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

Discussion

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

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

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

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

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

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

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

Conclusion

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

References

Baker EA, Leaper DJ (2003) The plasminogen activator and matrix metalloproteinase systems in colorectal cancer: relationship to tumour pathology. Eur J Cancer 39:981–988

Chen MJ, Chiou YY, Wu SL (2000) Lifestyle habits and gastric cancer in hospital-based case-control study in Taiwan. Am J Gastroenterol 95:3242–3249

Forget MA, Desrosiers RR, Beliveau R (1999) Physiological roles of matrix metalloproteinases: implications for tumor growth and metastasis. Can J Physiol Pharmacol 77:465–480

Furuya Y, Ichikura T, Mochizuki H (1999) Interleukin-1alpha concentration in tumors as a risk factor for liver metastasis in gastric cancer. Surg Today 29:288–289

Ghilardi G, Biondi ML, Mangoni J, Leviti S, DeMonti M, Guagnellini E, Scorza R (2001) Matrix metalloproteinase-1 promoter polymorphism 1G/2G is correlated with colorectal cancer invasiveness. Clin Cancer Res 7:2344–2346

Hewitt RE, Leach IH, Powe DG, Clark IM, Cawston TE, Turner DR (1991) Distribution of collagenase and tissue inhibitor of metalloproteinases (TIMP) in colorectal tumours. Int. J Cancer 49:666–672

Inoue T, Yashiro M, Nishimura S, Maeda K, Sawada T, Ogawa Y, Sowa M, Chung KH (1999) Matrix metalloproteinase-1 expression is a prognostic factor for patients with advanced gastric cancer. Int J Mol Med 4:73–77

Ito R, Kitadai Y, Kyo E, Yokozaki H, Yasui W, Yamashita U, Nikai H, Tahara E (1993) Interleukin 1 alpha acts as an autocrine growth stimulator for human gastric carcinoma cells. Cancer Res 53:4102–4106

Ito T, Ito M, Shiozawa J, Naito S, Kanematsu T, Sekine I (1999) Expression of the MMP-1 in human pancreatic carcinoma: relationship with prognostic factor. Mod Pathol 12:669–674

Japanese Gastric Cancer Association (1998) Japanese Classification of Gastric Carcinoma – 2nd English Edition. Gastric Cancer 1:10–24

Kanamori Y, Matsushima M, Minaguchi T, Kobayashi K, Sagae S, Kudo R, Terakawa N, Nakamura Y (1999) Correlation between expression of the matrix metalloproteinase-1 gene in ovarian cancers and an insertion/deletion polymorphism in its promoter region. Cancer Res 59:4225–4227

Kohn EC, Liotta LA (1995) Molecular insights into cancer invasion: strategies for prevention and intervention. Cancer Res 55:1856–1862

Lauren P (1965) The two histological main types of gastric carcinoma. Diffuse and so-called intestinal type carcinoma: an attempt at histological classification. Acta Pathol Microbiol Scand 64:31–49

Liotta LA, Steeg PS, Stetler Stevenson WG (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 64:327–336

Marbaix E, Donnez J, Courtoy PJ, Eeckhout Y (1992) Progesterone regulates the activity of collagenase and related gelatinases A and B in human endometrial explants. Proc Natl Acad Sci USA 89:11789–11793

McDonnel S, Matrisian LM (1991) Stromelysin in tumor progression and invasion. Cancer Metastasis Rev 9:305–319

Migita T, Sato E, Saito K, Mizoi T, Shiiba K, Matsuno S, Nagura H, Ohtani H (1999) Differing expression of MMPs-1 and -9 and urokinase receptor between diffuse- and intestinal-type gastric carcinoma. Int J Cancer 84:74–79

Murray GI, Duncan ME, Arbuckle E, Melvin WT, Fothergill JE (1998a) Matrix metalloproteinases and their inhibitors in gastric cancer. Gut 43:791–797

Murray GI, Duncan ME, O'Neil P, Mckay JA, Melvin WT, Fothergill JE (1998b) Matrix metalloprotease-1 is associated with poor prognosis in oesophageal cancer. J Pathol 185:256–261

Nishioka Y, Kobayashi K, Sagae S, Ishioka S, Nishikawa A, Matsushima M, Kanamori Y, Minaguchi T, Nakamura Y, Tokino T, Kudo R (2000) A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter in endometrial carcinomas. Jpn J Cancer Res 91:612–615

Ochiai A, Yasui W, Tahara E (1985) Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. Jpn J Cancer Res 76:1064–1071

Otani Y, Kubota T, Sakurai Y, Igarashi N, Yokoyama T, Kimata M, Wada N, Kameyama K, Kumai K, Okada Y, Kitajima M (1999) Expression of matrix metalloproteinases in

gastric carcinoma and possibility of clinical application of matrix metalloproteinase inhibitor in vivo. Ann N Y Acad Sci 30:541–543
Park CC, Bissell MJ, Barcellos-Hoff MH (2000) The influence of the microenvironment on the malignant phenotype. Mol Med Today 6:324–329
Rutter JL, Mitchell TI, Buttice G, Meyers J, Gusella JF, Ozelius LJ, Brinckerhoff CE (1998) A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. Cancer Res 58:321–5325
Schneikert J, Peterziel H, Defossez PA, Klocker H, Launoit Y, Cato ACB (1996) Androgen receptor-Ets protein interaction is a novel mechanism for steroid hormone-mediated down-modulation of matrix metalloproteinase expression. J Biol Chem 271:23907–23913
Singer CF, Marbaix E, Kokorine I, Lemoine P, Donnez J, Eeckhout Y, Courtoy PJ (1997) Paracrine stimulation of interstitial collagenase (MMP-1) in the human endometrium by interleukin 1alpha and its dual block by ovarian steroids. Proc Natl Acad Sci USA 94:10341–10345
Sobin LH, Wittekind CH (eds) (2002) TNM classification of malignant tumors, 6th edn. Wiley-Liss, New York, pp 65–68
Stelzner S, Emmrich P (1997) The mixed type in Lauren's classification of gastric carcinoma. Histologic description and biologic behavior. Gen Diagn Pathol143:39–48
Templeton NS, Brown PD, Levy AT, Margulies IM, Liotta LA, Stetler-Stevenson WG (1990) Cloning and characterization of human tumor cell interstitial collagenase. Cancer Res 50:5431–5437
Tomimatsu S, Ichikura T, Mochizuki H (2001) Significant correlation between expression of interleukin-1alpha and liver metastasis in gastric carcinoma. Cancer 91:1272–1276
Wyatt CA, Coon CI, Gibson JJ, Brinckerhoff CE (2002) Potential for the 2G single nucleotide polymorphism in the promoter of matrix metalloproteinase to enhance gene expression in normal stromal cells. Cancer Res 62:7200–7202
Ye S, Dhillon S, Turner SJ, Bateman AC, Theaker JM, Pickering RM, Day I, Howell WM (2001) Invasiveness of cutaneous malignant melanoma is influenced by matrix metalloproteinase 1 gene polymorphism. Cancer Res 61:1296–1298
Yokozaki H (2000) Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathol Int 50:767–777
Zhu Y, Spitz MR, Lei L, Mills GB, Wu X (2001) A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter enhances lung cancer susceptibility. Cancer Res 61:7825–7829

Review Article

Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications

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Gastric cancer is one of the most common human cancers and is the second most frequent cause of cancer-related death in the world. Serial analysis of gene expression (SAGE) is a powerful technique to allow genome-wide analysis of gene expression in a quantitative manner without prior knowledge of the gene sequences. SAGE on 5 samples of gastric cancer with different histology and clinical stages have created large SAGE libraries of gastric cancer that enable us to identify new cancer biomarkers. Commonly up-regulated genes in gastric cancer in comparison with normal gastric epithelia included *CEACAM6*, *APOC1* and *YF13H12*. By comparing gene expression profiles of gastric cancers at early and advanced stages, several genes differentially expressed by tumor stage were also identified, including *FUS*, *CDH17*, *COL1A1* and *COL1A2*, which should be novel genetic markers for high-grade malignancy. Regenerating gene type IV (*REGIV*) is one of the most up-regulated genes in a SAGE library of a scirrhous-type gastric cancer. *In vitro* studies using RegIV-transfected cells revealed that RegIV is secreted by cancer cells and inhibits apoptosis, suggesting that RegIV may serve as a novel biomarker and therapeutic target for gastric cancer. Production of RNA aptamers could be a useful approach to establish a detection system in blood. A custom-made array, named Ex-STOMACHIP, consisting of 395 genes, including highly differentially expressed genes identified by our SAGE and other known genes related to carcinogenesis and chemosensitivity, is useful to study the molecular pathogenesis of gastric cancer and to obtain information about biological behavior and sensitivity to therapy in the clinical setting. Combined analyses of gene expression profile, genetic polymorphism and genetic instability will aid not only cancer detection, but also characterization of individual cancers and patients, leading to personalized medicine and cancer prevention. (Cancer Sci 2004; 95: 385–392)

According to the World Health Organization, gastric cancer is the fourth most common malignancy in the world, with some 870,000 new cases every year, and mortality from gastric cancer is second only to lung cancer.[1] The incidence of gastric cancer is declining worldwide. This trend is mainly due to decreased consumption of salt-preserved food, avoidance of high-salt diet and availability of fresh fruit and vegetables throughout the year. Another reason for the high incidence of gastric cancer in Japan is a high rate of *Helicobacter pylori* infection among Japanese. *Helicobacter* causes chronic active or atrophic gastritis and intestinal metaplasia, which are believed to be precancerous or predisposing conditions for gastric cancer. Advances in diagnosis and treatment have resulted in excellent long-term survival for patients with early cancer, but the prognosis of advanced cancer remains poor.

Cancer is a chronic proliferative disease with multiple genetic and epigenetic alterations; that is, it is a disease with altered gene expression. Integrated research in molecular pathology over the past 15 years has uncovered many of the molecular mechanisms of the development and progression of gastric cancer.[2–6] Genetic polymorphism is an important endogenous cause and a fundamental factor influencing cancer risk. Genetic instability, DNA hypermethylation and histone hypoacetylation are early events. Multiple alterations accumulate, including inactivation of tumor suppressor genes, activation of oncogenes and abnormalities of cell cycle regulators and growth factors. Some of these changes occur commonly in both differentiated and undifferentiated types and some differ depending on the histological type. A better knowledge of changes in gene expression during stomach carcinogenesis may lead to new paradigms and possible improvements in cancer diagnosis, treatment and prevention. Although several large-scale gene expression studies using microarray techniques have been performed on gastric cancer,[7–9] they have utilized different platforms that varied in the number and identity of the genes printed on them. Besides the microarray technique, serial analysis of gene expression (SAGE) is a powerful technique for global analysis of gene expression in a quantitative manner without prior knowledge of the gene sequences.[10]

This review presents an outline of our approach to search for new genes of gastric cancer through SAGE and discusses its implications for diagnosis, treatment and prevention.

