genotype.
CI, confidence interval; OR, odds ratio.

specific manner. Association between claudins and cancer has previously been suggested.[66] *CLDN18* was first identified as a downstream target of the T/EBP/NKX2.1 homeodomain transcription factor.[67] There are two variants: variant 1 is expressed in the lung and variant 2 is expressed in the stomach.

RT-PCR in human samples confirmed that variant 1 was expressed in the lung and variant 2 was expressed in the stomach and small intestine, while other tissues including the colon, liver, pancreas and so on, did not express either variant.[64] More than 50% of gastric cancers had lost the expression of variant 2, whereas all samples of the corresponding non-neoplastic gastric mucosa expressed variant 2 at a good level. From immunostaining, claudin-18 was expressed on the cell membrane of all epithelial cells of the normal gastric mucosa, and Paneth cells of the duodenum.[64] Expression of claudin-18 was reduced in some of the intestinal metaplasia, most gastric adenomas, and approximately 60% of gastric cancers. In addition, advanced gastric cancer patients with reduced claudin-18 expression had poorer prognosis than those with preserved claudin-18 expression, while no association was found between claudin-18 expression and other clinicopathological characteristics. The analysis of mucin phenotype and claudin-18 expression demonstrated that reduced expression of claudin-18 was significantly associated with intestinal phenotype. A typical example is shown in Fig. 10. The expression of claudin-18 was lost in the MUC2-positive I type, while the MUC5AC-positive G type had preserved claudin-18 expression. Recently, it was reported that the protein kinase C (PKC)/mitogen-activated protein kinase (MAPK)/activator protein-1 (AP-1) dependent pathway regulated the expression of variant 2 of claudin-18 in gastric cancer cells.[68] While it is still necessary for a functional analysis to be performed, downregulation of claudin-18 may be involved in gastric cancer with an intestinal phenotype, and may participate in stomach carcinogenesis at an early stage, and also in progression of the cancer and poor survival rate for patients.

Polymorphism of cancer-related genes is known to affect cancer development and progression.[69] It is known that

Figure 11 Polymerase chain reaction–restriction fragment length polymorphism analysis for G/A genotype in the promoter of *CLDN-18* variant 1. See Table 2 for information on genotype distribution.

certain genetic polymorphisms also alter chemosensitivity and toxicity.[70] There is a single nucleotide polymorphism (SNP), guanine (G) to adenine (A) substitution at position −191 bp from the transcriptional start site within the promoter of *CLDN-18* variant 1. This SNP was analyzed on PCR–restriction fragment length polymorphism in peripheral blood DNA samples taken from gastric cancer patients and control subjects under informed consent. The case–control study demonstrated that genotypes G/G and G/A were significantly more frequent in gastric cancer patients than in control subjects, and that the odds ratio of the G/G and G/A genotype then indicated a significantly enhanced risk for gastric cancer of 5.17 (95% confidence interval: 2.01–13.3) compared to the A/A genotype (Fig. 11; Table 2). This information could be directly connected with personalized cancer prevention planning.

CDH17

By comparing SAGE libraries between early and advanced gastric cancers of an intestinal phenotype (GSM8505 and GSM8867),[14] *CDH17* was extracted as one of the most upregulated genes in advanced cancer.[71] *CDH17* encodes cadherin-17, also called liver–intestine (LI)-cadherin, and is a structurally unique member of the cadherin superfamily.[72,73] Cadherin-17 has only 20 amino acids in the cytoplasmic domain, while classic cadherins have a highly conserved cytoplasmic domain that consists of 150–160 amino acids. *CDH17* expression is regulated by CDX2 in the gastrointes-

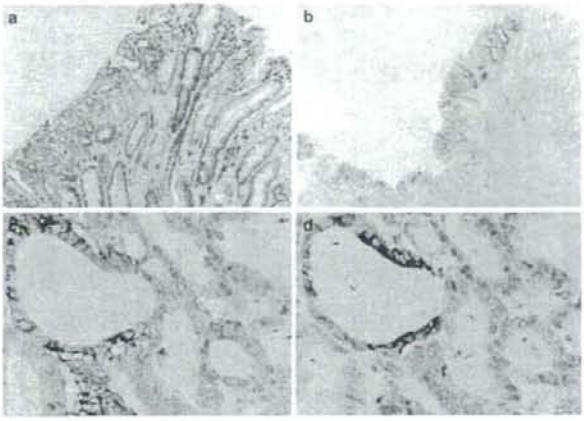

Figure 10 Immunostaining of claudin-18 in gastric cancer and its relation with mucin phenotype. (**a**,**b**) Claudin-18 expression is lost in MUC2-positive I-type gastric cancer, while (**c**,**d**) MUC5AC-positive G type had preserved claudin-18 expression. (**a**,**c**, claudin-18; **b**, MUC2; **d**, MUC5AC.)

