is no mutation in the *hBub1* gene in gastric cancer.

3.3 Abnormal Growth Factor/Cytokine Network in Gastric Cancer

Gastric cancer cells express a broad spectrum or growth factors, cytokines or both, including TGF-α, TGF-β, EGF, amphiregulin (AR), cripto, heparin binding (HB)-EGF, PDGF, IGF II, basic fibroblast growth factor (bFGF), IL-1α, IL-6, IL-8, and OPN. These growth factors and cytokines function as autocrine, paracrine, and juxtacrine modulators for the growth of cancer cells, and they organize the complex interaction between cancer cells and stromal cells which play a key role in morphogenesis, invasion, neovascularization, and metastasis (Fig. 4). Interestingly, the expression pattern of these growth factors and cytokines by cancer cells differs in the two histological types of gastric carcinomas. The EGF family including EGF, TGFα, cripto and AR are commonly overexpressed in intestinal type carcinoma, whereas TGFβ, IGF II, and bFGF are predominantly overexpressed in diffuse type carcinoma. Coexpression of EGF/TGF-α, EGFR and cripto correlates well with the biological malignancy, as these factors induce metalloproteinases. Overexpression

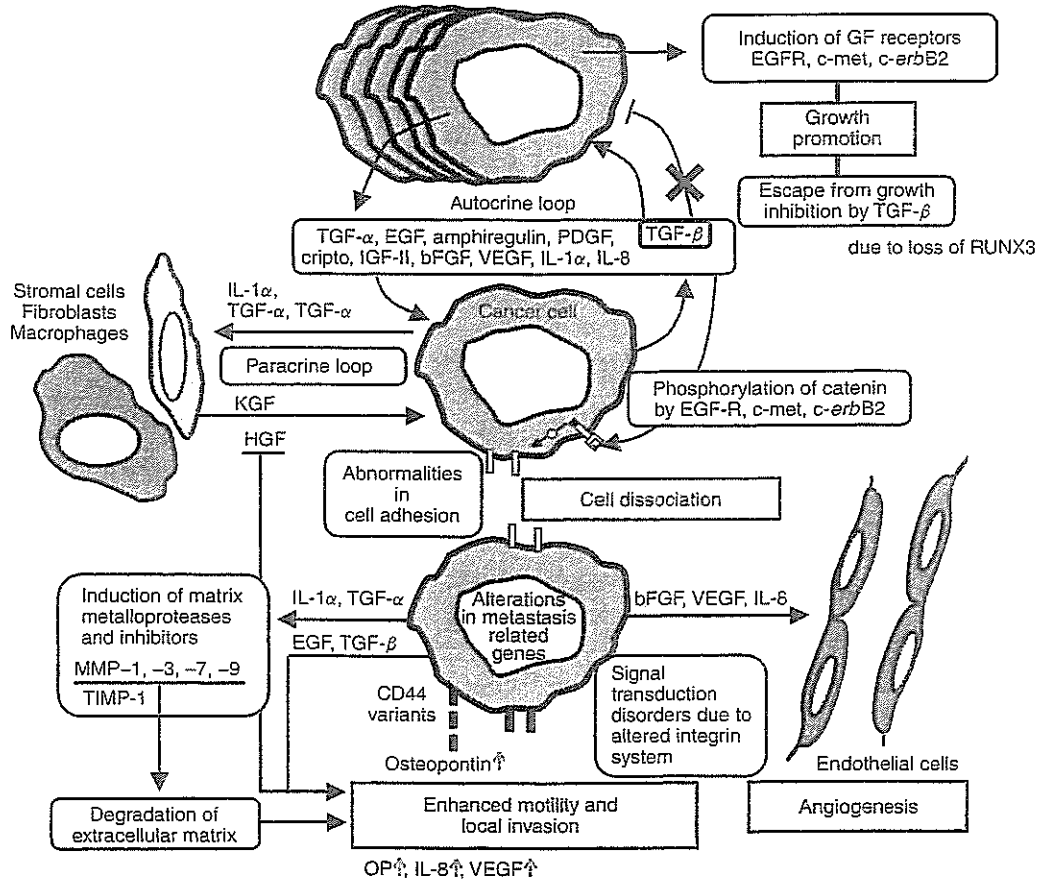

Fig. 4 Cancer-stromal interaction in gastric cancer through growth factors and cytokines.

of cripto is frequently associated with intestinal metaplasia and gastric adenoma. Akagi et al. have recently shown that gastric cancer cells express neutrophilin-1(NRP-1), which acts as a coreceptor for VEGF-165 and increases its affinity for VEGF receptor 2 endothelial cells. EGF induces both NRP-1 and VEGF expression, suggesting that regulation of NRP-1 expression in gastric cancer is intimately associated with EGF/EGFR system.