Advantage of SAGE in global analysis of gene expression

SAGE is based on the following two principles.[10] First, a short nucleotide sequence tag (about 10 base pairs) contains sufficient information to uniquely identify a transcript, provided it is isolated from a defined position within the transcript. Second, concentration of short sequence tags allows the efficient analysis of transcripts in a serial manner by the sequencing of multiple tags within a single clone. SAGE analyzes tags of about 10 bp derived from a defined position, near the polyA tail of the cDNAs, downstream of the CATG sequence. Because the SAGE tag numbers directly reflect the abundance of the mRNAs, SAGE data are highly accurate and quantitative. Completion of the human genome sequence has facilitated the mapping of specific genes to individual tags. Large numbers of normal and tumor tissues and cells have been analyzed by SAGE, creating large databases. Now, database including about 250 SAGE libraries are online and available to the public. Since the database contains accurate distribution and frequency data of

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the genes and tags, we can study the expression of genes of interest in other tissues in the database by Virtual northern analysis.[11] Molecular characterization has been performed by SAGE in cancers of the lung, breast, colon, ovary and prostate, tumor endothelium, and other organs with the aim of developing diagnostic and therapeutic strategies.[12–16] Although SAGE is thus useful for global analysis of gene expression and to identify genes up-regulated or down-regulated in tissues and cells of interest, it is not suitable to study gene expression in large numbers of cases because at least 10,000 tags should be sequenced to obtain a SAGE library. To verify whether gene expression data obtained by SAGE reflect general phenomena, real-time RT-PCR or other methods must be combined.

Strategy to search for novel genes associated with gastric cancer by SAGE and its clinical implication

Our strategy to search for novel biomarkers using SAGE and to apply the results to clinical diagnostics, treatment and prevention is shown in Fig. 1. First, we perform SAGE on typical gastric cancer tissues, compare gene expression profiles among them or with those in normal gastric tissue and identify specifically up-regulated or down-regulated genes. The expression of these genes is confirmed in large numbers of cases by real-time RT-PCR and immunohistochemistry if antibodies are available. With the specific genes identified by SAGE, known genes participating in the development and progression of gastric cancer and known genetic markers for chemosensitivity, we prepare a custom-made cDNA microarray. If a specific gene encodes a secretory protein, this may be detected in the blood and should be a novel biomarker of gastric cancer. For such a molecule, we produce an RNA aptamer or antibody and establish a measuring system such as ELISA for blood. Genetic polymorphism is an important determinant among endogenous causes of cancer. The majority of genetic variation between individual humans is believed to be due to single nucleotide polymorphisms (SNPs), and 1% of all SNPs results in functional variation in proteins and alters cancer predisposition.[17,18] Polymorphisms of genes whose expression is highly altered in cancer may be candidates for novel risk factors, and this information will be useful for cancer prevention. By functional analysis, we can understand the molecular mechanisms of stomach carcinogenesis in more detail and determine whether the genes can be novel therapeutic targets.

Generation of gene expression profiles of gastric cancer by SAGE

So far, three SAGE studies of gastric cancer have been reported that identified several up-regulated and down-regulated genes.[19–21] Up-regulated genes include *S100A* calcium-binding protein family and *TFF3*. However, the reported studies examined only one[19] or two samples[20,21] of gastric cancer. We have performed SAGE analysis on 5 samples of gastric cancer of different stages and histologies from 4 patients.[22] They included early and advanced cancers of well-differentiated type (tubular adenocarcinoma of well-differentiated type; tub1), primary and metastatic tumors of poorly differentiated adenocarcinoma of solid type (por1) and one poorly differentiated adenocarcinoma of non-solid type (por2; scirrhous) gastric cancer. Histological classification was made according to the Japanese Classification of Gastric Cancer.[23] SAGE was carried out according to SAGE protocol version 1.0e, June 23, 2000. Tags were extracted from the raw sequence data with SAGE2000 analysis software version 4.12, kindly provided by Dr. Kenneth W. Kinzler (Johns Hopkins University School of Medicine). Our SAGE generated a total of 137,706 tags including 38,903 unique tags. Our SAGE libraries are 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) (http://www.ncbi.nlm.nih.gov/SAGE/). Besides our libraries, five other SAGE libraries of gastric tissue are available from SAGEmap. Those include two gastric cancers (GSM757, SAGE_gastric_cancer-G234 and GSM2385, SAGE_gastric_cancer-G189) and normal gastric epithelia (GSM874, SAGE_normal_gastric_body_epithelia).[19] As already mentioned, SAGE is useful for comprehensive gene expression analysis and an SAGE database contains accurate data on the distribution and frequency of tags, so we can directly

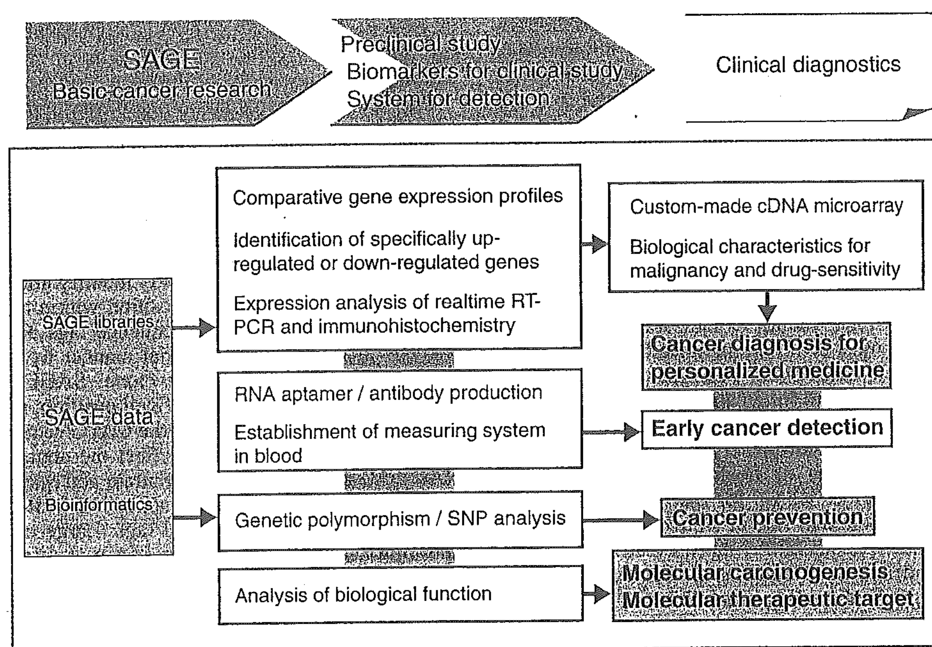

Fig. 1. Strategy to search for novel genes associated with gastric cancer through SAGE, and its clinical implication.

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

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

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

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

Identification of gastric cancer-specific genes by SAGE

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

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

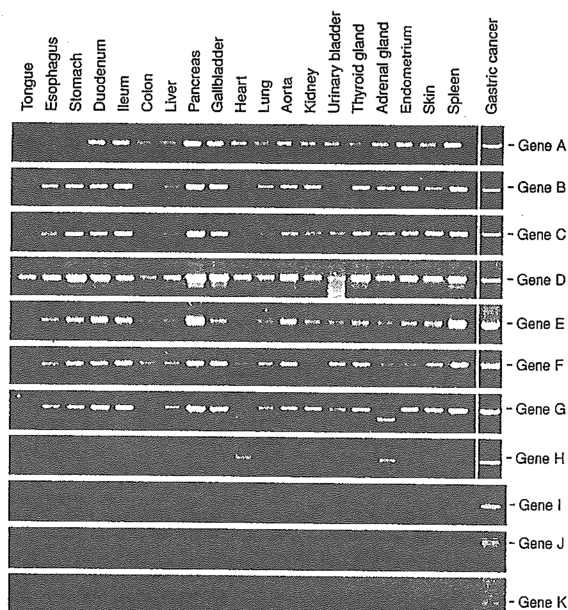

Fig. 2. Expression of genes detected in SAGE libraries of gastric cancer, but not in the libraries of 15 normal tissues. Semi-quantitative RT-PCR on various normal human tissues revealed that genes H, I, J and K are not expressed significantly in normal tissues, whereas they are detected in gastric cancer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

Identification of genes involved in invasion and metastasis by SAGE

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

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

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

Candidate novel biomarkers of gastric cancer

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

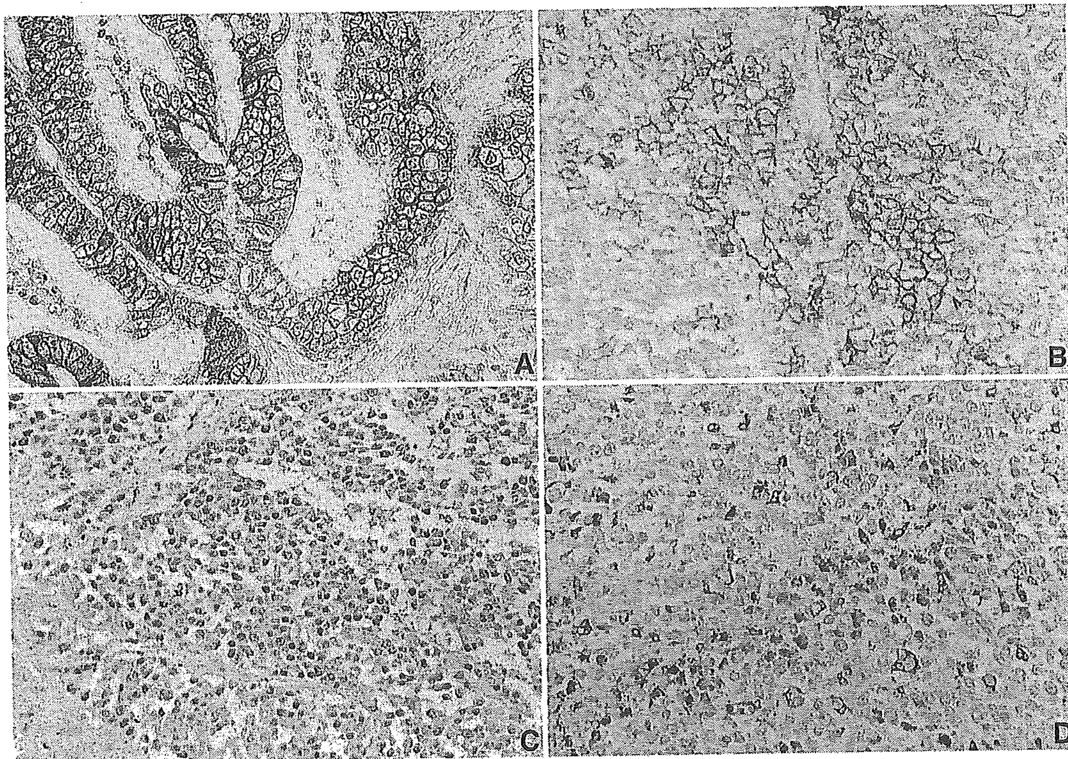

Fig. 3. Expression of cadherin 17 and apolipoprotein E in gastric carcinomas (immunohistochemical analysis). Cadherin 17 is expressed at the cell surface and cell-cell border of well-differentiated-type gastric adenocarcinoma with intestinal phenotype (A) and poorly differentiated solid-type gastric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