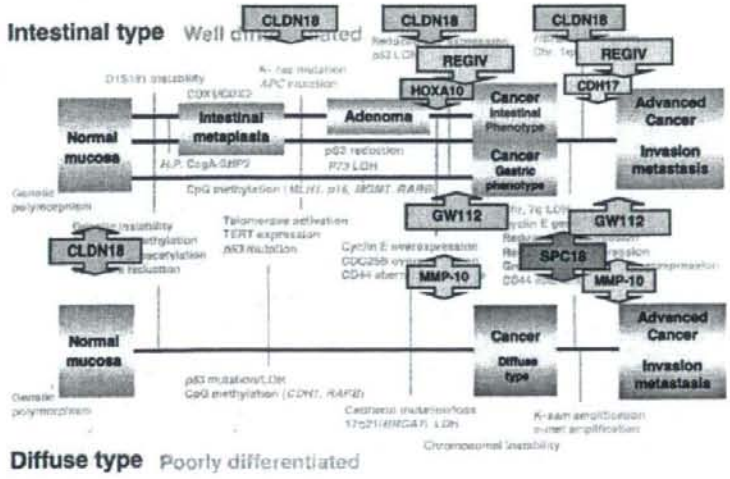

Figure 12 Role of novel genes and molecules identified on transcriptome dissection in stomach carcinogenesis.

tinal tract.[54] There are reports that *CDH17* expression is correlated with lymph node metastasis of gastric cancer.[74,75] A validation study of gastric cancer on quantitative RT-PCR and immunostaining confirmed that the expression of cadherin-17 was associated with depth of tumor invasion and a poor prognosis for the patient.[71] Furthermore, significant association was observed between cadherin-17 expression and CDX2 expression as well as MUC2-positive I type gastric cancer. We have recently found that EGF induced cadherin-17 expression in gastric cancer cells. Therefore, cadherin-17 may participate in the development and progression of gastric cancers of an intestinal phenotype, partly through the EGFR pathway.

Novel genes and stomach carcinogenesis

The role of genes and molecules identified on transcriptome dissection introduced here in stomach carcinogenesis is summarized in Fig. 12. Increased expression of Reg IV and HOXA10, and reduced expression of claudin-18 may participate in the development and differentiation of gastric cancer with an intestinal phenotype. Reg IV may be involved in late stages of carcinogenesis. In contrast, GW112 may play a role in the development and progression of histologically well-differentiated gastric cancer independently from Reg IV. SPC18 may participate in tumor growth and invasion regardless of histological type and mucin phenotype. Importantly, all

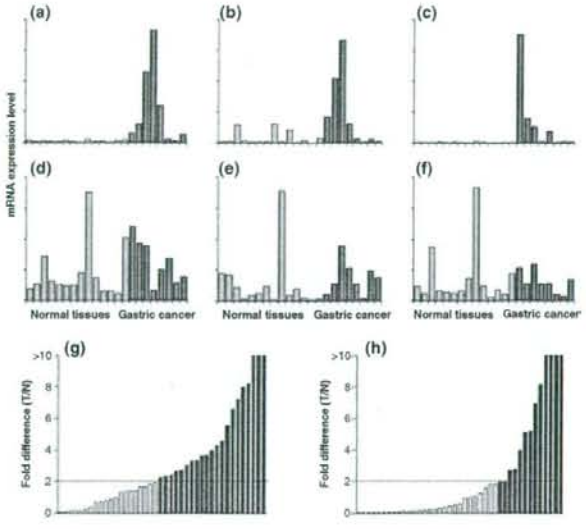

Figure 13 Representative results of quantitative reverse transcription–polymerase chain reaction of various normal tissues (brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, spleen, leukocytes) and gastric cancers for genes extracted by the *Escherichia coli* ampicillin trap (CAST) method. (**a**) Gene A; (**b**) gene B; (**c**) gene C; (**d**) gene D; (**e**) gene E; (**f**) gene F. (**g**,**h**) Ratio of each mRNA in gastric cancer (T) to that in corresponding non-neoplastic mucosa (N), (fold difference): (**g**) gene A: T/N > 2, 21/41 (51%); (**h**) gene C: T/N > 2, 13/41 (32%).

the genes and molecules identified on transcriptome dissection could be novel diagnostic and/or therapeutic targets.