IL-1α is a cytokine mainly produced by activated macrophages through NF-κB activation and mediates many of the local and systemic responses to infection and inflammation. Gastric cancer cells also produce it. We have found that IL-1α acts as an autocrine growth factor for oral and gastric carcinoma cells and plays a pivotal role as a trigger for induction of EGF and EGFR expression. The expression of IL-1α by tumor cells is induced by either IL-1α, EGF, or TGF-α, while IL-α upregulates the expression of TGF-α and EGFR by tumor cells themselves, indicating that an intimate interplay between IL-1α and EGF/receptor system stimulates the growth of gastric cancer cells. In addition to IL-1α, IL-6 is also an autocrine growth stimulator for gastric cancer cells. The expression of IL-1α by tumor cells is induced by IL-6, while IL-1α increases the expression of IL-6 by tumor cells themselves. Currently, Fukayama's group reported that IL-1β may act as an autocrine growth factor in a human Epstein-Barr virus-associated gastric carcinoma.

IL-8, a member of the CXC chemokine family, induces haptotactic migration and proliferation of melanoma cells and angiogenesis. More importantly, gastric cancer cell lines express mRNA and protein for IL-8 and IL-8 receptors (IL-8RA and IL-8RB). More than 80% of primary tumors coexpress IL-8 and IL-8 receptor; this coexpression correlates directly with tumor vascularity and tumor progression. IL-8 enhances the expression of EGFR, type IV collagenase (MMP-9), VEGF, and IL-8 itself by tumor cells, while IL-8 decreases expression of E-cadherin. Moreover, IL-8 increases MMP-9 activity and the ability of gastric cancer cells to invade through Matrigel. IL-8 may play an important role in the growth and progression of gastric carcinoma by autocrine and paracrine mechanisms.

In addition to IL-8, VEGF and bFGF participate mainly in neovascularization in gastric cancer. We have shown that eight gastric cancer cell lines secrete VEGF into conditioned media. EGF or IL-1α upregulates VEGF expression by tumor cells, whereas interferon-γ downregulates it. VEGF promotes angiogenesis and the progression of gastric carcinoma, especially intestinal type. VEGF-C produced by tumor cells participates in the development of lymph node metastasis. On the other hand, bFGF produced by tumor cells is frequently associated with angiogenesis and extensive fibrosis in diffuse type carcinoma, particularly those of the scirrhous type. Interestingly, Nakazawa et al. reported that keratinocyte growth factor (KGF) produced by gastric fibroblasts specifically binds to K-sam on tumor cells and then stimulates proliferation of cancer cells, resulting in the development of the scirrhous type of gastric cancer. KGF from gastric fibroblasts may underline the remarkable proliferation of scirrhous gastric cancer cells in a paracrine manner.

Stromal cells, especially fibroblasts stimulated by growth factors or cytokines such as IL-1α, TGF-α, and TGFβ, secrete HGF/SF, which can function in a paracrine manner as a morphogen or mitogen of tumor cells. For example, in the case

of a cancer cell clone maintaining expression of cell adhesion molecules. HGF/SF promotes tubular formation of tumor cells, resulting in intestinal type gastric cancer. Conversely, in the case of a clone with reduced expression of cell adhesion molecules, HGF/SF can act as a mitogen and induce scattering of tumor cells, resulting in diffuse type gastric cancer.

OPN, also termed Eta-1 (early T-lymphocyte activation-1), which is a reported protein ligand of CD44, is overexpressed in 73% of gastric cancer. The coexpression of OPN and CD44 v9 in tumor cells correlates with the nodal metastasis in diffuse type gastric cancer. The *CD44* gene contains at least 20 exons, 12 of which can be alternatively spliced to make up a wide variety of molecular variants. All gastric cancer cell lines and primary tumors show overexpression of abnormal CD44 transcripts containing the intron 9 sequence. The intestinal metaplasia also expresses these variants but normal gastric mucosa does not express them. Currently, Medico et al. reported that OPN is an autocrine mediator of HGF induced invasive growth.