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

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

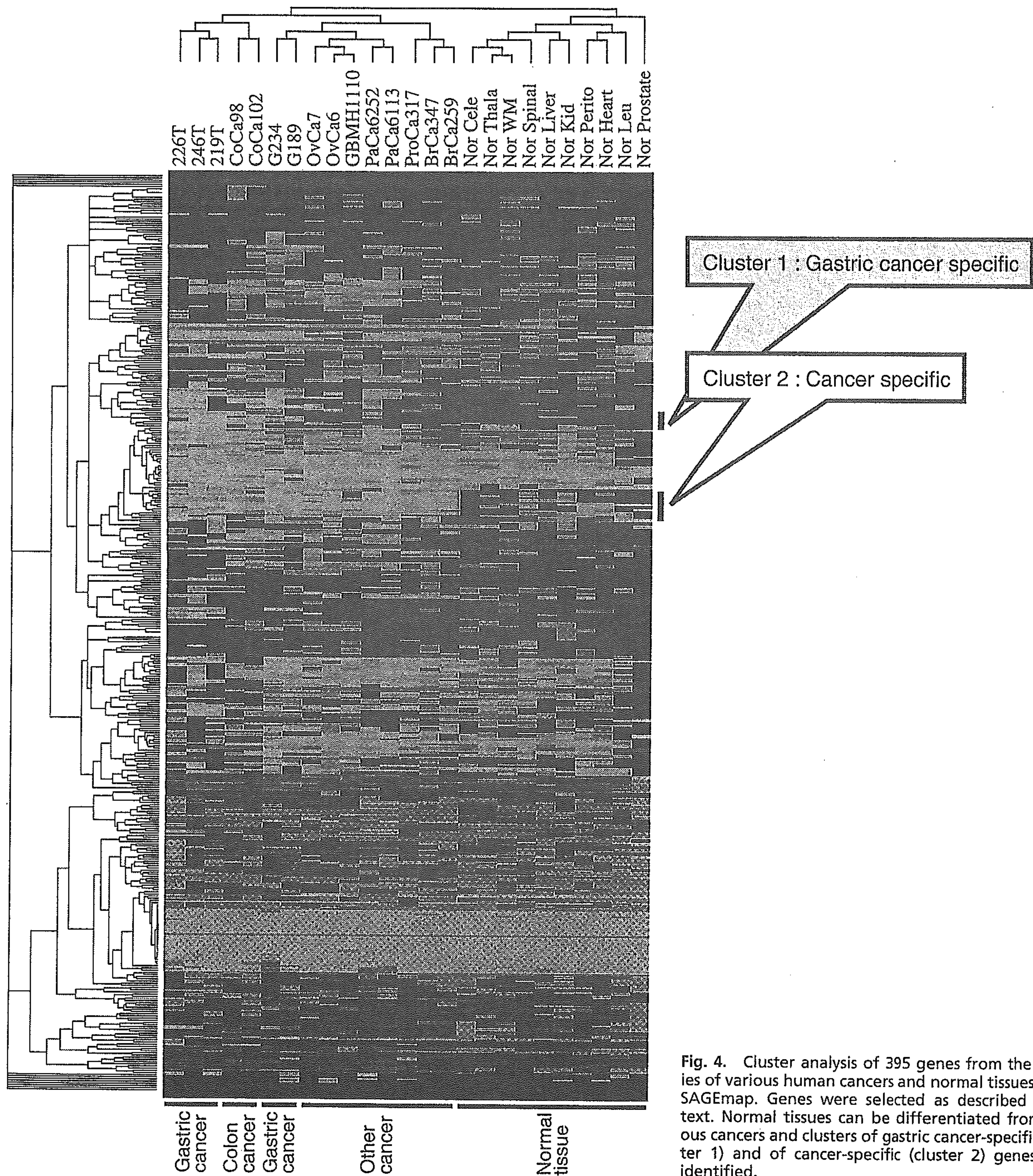

Fig. 4. Cluster analysis of 395 genes from the libraries of various human cancers and normal tissues in the SAGEmap. Genes were selected as described in the text. Normal tissues can be differentiated from various cancers and clusters of gastric cancer-specific (cluster 1) and of cancer-specific (cluster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

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

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

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

New strategy of gene diagnosis of gastric cancer

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

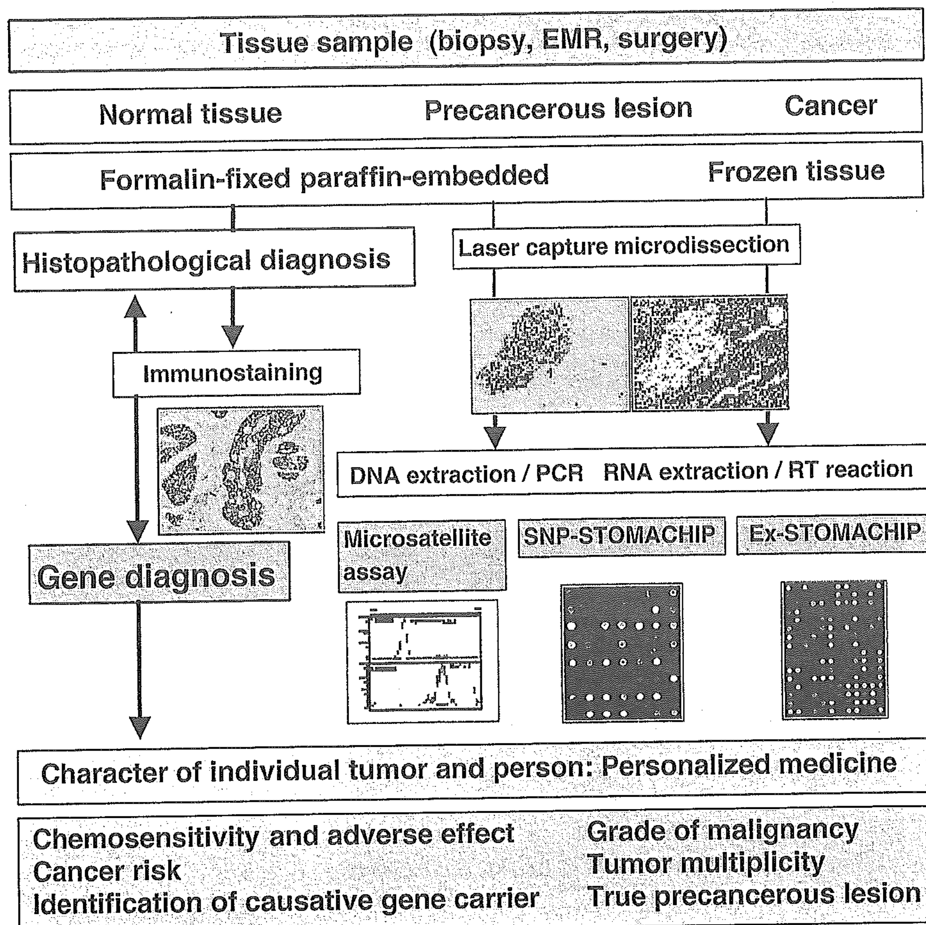

Fig. 5. New strategy of gene diagnosis of gastric cancer using pathology samples.

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

Conclusion

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

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1. Ohgaki H, Matsukura N. Stomach cancer. In: Stewart BW, Kleihues P, editors. World cancer report. Lyon: IARC Press; 2003. p. 197–7.
2. Tahara E. Molecular mechanism of stomach carcinogenesis. *J Cancer Res Clin Oncol* 1993; **119**: 265–72.
3. Yasui W, Oue N, Kuniyasu H, Ito R, Tahara E, Yokozaki H. Molecular diagnosis of gastric cancer: present and future. *Gastric Cancer* 2001; **4**: 113–21.
4. Ohgaki H, Yasui W, Yokota J. In: Vainio H, Hietanen E, editors. Handbook of experimental pharmacology. Mechanisms in carcinogenesis and cancer research. Heidelberg: Springer-Verlag; 2003. p. 25–39.
5. Yokozaki H, Yasui W, Tahara E. Genetic and epigenetic changes in stomach cancer. *Int Rev Cytol* 2001; **204**: 49–95.
6. Yasui W, Oue N, Ono S, Mitani Y, Ito R, Nakayama H. Histone acetylation and gastrointestinal carcinogenesis. *Ann N Y Acad Sci* 2003; **983**: 220–31.
7. El-Rifai W, Frierson HF Jr, Harper JC, Powell SM, Knuutila S. Expression profiling of gastric adenocarcinoma using cDNA array. *Int J Cancer* 2001; **92**: 832–8.
8. Hasegawa S, Furukawa Y, Li M, Satoh S, Kato T, Watanabe T, Katagiri T, Tsunoda T, Yamaoka Y, Nakamura Y. Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. *Cancer Res* 2002; **62**: 7–12.
9. Hippo Y, Taniguchi H, Tsutsumi S, Machida N, Chong JM, Fukayama M, Kodama T, Aburatani H. Global gene expression analysis of gastric cancer by oligonucleotide microarrays. *Cancer Res* 2002; **62**: 233–40.
10. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995; **270**: 484–7.
11. Argani P, Rosty C, Reiter RE, Wilentz RE, Murugesan SR, Leach SD, Ryu B, Skinner HG, Goggins M, Jaffee EM, Yeo CJ, Cameron JL, Kern SE, Hruban RH. Discovery of new markers of cancer through serial analysis of gene expression: prostate stem cell antigen is overexpressed in pancreatic adenocarcinoma. *Cancer Res* 2001; **61**: 4320–4.
12. Nacht M, Dracheva T, Gao Y, Fujii T, Chen Y, Player A, Akmaev V, Cook B, Dufault M, Zhang M, Zhang W, Guo M-Z, Curran J, Han S, Sidranbsky D, Buetow K, Madden SL, Jen J. Molecular characteristics of non-small cell lung cancer. *Proc Natl Acad Sci USA* 2001; **98**: 15203–8.
13. Porter DA, Krop IE, Nasser S, Sgroi D, Kaelin CM, Marks JR, Riggins G, Polyak K. A SAGE (serial analysis of gene expression) view of breast tumor progression. *Cancer Res* 2001; **61**: 5697–702.
14. Buckhaults P, Rago C, St Croix B, Romans KE, Saha S, Zhang L, Vogelstein B, Kinzler KW. Secreted and cell surface genes expressed in benign and malignant colorectal tumors. *Cancer Res* 2001; **61**: 6996–7001.
15. Hough CD, Sherman-Baust CA, Pizer ES, Montz FJ, Im DD, Rosenshein NB, Cho KR, Riggins GJ, Morin PJ. Large-scale serial analysis of gene expression revealed gene differentially expressed in ovarian cancer. *Cancer Res* 2001; **61**: 3869–76.
16. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW. Gene expressed in human tumor endothelium. *Science* 2000; **289**: 1197–202.
17. The International SNP Map Working Group. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 2001; **409**: 92841.
18. Gonzalez CA, Sala N, Capella G. Genetic susceptibility and gastric cancer risk. *Int J Cancer* 2002; **100**: 249–60.
19. El-Rafai W, Moskaluk CA, Abdrabbo MK, Harper J, Yoshida C, Riggins GJ, Frieson HF Jr, Powell SM. Gastric cancers overexpress S100A calcium-binding proteins. *Cancer Res* 2002; **62**: 6823–6.
20. Oien KA, Vass JK, Downie I, Fullarton G, Keith WN. Profiling, comparison and validation of gene expression in gastric carcinoma and normal stomach. *Oncogene* 2003; **22**: 4287–300.
21. Lee J-Y, Eom E-M, Kim D-S, Ha-Lee YM, Lee D-H. Analysis of gene expression profiles of gastric normal and cancer tissues by SAGE. *Genomics* 2003; **82**: 78–85.
22. Oue N, Hamai Y, Mitani Y, Matsumura S, Oshimo Y, Aung PP, Kuraoka K, Nakayama H, Yasui W. Gene expression profile of gastric carcinoma: identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. *Cancer Res* 2004; **64**: 2397–405.
23. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma. 13th ed. Tokyo: Kanehara Publishers; 1999.
24. Yu J, Hwang PM, Rago C, Kinzler KW, Vogelstein B. Identification and classification of p53-regulated genes. *Proc Natl Acad Sci USA* 1999; **96**: 14517–22.
25. International Union Against Cancer. TNM classification of malignant tumors. 6th ed. New York: Wiley-Liss; 2002.
26. Hicks GG, Singh N, Nashabi A, Mai S, Bozek G, Klewes L, Arapovic D, White EK, Koury MJ, Oltz EM, Van Kaer L, Ruley HE. Fus deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and perinatal death. *Nat Genet* 2000; **24**: 175–9.
27. Grotzinger C, Kneifel J, Patschan D, Schnoy N, Anagnostopoulos I, Faiss S, Tauber R, Wiedenmann B, Gessner R. LI-cadherin: a marker of gastric metaplasia and neoplasia. *Gut* 2001; **49**: 73–81.
28. Hartupee JC, Zhang H, Bonaldo MF, Soares MB, Dieckgraefe BK. Isolation and characterization of a cDNA encoding a novel member of the human generating protein family. *Biochim Biophys Acta* 2001; **1518**: 287–93.
29. Violette S, Festor E, Pandrea-Vasile I, Mitchell V, Adida C, Dussaulx E, Lacorte JM, Chambaz J, Lacasa M, Lesuffleur T. Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. *Int J Cancer* 2003; **103**: 185–93.
30. Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin Chem* 1999; **45**: 1628–50.
31. Cerchia L, Hamm J, Libri D, Tavitian B, de Franciscis V. Nucleic acid aptamers in cancer medicine. *FEBS Lett* 2002; **528**: 12–6.