NEW APPROACHES FOR IDENTIFYING MEMBRANE-BOUND OR SECRETED PROTEINS AS DIAGNOSTIC AND THERAPEUTIC TARGETS

Cell surface and secreted proteins are important both in basic science and clinical applications. These proteins, when overexpressed in cancer, are potential drug targets and tumor markers. We have been searching for such molecules using a novel signal sequence trap method called the *Escherichia coli* ampicillin trap (CAST) method, which was developed by Ferguson *et al.*[76] The principle is as follows: the mechanisms for protein translocation across prokaryotic and eukaryotic membranes are relatively conserved, and mammalian signal sequences can functionally replace those of prokaryotic genes. pCAST is a plasmid with a mutant *β-lactamase* lacking the endogenous signal peptide. A *Bam*HI site is placed upstream of, and in-frame with, the mutant *β-lactamase*, and an *Eco*RI site is included for directional cloning. When transformed with pCAST, bacteria do not grow on ampicillin-supplemented media. Survival on ampicillin is observed only when various cDNA fragments encoding a signal sequence have been inserted into this site in pCAST.

Random-primed cDNA libraries were generated from gastric cancer cell lines and normal gastric mucosal tissue, ligated into pCAST (kindly provided by Dr Jonathan Graff, University of Texas Southwestern Medical Center at Dallas), and >1000 of the randomly selected ampicillin-resistant clones were sequenced. These included many genes encoding secreted and transmembrane proteins. By comparing the list of identified genes from gastric cancer cell lines with normal gastric mucosa, candidates overexpressed in cancer were selected. The expression was then validated on quantitative RT-PCR in gastric cancer tissues and corresponding non-neoplastic gastric mucosa in the same way as the identification through SAGE data analysis (Fig. 13). It was found that several genes for secretory and membrane proteins were definitely overexpressed in gastric cancer, and should thus be studied for their function in protein expression and cancer. From these, it is expected that further novel diagnostic and therapeutic targets will be identified.

CONCLUSIONS

Transcriptome dissection is a detailed analysis of entire expressed transcripts in affected tissues, to facilitate an understanding of the precise molecular mechanisms of pathogenesis. Transcriptome dissection of gastric cancer through SAGE-based analysis uncovered several novel genes that were specifically upregulated or downregulated in gastric cancer. These include *Reg IV, GW112, SPC18, HOXA10, and CLDN18* and so on. The information obtained from transcriptome dissection greatly contributes to development of new tools for diagnosis, treatment and prevention of cancer. In a tide of translation from the discovery of seeds to the clinical stage, we believe that our approach is in the right direction of pathology, which is located in the center of diag-

nosis and which provides a bridge between genomic research, morphological analysis and clinics.

ACKNOWLEDGMENTS

The authors would like to thank Emeritus Professor Eiichi Tahara (Hiroshima University) for invaluable advice and continuous encouragement and Professor Hisao Ito (Tottori University) and Emeritus Professor Hiroki Kajihara (Hiroshima University) for their sincere support. The authors wish to thank Professor Hiroshi Yokozaki (Kobe University), Professor Hiroki Kuniyasu (Naka Medical University), and all former and present members of the Department of Molecular Pathology, Hiroshima University. Also, they are grateful to Professor Koki Inai, Professor Shinji Tanaka, Dr Yasuhiko Kitadai, Dr Koji Arihiro, Professor Takashi Takata and Professor Tetsuya Toge (Hiroshima University), Professor Kazuhiro Yoshida (Gifu University), Dr Kazuyoshi Yanagihara and Dr Hiroki Sasaki (National Cancer Center Research Institute), Dr Tsuyoshi Noguchi (Oita University), Dr Kei Nakachi (Radiation Effects Research Foundation) and all collaborators who kindly supported the research. The authors also thank members of Fujirebio and Mitsubishi Rayon for their technical assistance. This review article was prepared based on the data presented at the 97th Annual Meeting of the Japanese Society of Pathology, Kanazawa, 15 May 2008 by Wataru Yasui for the Japan Pathology Award. The experimental work presented here was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan for the Third Term Comprehensive 10 Year Strategy for Cancer Control and for Cancer Research, grants for medical research from the Tsuchiya Foundation, and a research grant from the Princess Takamatsu Cancer Research Fund.

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Characteristic gene expression in stromal cells of gastric cancers among atomic-bomb survivors

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Int. J. Cancer: **124,** 1112–1121 (2009)

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To elucidate the mechanism of radiation-induced cancers, molecular analysis of cancers in atomic-bomb survivors is important. In our study, we developed a custom oligonucleotide array of 208 genes. We analyzed gene expression profiles of gastric cancers (GCs) from atomic-bomb survivors and identified 9 genes with significantly lower expression in GCs from exposed patients than in GCs from nonexposed patients. Among these 9 genes, expression of versican and osteonectin was investigated in greater detail using immunohistochemistry in 116 GCs from 64 exposed and 52 nonexposed patients who developed GC after the bombing. In the Stage I/II GCs, the clinicopathologic, phenotypic and proliferative characteristics of GCs from exposed and nonexposed patients did not differ significantly; however, versican and osteonectin were expressed at much lower levels in the area of tumor-associated stroma of exposed patients than in nonexposed patients ($p = 0.026$ and $p = 0.024$, respectively). These results suggest that the characteristics of tumor-associated stromal cells differ between GCs from exposed and nonexposed patients.