4 Genetic and Epigenetic Alterations and Abnormal Growth Factor/Cytokine Network in Colorectal Cancer

Cancer of the colon and rectum is the fourth most common cancer in the world. In 1996, an estimated 875 000 new cases were diagnosed worldwide, accounting for 8.5% of all new cancers. Approximately 98% of malignant colorectal tumors are adenocarcinoma. Rectal tumors account for about 27%, while almost 50% occur proximal to the splenic flexure.

The accumulation of multiple genetic and epigenetic alterations in tumor-suppressor genes, oncogenes and DNA mismatch repair genes takes place in the multistep process of colorectal carcinogenesis. Inactivation of APC, p53, and DCC and K-ras mutations are involved in a major genetic pathway for colorectal tumorigenesis showing the course of malignant progression from normal mucosal cells through adenomas (adenoma–carcinoma sequence) (Fig. 5). This section will make an overview of multiple

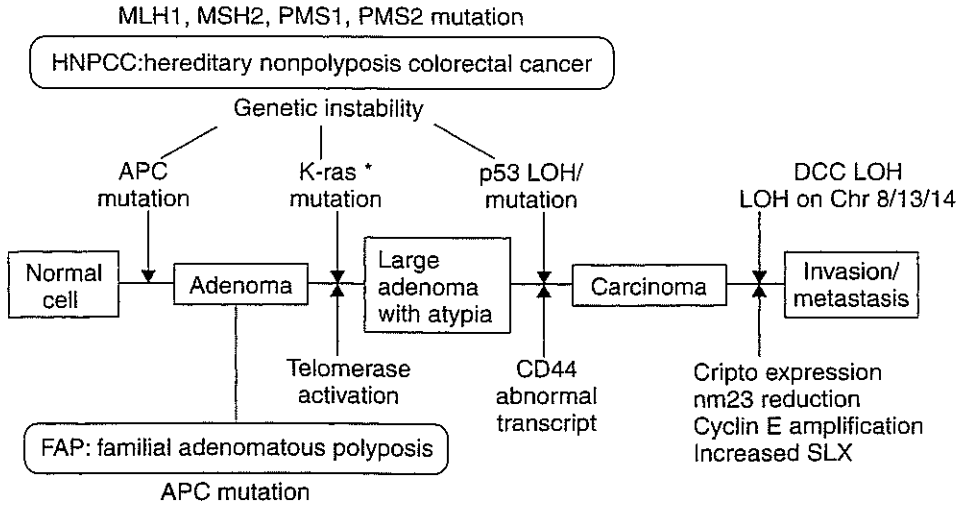

Fig. 5 A major genetic pathway for colorectal carcinogenesis. (*K-ras mutation is infrequent in flat adenoma)

genetic and epigenetic alterations responsible for colorectal carcinogenesis and abnormal growth factor/cytokine network, which is implicated in the progression and metastasis of colorectal cancer.

4.1 Genetic and Epigenetic Alterations in Colorectal Cancer

The *APC* gene, first isolated as a causative gene for familial adenomatous polyposis (FAP), encodes a large protein of 2843 amino acids, which forms a complex with α-and β-catenins and may mediate cell adhesion, cytoskeletal anchoring, and signal transduction. The *APC* gene is abnormal in the germline of FAP patients. LOH and mutations of the *APC* gene occur in 60% of sporadic colorectal adenomas and adenocarcinomas. These tumors harbor loss of the *APC* gene in one allele and mutation of the gene in the remaining allele, supporting Knudson's two-hit theory. The characteristic of the mutation is a base substitution that leads to a stop codon (nonsense mutation), which occurs in about 70% of FAP kindred. The APC alterations are found even in small adenomas with mild atypia. The tumor-suppressor function of APC has also been demonstrated in mouse models. These results and animal models show that inactivation of APC is an initiating genetic event for colorectal tumorigenesis.