32. Inoue H, Matsuyama A, Mimori K, Ueo H, Mori M. Prognostic score of gastric cancer determined by cDNA microarray. *Clin Cancer Res* 2002; **8**: 3475–9.
33. Zembutsu H, Ohnishi Y, Tsunoda T, Furukawa Y, Katagiri T, Ueyama Y, Tamaoki N, Nomura T, Kitahara O, Yanagawa R, Hirata K, Nakamura Y. Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. *Cancer Res* 2002; **62**: 518–27.
34. Watters JW, McLeod HL. Cancer pharmacogenomics: current and future applications. *Biochim Biophys Acta* 2003; **1603**: 99–111.
35. Sawiris GP, Sherman-Baust CA, Becker KG, Cheadle C, Teichberg D, Morin PJ. Development of a highly specialized cDNA array for the study and diagnosis of epithelial ovarian cancer. *Cancer Res* 2002; **62**: 2923–8.
36. Yasui W, Yokozaki H, Shimamoto F, Tahara H, Tahara E. Molecular-pathological diagnosis of gastrointestinal tissues and its contribution to cancer histopathology. *Pathol Int* 1999; **49**: 763–74.

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FREQUENT EPIGENETIC INACTIVATION OF *RIZ1* BY PROMOTER HYPERMETHYLATION IN HUMAN GASTRIC CARCINOMA

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The retinoblastoma protein-interacting zinc finger gene, *RIZ1* (GenBank accession number U17838), is involved in chromatin-mediated gene expression and is also a target for frameshift mutation in microsatellite-unstable cancers. Methylation of the *RIZ1* promoter CpG island has been shown to be a common mechanism in inactivating the *RIZ1* gene in human liver and breast cancers. We investigated levels of *RIZ1* mRNA in 45 gastric carcinoma tissues by quantitative RT-PCR and in gastric carcinoma cell lines by RT-PCR. In addition, we examined CpG island methylator phenotype (CIMP) status, *p53* mutation status, and the correlation between promoter methylation status and *RIZ1* mRNA expression. CIMP status was investigated by examining the methylation status of *MINT1*, *MINT2*, *MINT12*, *MINT25* and *MINT31*. *p53* mutation status was examined by PCR-single strand conformation polymorphism and promoter methylation status was examined by methylation-specific PCR. Promoter hypermethylation of the *RIZ1* gene was found in 31 (69%) of 45 gastric carcinoma tissues and in 3 (21%) of 14 corresponding non-neoplastic mucosae, the incidence being significantly different ($p = 0.002$). None of the 12 normal gastric tissues from young non-cancer individuals showed hypermethylation. Promoter hypermethylation was associated with reduced *RIZ1* expression in gastric carcinoma tissues ($p = 0.029$). Promoter hypermethylation of the *RIZ1* gene was significantly associated with CIMP ($p = 0.002$). Mutation status of the *p53* gene was not associated with methylation status or *RIZ1* expression in gastric carcinoma. In gastric carcinoma cell lines MKN-28 and KATO-III, the *RIZ1* promoter was hypermethylated and *RIZ1* transcription was inactive. Treatment of these cells with demethylating agent 5-aza-2'-deoxycytidine restored *RIZ1* transcription. Our results suggest that transcriptional inactivation of the *RIZ1* gene by promoter hypermethylation may participate in stomach carcinogenesis.
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Key words: *DNA methylation; gastric carcinoma; RIZ1; CIMP; p53*

Epigenetic mechanisms, including DNA methylation and alteration of chromatin structure, are important ways to silence many genes, especially defective tumor suppressor genes, involved in human cancers.[1,2] Recent studies have shown that promoter hypermethylation is a crucial mechanism in transcriptional silencing of tumor suppressor genes in gastric cancer.[3–13] We also showed that DNA methylation occurs for *MGMT*,[14] *p16^{INK4a}*, *RAR-beta*, *CDH1*,[15] *TSP1*,[16] *HLTF*,[17] and *cyclin D2*[18] in gastric carcinomas.

The retinoblastoma protein-interacting zinc finger gene *RIZ* was isolated with a functional screen for Rb-binding protein.[19] Domain analysis suggests that *RIZ1* is a putative methyltransferase. The PR (PRDI-BF1 and RIZ)/SET (Suvar3-9, Enhancer of zeste, Trithorax) domain is involved in chromatin-mediated gene expression[19,20] and plays an important role in human cancers as evidenced by genetic mutations of several family members.[21] The *RIZ* gene produces 2 mRNA and protein products through alternative promoters. *RIZ1* contains the PR domain, but *RIZ2* lacks this domain.[22] The *RIZ* gene is located on human chromosome 1p36, a region frequently deleted in many human cancers, including gastric cancer.[23,24] Expression of *RIZ1* but not *RIZ2* is frequently silenced in many human cancers, including carcinomas of the breast, colon and liver.[25–27] The *RIZ* gene is also a target for frameshift mutations in microsatellite-unstable cancers of the colon, stomach, endometrium and pancreas.[27–29] Missense mutations of *RIZ1* are common in human diffuse large B cell lymphoma but not in other tumors, including gastric carcinoma.[30] *RIZ1* is considered to be a tumor suppressor gene because *RIZ1* can induce G_2-M arrest and apoptosis in breast cancer, liver cancer and microsatellite-unstable colon cancers.[25–27,31] Moreover, a knockout study showed that *RIZ1* is a tumor susceptibility gene in mice.[30] *RIZ1* and *p53* deficiencies are likely to cooperate in tumor formation in mice and are expected to occur in human cancers as well.[30] Many sporadic human cancers carry both *p53* mutation and silenced *RIZ1* gene.[25,30] Recently, methylation of the *RIZ1* promoter CpG island has been shown to be a common mechanism in inactivating the *RIZ1* gene in human liver and breast cancers.[32] Although frameshift mutations of *RIZ* have been found in some microsatellite-unstable gastric cancers,[28,29] little is known about correlation between *RIZ1* expression, and *RIZ1* methylation status, clinicopathological features and *p53* mutation status in gastric carcinoma.

Gastric carcinomas frequently have CpG island methylator phenotype.[33] Another common phenotype after the CpG island methylator phenotype (CIMP) is CIMP in bracelets. These gastric carcinomas, designated for the CIMP-positive, show methylation at more than 3–5 loci (*methylated in tumors* [*MINT*]*1*, *MINT2*, *MINT12*, *MINT25* and *MINT31*). CIMP-positive gastric carcinomas are frequently associated with promoter methylation of *p16^{INK4a}*[33] and *hMLH1*,[34] suggesting that CIMP is an important pathway involved in stomach carcinogenesis. Association between promoter hypermethylation of *RIZ1* and CIMP was found in colon carcinoma,[35] although it remains unclear in gastric carcinoma.

We investigated promoter methylation status and expression levels of the *RIZ1* gene in primary gastric carcinoma tissues as well as corresponding non-neoplastic mucosa and gastric carcinoma cell lines. We also examined promoter methylation status of the *RIZ1* gene in normal gastric mucosa obtained endoscopically from young healthy individuals to investigate whether methylation of the *RIZ1* gene is associated with aging.[1,2,36,37] To determine whether transcriptional silencing of the *RIZ1* gene is caused by promoter hypermethylation, we compared the methylation status

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with mRNA expression levels of the *RIZ1* gene in 8 gastric carcinoma cell lines and 45 gastric carcinoma tissues. We compared methylation status and the expression levels of the *RIZ1* gene to clinicopathological features. We also examined the relation of *RIZ1* methylation status with CIMP status and *p53* mutation status.

MATERIAL AND METHODS

Cell lines

Eight cell lines derived from human gastric carcinomas were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma.[38] Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7; MKN-28; MKN-74, well differentiated adenocarcinoma; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki. KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinomas, were kindly provided by Dr. M. Sekiguchi and Dr. K. Yanagihara, respectively. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% FBS (Whittaker, Walkersville, MA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Drug treatment

Cells were treated with a final concentration of 1 μM 5-aza-2′-deoxycytidine (Aza-dC, Sigma Chemical Co., Tokyo, Japan) for 5 days or with 300 nM of Trichostatin A (TSA, Sigma Chemical Co.) for 1 day before cells were harvested for RNA extraction.

Tissue samples

Forty-five gastric carcinoma tissue specimens from 45 patients and 14 corresponding non-neoplastic mucosae were analyzed for promoter methylation of *RIZ1*. Total RNA was available for 45 pairs of gastric carcinomas and corresponding non-neoplastic mucosae to study expression of *RIZ1*. Tumors and corresponding non-neoplastic mucosae were surgically removed, immediately frozen in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the tumor tissue specimens consisted mainly of carcinoma tissue and that non-neoplastic mucosa did not exhibit any tumor cell invasion or show significant inflammatory involvement. Histological classification was carried out according to the Lauren classification system.[39] Diffuse-type gastric carcinomas were further classified into diffuse-adherent and diffuse-scattered subtypes.[40] In addition, gastric carcinomas were classified into 2 types: carcinoma with either intestinal or diffuse-type components (pure type) and carcinoma with coexistence of both types of components (mixed type).[41] Tumor staging was carried out according to the TNM stage grouping.[42] In addition, we examined methylation status of the *RIZ1* gene in 12 samples of normal gastric mucosae obtained endoscopically from 12 healthy young individuals (age range = 22–35 years; average = 25.4 years) with no clinical symptoms and no microscopic changes.