Key words: gastric cancer; radiation carcinogenesis; atomic bomb; versican; osteonectin

More than 60 years have passed since atomic-bomb (A-bomb) exposure in Hiroshima and Nagasaki. A prospective cohort study [Life Span Study (LSS)] of 120,000 subjects has been conducted by the Radiation Effects Research Foundation (RERF).[1] Solid cancers, including breast, colon, lung and stomach, have a long latency period, and the excess relative risks of solid cancers remain high, specifically among those exposed when young.[1] Although approximately half of the LSS members have already deceased, cancer mortality in the LSS has continued to increase with the aging of this population, and it is anticipated to peak in 2015. Previous studies conducted to elucidate the mechanism of radiation-associated carcinogenesis mainly used formalin-fixed and paraffin-embedded archival tissues,[2,3] which are not suitable for selected molecular analyses (*e.g.* quantitative assays of gene expression) because of degradation of RNA.

According to the World Health Organization, gastric cancer (GC) is the fourth most common malignancy world wide, with ~870,000 new cases occurring yearly. Cancer develops as a result of multiple genetic and epigenetic alterations.[4,5] Better knowledge of changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment and prevention of GC. Thus far, the effect of radiation on GC development has been estimated on the basis of the LSS, in which both mortality and incidence were used as end points. The excess relative risks per gray (Gy) were 1.20 for mortality[6] and 1.32 for incidence.[1] Although several genetic alterations, including mutations in *TP53* and *BRAF*, have been reported in selected cancers of A-bomb survivors,[2,3,7] changes in gene expression have not been investigated. Furthermore, specific mutations for radiation-associated cancers have not been reported.

In our study, we performed custom array analysis of GCs from A-bomb survivors. We found reduced expression of versican and osteonectin in GCs from exposed patients. Versican, a large chondroitin sulfate proteoglycan, belongs to the aggrecan gene family.[8] Versican is a component of the extracellular matrix (ECM) of various soft tissues and is involved in a number of pathologic processes including cancer, atherosclerotic vascular diseases and so on.[9] Versican represses cell adhesion and promotes proliferation, migration, and invasion.[10,11] Increased stromal versican deposition correlates with breast cancer relapse and prostate cancer progression.[12,13] Osteonectin, a matricellular glycoprotein, modulates the interaction of cells with the ECM through its regulation of cell adhesion and matrix assembly.[14] Increased expression of osteonectin has been reported in several human cancers, and stromal osteonectin expression has been shown to correlates with tumor progression and poor survival.[15,16] Osteonectin enhances the invasive capacity of prostate and breast cancer cells.[17,18] Although overexpression of versican and osteonectin has been reported in GC,[19,20] the relationship with radiation exposure history of patients has not been studied. Therefore, we performed immunohistochemical analysis of versican and osteonectin expression in 116 GCs from A-bomb survivors.

Material and methods

Tissue samples

Primary tumor samples from 136 patients with GC were collected. Patients were treated at Hiroshima University Hospital (Hiroshima, Japan) or at an affiliated hospital. All patients underwent curative resection. Only patients who did not undergo preoperative radio- or chemotherapy and did not have clinical evidence of distant metastasis were enrolled in the study.

For use in our oligonucleotide array analysis, 3 freshly frozen GC tissue samples from exposed patients (5, 7 and 18 mGy) were obtained during surgery at the Department of Surgical Oncology, Hiroshima University Hospital, between 2004 and 2005. These patients were A-bomb survivors (3 LSS cohort members, RERF) in Hiroshima, Japan, who developed GC after the bombing. Their corresponding nonneoplastic mucosa samples were also available. In addition, we analyzed 20 freshly frozen GC tissue samples from nonexposed patients, who underwent surgery between 1991 and 1998 at the Department of Surgical Oncology, Hiroshima University Hospital; they were neither A-bomb survivors nor the LSS cohort members. Of these 20 GC samples, 10 corresponding nonneoplastic mucosa samples were available. All 20 GC samples were obtained during surgery at Hiroshima University Hospital.

Grant sponsors: Ministry of Education, Culture, Science, Sports, and Technology of Japan; Ministry of Health, Labour and Welfare of Japan.
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Received 18 January 2008; Accepted after revision 29 September 2008
DOI 10.1002/ijc.24060
Published online 9 October 2008 in Wiley InterScience (www.interscience.wiley.com).