Bisulfite PCR and methylation-specific PCR

To examine the DNA methylation patterns of the promoters, genomic DNA was treated with sodium bisulfite as described previously.[43] A total of 2 μg of genomic DNA was denatured by treatment with 2 M NaOH and modified with 3 M sodium bisulfite for 16 hr. DNA samples were purified with Wizard DNA purification resin (Promega, Madison, WI), treated with 3 M NaOH, precipitated with ethanol, and resuspended in 25 μl water. Two-microliter aliquots were used as templates for PCR reactions. For analysis of DNA methylation of the *RIZ1* promoter (Fig. 1*a*), MSP was carried out with primers for *RIZ1* promoters as described previously.[32] For analysis of DNA methylation of *MINT1*, *MINT2*, *MINT12*, *MINT25*, and *MINT31*, we carried out bisulfite-PCR and then restriction digestion as described previously.[33] PCR products (15 μg) were loaded onto 8% nondenaturing polyacrylamide gels, stained with ethidium bromide and visualized under UV light. We considered cases with methylation at more than 3 of 5 loci

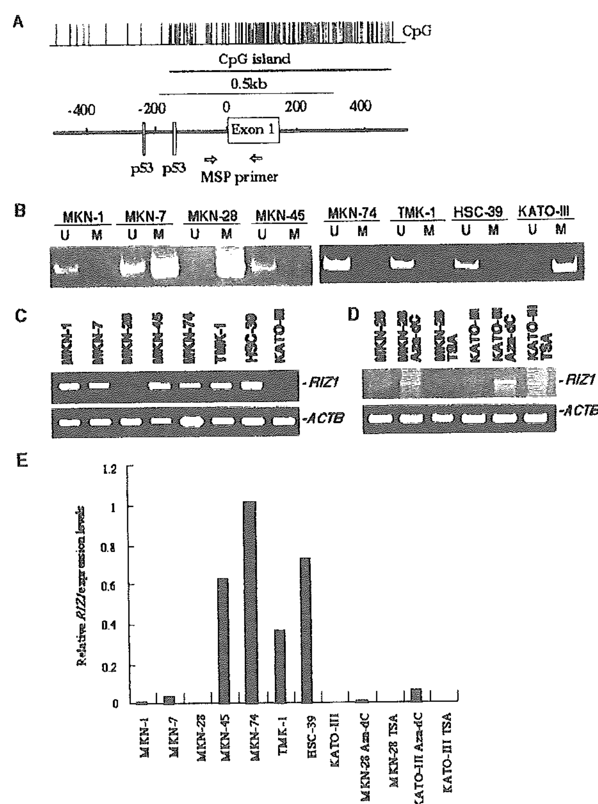

FIGURE 1 – Promoter methylation status and *RIZ1* mRNA expression in gastric carcinoma cell lines. (*a*) Map of the *RIZ1* promoter, depicting the CpG island, MSP primer, and *p53* consensus binding sites. Vertical lines show each CpG site. (*b*) MSP of *RIZ1* in gastric carcinoma cell lines. Primer sets were either unmethylated (U) or methylated (M). The methylated allele was detected in MKN-7, MKN-28, and KATO-III cell lines. (*c*) RT-PCR of gastric carcinoma cell lines. Expression of *RIZ1* was abolished in the MKN-28 and KATO-III cell lines. (*d*) Treatment of MKN-28 and KATO-III cells with the demethylating agent Aza-dC restored *RIZ1* expression. Treatment of these cells with TSA, a histone deacetylase inhibitor, was unable to reactivate *RIZ1* gene expression. (*e*) Quantitative real-time PCR of gastric carcinoma cell lines. The units are arbitrary, and *RIZ1* mRNA expression was calculated by standardization with 1 μg of total RNA from the MKN-74 cell line, which was assigned a *RIZ1* mRNA expression value of 1.0. As shown by RT-PCR analysis, expression of *RIZ1* was abolished in the MKN-28 and KATO-III cell lines. Treatment of MKN-28 and KATO-III cells with Aza-dC, the demethylating agent, restored *RIZ1* expression. Treatment of these cells with TSA, a histone deacetylase inhibitor, was unable to reactivate *RIZ1* gene expression. *RIZ1* expression in gastric carcinoma cell lines harboring *p53* abnormalities tended to be lower than that in gastric carcinoma cell lines harboring wild-type *p53*.

(*MINT1*, *MINT2*, *MINT12*, *MINT25* and *MINT31*) to be positive for CIMP.[33] The presence or absence of CIMP was determined previously in 39 of 45 gastric carcinoma samples.[34]

RT-PCR

RIZ1 expression in gastric carcinoma cell lines was analyzed by RT-PCR. Total RNA was extracted by RNeasy Mini Kit (QIAGEN, Tokyo, Japan), and 1 μg of total RNA was converted to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). To investigate *RIZ1* mRNA expression, RT-PCR was carried out with primers 5′-TGG CTG CGA TAT GTG AAT TG-3′ (sense) and 5′-CCT CTG AGC AGT CTT CAA GAG T-3′ (antisense). PCR conditions were 35 cycles at 94°C for 2 min, 60°C for 2 min and 72°C for 3 min. The amplification products were then analyzed by 1.5% agarose gel

electrophoresis with ethidium bromide and examined under UV light. *ACTB* -specific PCR products from the same RNA samples were amplified and these products served as internal controls.

Quantitative RT-PCR analysis

Total RNA was isolated with an RNeasy Mini Kit (QIAGEN, Tokyo, Japan) and 1 μg of total RNA was converted to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). PCRs were carried out with the SYBR Green PCR Core Reagents kit (Applied Biosystems, Tokyo, Japan). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNAs was carried out with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined by the threshold cycles for the *RIZ1* gene and the *ACTB* gene (internal control). Reference samples (gastric carcinoma cell line MKN-74) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. PCR amplification was carried out with a 96-well optical tray and caps in a final reaction mixture volume of 25 μl, according to the manufacturer's instructions. *RIZ1* primer sequences were 5′-ATT GAT GCC ACT GAT CCA GAG A-3′ (sense) and 5′-GCT CTG TTG ATT TCC AGT GGG A-3′ (antisense). *ACTB* primer sequences were 5′-TCA CCG AGC GCG GCT-3′ (sense) and 5′-TAA TGT CAC GCA CGA TTT CCC-3′ (antisense).

p53 *mutation analysis*

Exons 5–8 of the *p53* gene were examined for mutation by PCR single-strand conformation polymorphism (SSCP) analysis. Genomic DNA was PCR-amplified with 10 sets of primers. Primers for exon 5a were 5′-TGC CCT GAC TTT CAA CTC TGT-3′ (sense) and 5′-CAT GTG CTG TGA CTG CTT GTA-3′ (antisense). Primers for exon 5b were 5′-CTG TGC AGC TGT GGG TTG ATT-3′ (sense) and 5′-GCA ACC AGC CCT GTC GTC TCT-3′ (antisense). Primers for exon 6 were 5′-CAC TGA TTG CTC TTA GGT-3′ (sense) and 5′-AGT TGC AAA CCA GAC CTC-3′ (antisense). Primers for exon 7 were 5′-TAG GTT GGC TCT GAC TGT ACC-3′ (sense) and 5′-TGA CCT GGA GTC TTC CAG TGT-3′ (antisense). Primers for exon 8 were 5′-AGT GGT AAT CTA CTG GGA CGG-3′ (sense) and 5′-ACC TCG CTT AGT GCT CCC TG-3′ (antisense). PCR conditions for exon 5a and exon 6 were 35 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. PCR conditions for exon 5b were 45 cycles at 95°C for 1 min and 62°C for 1 min. PCR conditions for exons 7 and 8 were 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. PCR products were diluted 10-fold with formamide dye solution, denatured at 85°C for 10 min, and electrophoresed on 6% polyacrylamide gels. Gels were stained and visualized with a Silver Staining II kit (WAKO, Osaka, Japan). The presence or absence of *p53* mutation was determined previously in 30 of 45 gastric carcinoma samples.[16]

Statistical methods

Statistical analyses were carried out with Fisher's exact test and the Mann-Whitney *U* -test; *p* -values <0.05 were regarded as statistically significant.

RESULTS

Promoter methylation status and expression of RIZ1 *in gastric carcinoma cell lines*

To study whether promoter hypermethylation of *RIZ1* induces transcriptional inactivation, we carried out an *in vitro* analysis using 8 gastric carcinoma cell lines. As shown in Figure 1*b*, MSP showed promoter hypermethylation of *RIZ1* in MKN-7, MKN-28 and KATO-III cells and no promoter hypermethylation of *RIZ1* in MKN-1, MKN-45, MKN-74, TMK-1 and HSC-39 cells. In MKN-7 cells, *RIZ1* promoter was considered to be partially methylated, as described previously[32] because both methylated and unmethylated PCR products were detected. To investigate the relation between promoter methylation status and *RIZ1* expression levels, we carried out RT-PCR and quantitative RT-PCR analyses. Transcriptional inactivation was observed in MKN-28 and KATO-III cells with promoter hypermethylation (Fig. 1*c*,*e*). The other 6 cell lines expressed *RIZ1* as detected by RT-PCR analysis (Fig. 1*c*,*e*). To investigate whether methylation is responsible for transcriptional inactivation of *RIZ1*, we treated MKN-28 and KATO-III cells with Aza-dC, a demethylating agent, and carried out RT-PCR analysis. *RIZ1* mRNA expression appeared in these cells after 5 days of Aza-dC treatment (Fig. 1*d*,*e*). Treatment of these cells over 1 day with 300 nM TSA, a histone deacetylase inhibitor, was unable to reactivate *RIZ1* gene expression (Fig. 1*d*,*e*).

mRNA expression levels of RIZ1 *in gastric carcinoma*

We measured the levels of *RIZ1* mRNA expression using quantitative RT-PCR in 45 pairs of gastric carcinoma tissues and their corresponding non-neoplastic mucosae. The overall results of quantitative RT-PCR analysis are summarized in Table I. No association was found by Mann-Whitney *U* -test between mRNA levels of *RIZ1* in the tumor tissues and age ($p = 0.795$), gender ($p = 0.785$), T grade (depth of invasion, $p = 0.315$), N grade (lymph node metastasis, $p = 0.409$) or tumor stage ($p = 0.223$) (data not shown). No association was found between mRNA levels of *RIZ1* in the tumor tissues and histological classification (data not shown).

RIZ1 *promoter methylation status and mRNA expression levels in gastric carcinoma*

Methylation status of the *RIZ1* promoter was examined in a total of 45 gastric carcinoma tissue specimens from 45 patients and 14 corresponding non-neoplastic mucosae. Promoter hypermethylation of the *RIZ1* gene was found in 31 (69%) of 45 gastric carcinoma tissues and in 3 (21%) of 14 corresponding non-neoplastic mucosae. The incidence of hypermethylation between the carcinomas and the non-neoplastic mucosae were significantly different ($p = 0.002$, Fisher's exact test). Representative results of MSP for *RIZ1* are shown in Figure 2*a* ,*b* and the overall results of MSP in the tumors are summarized in Tables I and II. No association was detected by Fisher's exact test between methylation status of *RIZ1* and age ($p = 0.143$), gender ($p = 0.492$), T grade ($p = 0.885$), N grade ($p = 0.497$), tumor stage ($p = 0.478$) or pure and mixed types of histological classification ($p = 0.520$) (Table II). When we further classified pure type gastric carcinomas into diffuse-scattered type and intestinal and diffuse-adherent type, however, methylation of the *RIZ1* gene was found more frequently in intestinal and diffuse-adherent type than in diffuse-scattered type ($p = 0.047$, Fisher's exact test) (Table II). To determine whether transcriptional silencing of the *RIZ1* gene is caused by promoter hypermethylation, we compared the methylation status with mRNA expression of the *RIZ1* gene. As shown in Tables I and III, levels of *RIZ1* mRNA in tumor tissues with *RIZ1* hypermethylation (0.13 ± 0.04, mean ± SE) were significantly lower than those in tumor tissues without promoter hypermethylation (0.27 ± 0.10; $p = 0.029$, Mann-Whitney *U* -test) and those in corresponding non-neoplastic mucosae (0.50 ± 0.14; $p = 0.0009$, Mann-Whitney *U* -test). Levels of *RIZ1* mRNA in tumor tissues without *RIZ1* hypermethylation did not differ significantly from those in corresponding non-neoplastic mucosa ($p = 0.557$, Mann-Whitney *U* -test).

Methylation status of RIZ1 *in normal gastric mucosa from non-cancerous healthy individuals*

We examined methylation status of the *RIZ1* gene in a total of 12 normal gastric mucosae obtained endoscopically from non-cancerous individuals (age = 22–35 years; average, 25.4 years). Hypermethylation of the *RIZ1* gene was not found in any of these samples (Fig. 2*c*).

TABLE I – MRNA EXPRESSION LEVELS AND METHYLATION STATUS OF *RIZ1* IN GASTRIC CARCINOMAS

Methylation status in tumor tissue	*RIZ1* mRNA expression level in tumor tissue[1]	*RIZ1* mRNA expression level in non-neoplastic mucosa[1]	CIMP status	*p53* mutation status	Gender	Age	Stage[2]	T grade[3]	N grade[4]	Histology[5]
Unmethylated	1.365	0.053	Negative	Wild-type	F	76	IA	1	0	Intestinal
	0.570	1.173	Negative	Wild-type	M	41	IB	2b	0	Diffuse
	0.122	0.097	Negative	Wild-type	M	79	II	2b	1	Intestinal
	0.162	0.403	Negative	Wild-type	F	75	II	2b	1	Diffuse
	0.084	0.045	Negative	Wild-type	M	51	II	3	0	Intestinal
	0.036	0.077	Negative	Wild-type	F	66	IIIA	3	1	Diffuse
	0.079	0.013	Negative	Wild-type	M	50	IIIB	3	3	Diffuse
	0.096	5.426	Negative	Wild-type	M	34	IV	4	2	Intestinal
	0.747	0.271	Negative	Wild-type	M	66	IV	3	3	Intestinal
	0.093	0.097	Negative	Wild-type	F	74	IV	4	2	Diffuse
	0.183	1.890	Negative	Mutant-type	F	74	IB	2a	0	Diffuse
	0.128	0.086	Negative	Mutant-type	F	86	IB	2b	0	Intestinal
	0.032	0.036	Negative	Mutant-type	M	64	IV	3	3	Diffuse
	0.127	2.694	Positive	Mutant-type	M	59	IV	3	2	Intestinal
Methylated	0.002	0.019	Negative	Wild-type	M	75	IB	2b	0	Intestinal
	0.046	2.231	Negative	Wild-type	M	74	IB	2a	0	Intestinal
	0.008	0.025	Negative	Wild-type	M	73	IB	2a	0	Intestinal
	0.050	0.266	Negative	Wild-type	M	62	II	2b	1	Diffuse
	0.051	0.078	Negative	Wild-type	F	67	II	3	0	Diffuse
	0.147	0.075	Negative	Wild-type	F	64	IIIA	3	1	Diffuse
	0.064	0.102	Negative	Wild-type	M	55	IV	2b	3	Diffuse
	0.012	0.238	Negative	Wild-type	F	65	IV	3	3	Diffuse
	0.061	0.203	Negative	Mutant-type	M	85	IB	2b	0	Intestinal
	0.012	0.153	Negative	Mutant-type	M	72	II	3	0	Intestinal
	0.158	0.659	Negative	Mutant-type	F	46	IIIA	2b	2	Diffuse
	0.068	0.503	Negative	Mutant-type	M	57	IIIA	2b	2	Intestinal
	0.066	0.038	Negative	Mutant-type	F	75	IIIA	3	1	Intestinal
	1.246	0.139	Negative	Mutant-type	M	70	IIIB	3	2	Intestinal
	0.055	0.156	Positive	Wild-type	F	67	IB	2b	0	Intestinal
	0.002	0.602	Positive	Wild-type	M	57	II	2b	1	Diffuse
	0.032	0.082	Positive	Wild-type	F	81	II	2b	1	Diffuse
	0.659	0.152	Positive	Wild-type	M	62	IIIA	3	1	Intestinal
	0.176	0.158	Positive	Wild-type	M	69	IIIA	4	1	Diffuse
	0.004	0.011	Positive	Wild-type	M	81	IIIA	2b	2	Diffuse
	0.099	0.056	Positive	Wild-type	M	85	IIIA	3	1	Diffuse
	0.093	1.514	Positive	Wild-type	M	58	IIIB	3	2	Intestinal
	0.097	0.405	Positive	Wild-type	F	61	IIIB	3	2	Intestinal
	0.131	0.262	Positive	Wild-type	M	70	IIIB	3	2	Diffuse
	0.029	0.370	Positive	Wild-type	M	69	IV	3	3	Intestinal
	0.081	0.153	Positive	Wild-type	M	69	IV	4	2	Diffuse
	0.238	0.535	Positive	Wild-type	M	72	IV	3	3	Intestinal
	0.064	0.114	Positive	Mutant-type	M	61	IA	1	0	Intestinal
	0.100	0.135	Positive	Mutant-type	F	73	II	2b	1	Intestinal
	0.010	0.594	Positive	Mutant-type	F	67	IIIB	3	2	Diffuse
	0.275	0.143	Positive	Mutant-type	M	75	IV	4	3	Intestinal

[1]The units are arbitrary, and we calculated the *RIZ1* mRNA expression in tumor tissues and corresponding non-neoplastic mucosa by standardization with 1 μg of the MKN-74 gastric carcinoma cells, taken as 1.0. – [2]Stage was classified according to the criteria of the UICC TNM stage Grouping 6th edition, 2002, Stomach. – [3]T grade was classified according to the criteria of the UICC TNM stage Grouping 6th edition, 2002, Stomach. – [4]N grade was classified according to the criteria of the UICC TNM stage Grouping 6th edition, 2002, Stomach. – [5]Histology was classified according to the criteria of Lauren.

Association of RIZ1 *promoter hypermethylation with CIMP*

We analyzed CIMP by examining the methylation status of *MINT1*, *MINT2*, *MINT12*, *MINT25* and *MINT31* in 45 gastric carcinoma tissues. CpG island hypermethylation of the *MINT* loci was detected in 22 (49%) tissues for *MINT1* , 19 (42%) for *MINT2*, 21 (47%) for *MINT12*, 33 (73%) for *MINT25*, and 12 (27%) for *MINT31*. In total, 18 (40%) tissues were regarded as CIMP-positive (Table I). We then compared the promoter methylation status of the *RIZ1* gene with CIMP status. A significant association was found between the presence of CIMP and *RIZ1* promoter hypermethylation ($p = 0.002$, Fisher's exact test) (Table II).

Correlation between p53 *mutation status, methylation status and mRNA levels of the* RIZ1 *gene in gastric carcinoma*

To examine whether a correlation exists between *p53* mutation status and methylation status or mRNA level of the *RIZ1* gene, we examined *p53* mutation status of 45 gastric carcinoma tissues by PCR-SSCP analysis (Fig. 2*d*). In gastric carcinoma tissues, mutations in *p53* were observed in 14 (31%) tissues (Fig. 2*d*). No correlation was found between *p53* mutation status and methylation status of the *RIZ1* gene ($p = 0.805$, Fisher's exact test) (Tables I, II). Mutation status of *p53* also did not correlate with *RIZ1* mRNA levels in gastric carcinoma tissues (*RIZ1* mRNA expression level: wild-type *p53* , 0.18 ± 0.05 (mean ± SE); mutant *p53* , 0.18 ± 0.08; $p = 0.607$, Mann-Whitney U - test) (Tables I, III). *p53* is reported to be mutant in MKN-1, MKN-7, MKN-28, TMK-1 and HSC-39, wild-type in MKN-45 and MKN-74, and completely deleted in KATO-III.[44] Methylation of the *RIZ1* gene and abnormalities in *p53* coexisted in MKN-7, MKN-28 and KATO-III. Moreover, *RIZ1* mRNA levels in *p53* wild-type cell lines were higher than those in *p53* mutant cell lines except TMK-1 and HSC-39 (Fig. 1*e*).

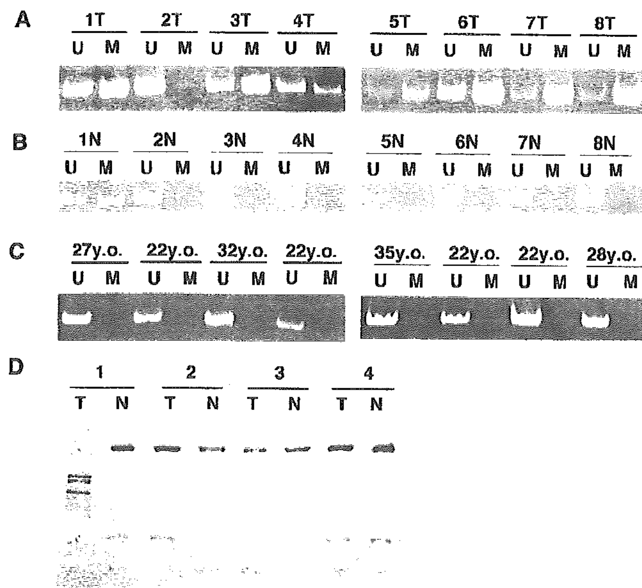

FIGURE 2 – MSP analysis of the *RIZ1* promoter and *p53* mutation analysis of gastric tissues. U, unmethylated PCR product; M, methylated PCR product. (*a*) Methylation status of *RIZ1* in gastric carcinoma tissues. The methylated allele was detected in 1T, 3T, 4T, 5T, 6T, 7T and 8T. (*b*) Methylation status of *RIZ1* in corresponding non-neoplastic mucosae. The methylated allele was detected in 1N. (*c*) Methylation status of *RIZ1* in normal gastric mucosae obtained from young healthy individuals (age = 22–35 years; average = 25.4 years). Promoter methylation of the *RIZ1* gene was not detected in any sample. (*d*) Mutation analysis of *p53* by SSCP in gastric carcinoma tissues. Mutation of *p53* exon 8 was observed in 1T.

TABLE II – ASSOCIATION BETWEEN *RIZ1* METHYLATION STATUS AND CLINICOPATHOLOGICAL FEATURES, CIMP STATUS AND P53 MUTATION STATUS IN GASTRIC CARCINOMAS

Feature	*RIZ1* methylation status: Positive	Negative	*p*-value[1]
Gender			
Male	21	8	0.492
Female	10	6	
Age			
<60	5	5	0.143
>61	26	9	
T grade			
1, 2	14	6	0.885
3, 4	17	8	
N			
N0	8	5	0.497
N1, N2, N3	23	9	
Stage			
I, II	12	7	0.478
III, IV	19	7	
Histology			
Intestinal	17	7	0.763
Diffuse	14	7	
Histology			
Mixed type	7	2	0.520
Pure type	24	12	
Intestinal & Diffuse-adherent type	21	7	0.047
Diffuse-scattered type	3	5	
CIMP			
Positive	17	1	0.002
Negative	14	13	
p53 mutation			
Positive	10	4	0.805
Negative	21	10	

[1]Fisher's exact test.

TABLE III – ASSOCIATION BETWEEN *RIZ1* MRNA EXPRESSION AND METHYLATION STATUS AND *P53* MUTATION STATUS IN GASTRIC CARCINOMAS

	RIZ1 expression levels[1]
Non-neoplastic mucosa ($n = 45$)	$0.50 \pm 0.14^{2,3}$
Tumor tissue without promoter hypermethylation ($n = 14$)	$0.27 \pm 0.10^{2,4}$
Tumor tissue with promoter hypermethylation ($n = 31$)	$0.13 \pm 0.04^{3,4}$
Tumor tissues with wild-type *p53* ($n = 31$)	0.18 ± 0.05^{5}
Tumor tissue with mutant-type *p53* ($n = 14$)	0.18 ± 0.08^{5}

[1]Mean ± SE.–$^2p = 0.557$.–$^3p = 0.0009$.–$^4p = 0.029$.–$^5p = 0.607$.

DISCUSSION

DNA methylation and alteration of chromatin structure are important ways of transcriptionally silencing many genes. In our present study, promoter hypermethylation of the *RIZ1* gene was detected in 3 cell lines (MKN-7, MKN-28 and KATO-III), which expressed undetectable levels of the *RIZ1* gene product except MKN-7 with partially methylated *RIZ1* promoter. Moreover, treatment of *RIZ1* mRNA-negative cells (MKN-28, KATO-III) with Aza-dC led to a reactivation of *RIZ1* expression. These results suggest that hypermethylation of the *RIZ1* promoter region plays an important role in transcriptional silencing of *RIZ1* in gastric carcinomas. In support of this conclusion, we found that the *RIZ1* gene was frequently targeted for methylation and silencing in gastric carcinoma tissues and that promoter hypermethylation of the *RIZ1* gene was associated with reduced expression. We found several tumor samples, however, with *RIZ1* gene hypermethylation that did not have low *RIZ1* gene expression. This result may be related to the extreme sensitivity of MSP, which can theoretically detect as little as 0.1% methylated cells.[43] Alternatively, a tumor may exhibit heterogeneity in *RIZ1* methylation. In this case, partial methylation of the *RIZ1* promoter region is likely to reduce the level of transcriptional repression. In contrast, several samples show low levels of *RIZ1* gene expression in the absence of *RIZ1* promoter methylation. Alternative inactivating pathways, such as hemizygous deletion and alteration of transcription factors, may account for low level of *RIZ1* gene expression.

The incidence of *RIZ1* gene promoter methylation was significantly different between gastric tumors and non-neoplastic mucosae; the *RIZ1* gene promoter was hypermethylated in 31 (69%) of 45 gastric carcinoma samples and in 3 (21%) of 14 corresponding non-neoplastic mucosa samples. This result suggests that methylation of the *RIZ1* gene promoter may contribute to gastric carcinogenesis. Methylation of the *RIZ1* gene also has been reported in non-malignant liver tissues.[32] Epigenetic changes including DNA methylation occur in premalignant and histologically normal gastric epithelium.[36,45,46] Furthermore, recent evidence suggests that methylation of certain genes such as E-cadherin and *p16* ^{INK4a} is associated with aging.[1,2,36,37] We confirmed that normal gastric mucosa from young healthy individuals was not hypermethylated in the *RIZ1* promoter region. Therefore, the aging mechanism can partly explain detection of methylation in non-neoplastic mucosae. Age-related methylation of the *RIZ1* gene may also explain the possible link between aging and increased risk for gastric cancer.

In addition to classifying tumors with the Lauren system, recent studies show the importance of histologically distinguishing between gastric tumors that are pure type or mixed type because patients with mixed type gastric tumors tend to have worse outcomes than those with pure types.[41] In our present study, methylation status of the *RIZ1* gene was not associated with histological classification by pure or mixed type. In the pure type of gastric carcinoma, however, methylation of the *RIZ1* gene was found more frequently in intestinal and diffuse-adherent types of carcinomas than in the diffuse-scattered type of carcinoma. Methylation of the *RIZ1* gene may primarily contribute to the intestinal and

diffuse-adherent types of gastric carcinomas. We also reported that methylation of $p16^{INK4a}$ and CIMP occurs more frequently in intestinal and diffuse-adherent types of gastric carcinomas than in the diffuse-scattered type of gastric carcinoma[34] and that methylation of *RAR-beta* and *CDH1* preferentially occurs in the diffuse-scattered type of gastric carcinoma.[15] Our findings suggest that promoter methylation of these genes must play a role in the morphogenesis of gastric carcinomas.

In our present study, a significant association was detected between the presence of CIMP and hypermethylation of the *RIZ1* promoter in gastric carcinomas. This result is consistent with that for colon cancer,[35] indicating that methylation of the *RIZ1* gene is not a random event in gastric carcinogenesis. CIMP is considered to lead to cancer formation and progression through the silencing of multiple tumor suppressor genes.[33] We suggest that the *RIZ1* gene is a likely target gene associated with CIMP. Because CIMP is associated with inactivation of $p16^{INK4a}$[33] and *hMLH1*,[34] unbridled cell cycle progression and genetic instability is likely to be involved in carcinogenesis of CIMP-positive carcinomas. Genetic instability may cause mutations of target genes such as *Bax*, *TGF-beta receptor type II*, and *RIZ1*, and loss of function of these genes is believed to contribute to carcinogenesis.[28,29,47] Microsatellite instability has been found in only 9–12% of gastric carcinomas,[33,48] and frameshift mutation of RIZ has been found in 36–48 % of microsatellite-unstable gastric carcinomas[28,29] but not in microsatellite-stable gastric carcinomas.[28] Although we did not examine frameshift mutations of *RIZ*, this mutation must be infrequent (5% or less) in gastric carcinoma. Taken together, these findings suggest that hypermethylation is the major mechanism of inactivation of the *RIZ1* gene in gastric carcinomas, especially in CIMP-positive carcinomas.

Abnormalities in *p53* and methylation-mediated silencing of the *RIZ1* gene coexist in MKN-28 and KATO-III, as described previously in other carcinoma cell lines.[25,30] No correlation was observed between *p53* mutation status and *RIZ1* methylation status in gastric carcinoma tissues. Molecular mechanisms underlying *RIZ1* and *p53* cooperation in tumor formation remain to be clarified.[30] Tumor suppressor p53 is a sequence-specific DNA-binding protein, and its biological effects are mediated by transactivation of various target genes.[49] A potential *p53* binding site exists within the promoter of *RIZ1* (Fig. 1*a*). Therefore, it is possible that *p53* directly binds to this potential binding site and activates *RIZ1* expression. In our present study, *RIZ1* mRNA levels were relatively high in MKN-45 and MKN-74, which both harbor wild-type *p53*, and relatively low in MKN-1, MKN-7 and MKN-28, all of which harbor mutant-type *p53* , and in KATO-III, with completely deleted *p53*. *RIZ1* mRNA levels in TMK-1 and HSC-39, both harboring mutant-type *p53* , were also relatively high. Moreover, there was no correlation between *p53* mutation status and *RIZ1* expression in gastric carcinoma tissues. Although *MGMT* and *TSP1* are regulated by promoter hypermethylation and *p53* , *p53* mutation status was not associated with methylation status or expression of these genes in gastric carcinoma.[14,16] Therefore, *RIZ1* is a likely *p53* -target gene, and further investigation is needed.

In conclusion, our results suggest that transcriptional inactivation of *RIZ1* by promoter hypermethylation associated with CIMP participates in the development of gastric carcinoma, especially of the intestinal and diffuse-adherent types.

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REFERENCES

1. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
2. Baylin SB. Herman JG. Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141–96.
3. Fleisher AS. Esteller M. Wang S. Tamura G, Suzuki H, Yin J. Zou TT, Abraham JM, Kong D, Smolinski KN. Shi YQ. Rhyu MG, et al. Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability. Cancer Res 1999;59:1090–5.
4. Leung SY. Yuen ST. Chung LP. Chu KM. Chan AS, Ho JC. hMLH1 promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability. Cancer Res 1999;59:159–64.
5. Shim YH, Kang GH, Ro JY. Correlation of p16 hypermethylation with p16 protein loss in sporadic gastric carcinomas. Lab Invest 2000;80:689–95.
6. Tamura G, Yin J, Wang S, Fleisher AS, Zou T, Abraham JM, Kong D, Smolinski KN, Wilson KT, James SP, Silverberg SG, Nishizuka S, et al. E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. J Natl Cancer Inst 2000;92:569–73.
7. Kikuchi T, Itoh F, Toyota M, Suzuki H, Yamamoto H, Fujita M, Hosokawa M, Imai K. Aberrant methylation and histone deacetylation of cyclooxygenase 2 in gastric cancer. Int J Cancer 2002;97:272–7.
8. Song SH, Jong HS, Choi HH, Inoue H, Tanabe T, Kim NK, Bang YJ. Transcriptional silencing of cyclooxygenase-2 by hyper-methylation of the 5′ CpG island in human gastric carcinoma cells. Cancer Res 2001;61:4628–35.
9. Byun DS, Lee MG, Chae KS, Ryu BG, Chi SG. Frequent epigenetic inactivation of RASSF1A by aberrant promoter hypermethylation in human gastric adenocarcinoma. Cancer Res 2001;61:7034–8.
10. Kang YH, Lee HS, Kim WH. Promoter methylation and silencing of PTEN in gastric carcinoma. Lab Invest 2002;82:285–91.
11. Sato K, Tamura G, Tsuchiya T, Endoh Y, Usuba O, Kimura W, Motoyama T. Frequent loss of expression without sequence mutations of the DCC gene in primary gastric cancer. Br J Cancer 2001;85:199–203.
12. Kaneda A, Kaminishi M, Nakanishi Y, Sugimura T, Ushijima T. Reduced expression of the insulin-induced protein 1 and p41 Arp2/3 complex genes in human gastric cancers. Int J Cancer 2002;100:57–62.
13. Kaneda A, Kaminishi M, Yanagihara K, Sugimura T, Ushijima T. Identification of silencing of nine genes in human gastric cancers. Cancer Res 2002;62:6645–50.
14. Oue N, Shigeishi H, Kuniyasu H, Yokozaki H, Kuraoka K, Ito R, Yasui, W. Promoter hypermethylation of MGMT is associated with protein loss in gastric carcinoma. Int J Cancer 2001;93:805–9.
15. Oue N. Motoshita J, Yokozaki H, Hayashi K, Tahara E, Taniyama K, Matsusaki K, Yasui W. Distinct promoter hypermethylation of p16INK4a, CDH1, and RAR-beta in intestinal, diffuse-adherent, and diffuse-scattered type gastric carcinomas. J Pathol 2002;198:55–9.
16. Oue N, Matsumura S, Nakayama H, Kitadai Y, Taniyama K, Matsusaki K, Yasui W. Reduced expression of the TSP1 gene and its association with promoter hypermethylation in gastric carcinoma. Oncology 2003;64:423–9.
17. Hamai H, Oue N, Mitani Y, Nakayama H, Ito R, Matsusaki K, Yoshida K, Toge T, Yasui W. DNA hypermethylation and histone hypo acetylation of the HLTF gene are associated with reduced expression in gastric carcinoma. Cancer Sci 2003;94:692–8.
18. Oshimo Y, Nakayama H, Ito R, Kitadai Y, Yoshida K, Chyama K, Yasui W. Promotor methylation of cyclin D2 gene in gastric carcinoma. Int J Oncol 2003;23:1663–70.
19. Buyse IM, Shao G, Huang S. The retinoblastoma protein binds to RIZ, a zinc finger protein that shares an epitope with the adenovirus EIA protein. Proc Natl Acad Sci USA 1995;92:4467–71.
20. Huang S, Shao G, Liu L. The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. J Biol Chem 1997;272:2984–91.
21. Jiang GL, Huang S. The *yin-yang* of PR domain family genes in tumorigenesis. Histol Histopathol 2000;15:109–17.
22. Liu L, Shao G, Steel-Perkins G, Huang S. The retinoblastoma interacting zinc finger gene RIZ produces a PR domain lacking product through an internal promoter. J Biol Chem 1997;272:2984–91.
23. Weith A, Brodeur GM, Bruns GA, Matise TC, Mischke D, Nizetic D, Seldin MF, van Roy N, Vance J. Report of the second international workshop on human chromosome 1 mapping 1995. Cytogenet Cell Genet 1996;72:114–44.
24. Koizumi Y, Tanaka S, Mou R, Koganei H, Kokawa A, Kitamura R, Yamauchi H, Ookubo K, Saito T, Tominaga S, Matsumura K, Shimada H, et al. Changes in DNA copy number in primary gastric

carcinomas by comparative genomic hybridization. Clin Cancer Res 1997;3:1067–76.
25. He L, Yu JX, Liu L, Buyse IM, Wang MS, Yang QC, Nakagawara A, Brodeur GM, Shi YE, Huang S. RIZ1, but not the alternative RIZ2 product of the same gene, is underexpressed in breast cancer, and forced RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis. Cancer Res 1998;58:4238–44.
26. Jiang GL, Liu L, Buyse IM, Simon D, Huang S. Decreased RIZ1 expression but not RIZ2 in hepatoma and suppression of hepatoma tumorigenicity by RIZ1. Int J Cancer 1999;83:541–7.
27. Chadwick RB, Jiang GL, Bennington GA, Yuan B, Johnson CK, Stevens MW, Niemann TH, Peltomaki P, Huang S, de la Chapelle A. Candidate tumor suppressor RIZ1 is frequently involved in colorectal carcinogenesis. Proc Natl Acad Sci USA 2000;97:2662–7.
28. Piao Z, fang W, Malkhosyan S, Kim H, Horii A, Perucho M, Huang S. Frameshift mutations of RIZ in human gastrointestinal and endometrial carcinomas with microsatellite instability. Cancer Res 2000; 60:4701–4.
29. Sakurada K, Furukawa T, Kato Y, Kayama T, Huang S, Horii A. RIZ, the retinoblastoma protein interacting zinc finger gene, is mutated in genetically unstable cancers of the pancreas, stomach, and colorectum. Genes Chromosomes Cancer 2001;30:207–11.
30. Steele-Perkins G, Fang W, Yang XH, Van Gele M, Carling T, Gu J, Buyse IM, Fletcher JA, Liu J, Bronson R, Chadwick RB, de la Chapella A, et al. Tumor formation and inactivation of RIZ1, an Rb-binding member of a nuclear protein-methyltransferase superfamily. Genes Dev 2001;15;2250–62.
31. Jiang GL, Huang S. Adenovirus expressing RIZ1 in tumor suppressor gene therapy of microsatellite-unstable colorectal cancers. Cancer Res 2001;61:1796–8.
32. Du Y, Carling T, Fang W, Piao Z, Sheu JC, Huang S. Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. Cancer Res 2001: 61:8094–9.
33. Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, Baylin SB, Issa J P. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res 1999;59:5438–42.
34. Oue N, Oshimo Y, Nakayama H, Ito R, Yoshida K, Matsusaki K, Yasui W. DNA methylation of multiple genes in gastric carcinoma: association with histological type and CpG island methylator phenotype. Cancer Sci 2003,94:901–5.
35. Whitehall VLJ, Wynter CVA, Walsh MD, Simms LA, Purdie D, Pandeya N, Young J, Meltzer SJ, Leggett BA, Jass JR. Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. Cancer Res 2002;62:6011–4.
36. Waki T, Tamura G, Tsuchiya T, Sato K, Nishizuka S, Motoyama T. Promoter methylation status of E-cadherin, hMLH1, and p16 genes in nonneoplastic gastric epithelia. Am J Pathol 2002;161:399–403.
37. Ahuja N, Issa JP. Aging, methylation and cancer. Histol Histopathol 2000;15:835–42.
38. Ochiai A, Yasui W, Tahara E. Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. Jpn J Cancer Res 1995; 76:1064–71.
39. Lauren P. The two histological main types of gastric carcinoma. Diffuse and so-called intestinal type carcinoma: an attempt at histological classification. Acta Pathol Microbiol Scand 1965;64:31–49.
40. Shimoyama Y, Hirohashi S. Expression of E- and P-cadherin in gastric carcinomas. Cancer Res 1991;51:2185–92.
41. Stelzner S, Emmrich P. The mixed type in Lauren's classification of gastric carcinoma. Histologic description and biologic behavior. Gen Diagn Pathol 1997;143:39–48.
42. Sobin LH, Wittekind CH, eds. TNM classification of malignant tumors, 6th ed. New York: Wiley-Liss Inc, 2002. pp 65–8.
43. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996;93:9821–6.
44. Yokozaki H. Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathol Int 2000;50:767–77.
45. Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG. CpG island methylation in premalignant stages of gastric carcinoma. Cancer Res 2001;61:2847–51.
46. To KF, Leung WK, Lee TL, Yu J, Tong JH, Chan MW, Ng EK, Chung SC, Sung JJ. Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. Int J Cancer 2002;102:623–8.
47. Iacopetta BJ, Soong R, House AK, Hamelin R. Gastric carcinomas with microsatellite instability: clinical features and mutations to the TGF-beta type II receptor, IGFII receptor, and BAX genes. J Pathol 1999;187:428–32.
48. Fang DC, Wang RQ, Yang SM, Yang JM, Liu HF, Peng GY, Xiao TL, Luo YH. Mutation and methylation of hMLH1 in gastric carcinomas with microsatellite instability. World J Gastroenterol 2003;9: 655–9.
49. Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev 1996;10: 1054–72.

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Dietary factors and cancer mortality among atomic-bomb survivors☆

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Abstract

Dietary factors such as fruit and vegetables are thought to reduce the risk of cancer incidence and mortality. We investigated the effect of a diet rich in fruit and vegetables against the long-term effects of radiation exposure on the risk of cancer. A cohort of 36,228 atomic-bomb survivors of Hiroshima and Nagasaki, for whom radiation dose estimates were currently available, had their diet assessed in 1980. They were followed for a period of 20 years for cancer mortality. The joint-effect of fruit and vegetables intake and radiation exposure on risk of cancer death was examined, in additive (sum of effects of diet alone and radiation alone) and multiplicative (product of effects of diet alone and radiation alone) models. In the additive model, a daily intake of fruit and vegetables significantly reduced the risk of cancer deaths by 13%, compared to an intake of once or less per week. Radiation exposure of 1 Sievert (Sv) increased significantly the risk of cancer death by 48–49%. The additive joint-effects showed a lower risk of cancer among those exposed to 1 Sv who had a diet rich in vegetables (49% − 13% = 36%) or fruit (48% − 13% = 35%). The multiplicative model gave similar results. The cancer risk reduction by vegetables in exposed persons went from 52% (effect of radiation alone) to 32% (product of effect of vegetables and radiation), and cancer risk reduction by fruit was 52% (radiation alone) to 34% (product of effect of fruit and radiation). There was no significant evidence to reject either the additive or the multiplicative model. A daily intake of fruit and vegetables was beneficial to the persons exposed to radiation in reducing their risks of cancer death.
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Keywords: Ionising radiation exposure; Dietary factors; Cancer mortality; Longitudinal study; Japan

1. Introduction

Ionising radiation exposure is a well-established carcinogenic risk factor. In a cohort of Japanese atomic-bomb survivors who received a broad range of doses (mean 0.1 Sv), radiation accounts for 5% of cancer deaths [1]. A diet rich in fruit and vegetables is thought to reduce the cancer risks, especially digestive cancers [2]. This protective effect was indeed observed among the cited cohort of A-bomb survivors, where the risks of all solid cancer deaths were significantly reduced by 8% for those with a high vegetables consumption, and by 12% for those with a high fruit intake [3,4]. Fruit and vegetables

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are a rich source of antioxidants and numerous other compounds, and have shown anti-carcinogenic effects in epidemiological studies [5,6]. However, clinical trials on dietary antioxidant supplementation did not report convincing protective effects against cancer incidence or mortality [7–9]. Yet, the protective effects might also be explained by other micronutrients such as isothiocyanates, allium compounds, isoflavones, protease inhibitors, saponins, and dietary fiber [10].

The potential of dietary antioxidants to reduce the cellular damage induced by ionising radiation has been recently reviewed [11]. Numerous laboratory studies have described the protective effects of vitamins and other dietary factors supplemented *before* radiation exposure, examining their radio-protective effects. However, few have reported their effects *after* irradiation. Dietary factors such as flavorings (anisaldehyde, cinamaldehide, coumarin, and vanillin) have shown to be effective even when applied after X-irradiation to reduce radiation-induced mutations [12], and chromosome aberrations [13]. These compounds have also been effective in mice [14]. Ascorbic acid and beta-carotene have reduced the radiation effects on the frequency of mutations and morphological transformations in cultured mammalian cells [15] and in vivo studies [16,17], even when added after irradiation.

As compared to laboratory experiments, human studies are very limited. Clinical trials involving persons exposed to ionising radiation during medical treatment or for professional reasons have evaluated the early effects of a supplementation of antioxidants on chronic radiation damage [11]. The findings showed a regression of the symptoms at high doses of combined antioxidants. However, little is known of the late protection effect of antioxidants on carcinogenesis, when the cellular damage was initiated by earlier radiation exposure. In animal studies, antioxidants such as selenium and Vitamin D have been suggested to prevent cancer promotion, but the effect of such antioxidants on cancer progression in humans is still unknown [2].

We have previously reported that intake of fruit and vegetables reduced significantly the risk of death from digestive and respiratory cancers [3]. In the present study, diet was assessed 35 years after atomic-bomb radiation exposure, among A-bomb survivors of Hiroshima and Nagasaki. The aim of the present study was to analyse the effect of a daily consumption of fruit and vegetables on the long-term effects of radiation on cancer risk.

2. Study subjects and methods

2.1. Study population

The life span study (LSS) is a longitudinal study of 120,321 persons who have been exposed to the atomic bombings in Hiroshima and Nagasaki, and non-exposed controls. The cohort follow-up was initiated in 1950 by the Atomic Bomb Casualty Commission (ABCC), followed by the Radiation Effects Research Foundation [18].

The part of the LSS cohort used in the present study included 36,228 individuals who were alive as of September 1, 1978, and responded to a lifestyle mail questionnaire in 1980, and for whom radiation dose estimates were currently available [19]. The prevalent cases of cancer at baseline ($n = 1159$), and the death occurring within the first 2 years of follow-up ($n = 251$) were excluded from the analyses. The total study population consisted of 34,818 persons.

Among them, 12,839 persons had dose estimates of less than 0.005 Sv (36.9% of the population). The mean dose for the remaining 21,979 cohort participants with dose estimates higher than 0.005 Sv was 0.20 Sv. The distribution of the participants by radiation dose and city is shown in Table 1.

2.2. Dosimetry/radiation dose assessment

Individual doses for survivors were estimated based on detailed models of the dose–distance relationship and of how doses are affected by shielding [18]. The dosimetry system DS86 was used, giving individual estimates of gamma ray and neutron exposures [20]. The weighted organ dose (in Sievert or Sv) was calcu-

Table 1
Number of subjects by radiation dose and city

City	Total	DS86 weighted colon dose (Sv)			
		0 (≤0.005)	0.005–0.5	0.5–1.0	>1.0
Hiroshima	26,451	9,815	15,081	950	605
Nagasaki	8,367	3,024	4,382	600	361
Total	34,818	12,839	19,463	1,550	966