The major tumor-suppression activity of APC is regulation of β-catenin. APC's association with β-catenin and promotion of the degradation of β-catenin are most relevant to its tumor-suppressor function. APC binds not only β-catenin but also glycogen synthase kinase-3β (GSK3β) and Axin. APC and Axin serve as a scaffold to facilitate the phosphorylation of β-catenin by GSK3β. The phosphorylated β-catenin is ubiquitinated by β-Trcp and the ubiquitinated β-catenin is then degraded by proteasome. Mutant APC proteins cannot regulate the degradation of β-catenin, resulting in excessive β-catenin that interacts with T-cell factor (TCF)-4 and translocates into the nucleus. The β-catenin/TCF-4 complex then activates the expression of many genes including *c-myc*, matrix metalloproteinase-7 (*MMP-7*), peroxisome proliferator-activated receptor δ (*PPARδ*) and cyclin D1, leading to promotion of colon tumorigenesis. In fact, overexpression of *c-myc*, *PPARδ* and cyclin D1 is observed in colorectal cancer. A recent study of PPARδ deficient (Ppard−/−) mice has shown that colon tumor formation is significantly greater in mice nullizygous for PPAR, suggesting that PPARδ attenuates colon carcinogenesis. It is contrary to previous reports suggesting that activation of PPARδ is causally associated with colon polyp formation. Further work is necessary to clarify the role of PPARδ in colon carcinogenesis.

On the other hand, somatic mutations of β-catenin are detected in both human and rodent colorectal tumors that do not have the APC mutation. All the β-catenin mutations found in colorectal cancers occur at the critical region for phosphorylation of β-catenin by GSK3β. The mutant β-catenin is resistant to APC-mediated degradation.

Mutation in the *K-ras* oncogene is involved in the progression from small adenoma with mild atypia to large adenoma with severe atypia. About 40 to 50% of large adenomas with severe atypia and adenocarcinomas contain K-ras point mutations at codon 12 or 13, compared to only 10% of small adenomas with mild atypia. Conversely, the frequency of K-ras mutation is lower (<10%) in superficial-type or flat adenomas even in the presence

of significant atypia. Much evidence indicates that mutation of K-ras alone can bring about a hyperplastic lesion that has a limited potential to progress to larger tumors. Mutant K-ras can, however, promote tumor progression in lesions initiated by APC mutation.

LOH of the *p53* gene locus is detected in about 80% of colorectal adenocarcinoma, and most harbor inactivation of the *p53* gene in both alleles. Because only 5 to 20% of the adenomas have p53 inactivation, it must play a crucial key in the transition from adenoma to adenocarcinoma. There are hot spots for point mutations in the highly conserved regions such as codon 175, 248, and 273, where G:C to A:T transitions occur. Abnormal accumulation of p53 protein detected by immunohistochemistry is frequently associated with deeply invasive carcinomas and carcinomas with metastasis. Almost all mutant p53 proteins derived from cancers have altered sequence-specific DNA binding and transcription activities.

Overexpression of *c-erbB2* has been reported in 80 to 100% of colorectal cancers. The amplification of *c-erbB2*, which is common in intestinal type gastric cancer, occurs in about 10% of colorectal carcinomas. Moreover, the cyclin E gene, a positive regulator of cell cycle progression, is amplified in about 10% of colorectal carcinomas. The overexpression of cyclin E is detected in 5% of adenomas and in 20% of adenocarcinomas. Among adenomas, a significant correlation is observed between cyclin E expression and the grade of atypia. Overexpression of cyclin E is prominent in carcinoma invading the submucosa or deeper compared to those limited to the mucosal layer. Cyclin E expression is thus a candidates molecular biomarker for predicting malignant progression of colorectal as well as gastric cancers. Reduction in p27 expression participates in progression and poor prognosis of colorectal cancer as well.

LOH of 18q including *DCC*, *Smad4*, and *Smad2* genes is frequently associated with advanced colorectal cancer. Among them, *DCC* and *Smad4* play important roles in colon cancer progression. LOH of the *DCC* gene is rare in adenoma but frequent (about 70%) in adenocarcinoma. LOH of *DCC* increases as the tumor invades deeply, and almost all the metastatic liver tumors show this LOH. Moreover, reduced expression of *DCC* in colorectal cancer is correlated with a poor prognosis. *DCC* encodes a receptor for netrin-1, but its function in normal colon epithelial cells remains unclear. *Smad4* encodes a protein that plays a critical role in the TGF-β signal transduction pathway. Although *Smad4* was isolated as a tumor-suppressor gene for pancreatic cancer, somatic mutations of *Smad4* frequently take place in advanced colon cancer, suggesting that Smad4 inactivation confers progression of colorectal cancer. In addition, *Smad4* germline mutations are responsible for juvenile polyposis, an autosomal disease that has high susceptibility for hamartomatous polyposis and gastrointestinal cancer.

In addition to these molecular events, and as mentioned for gastric cancer, telomere reduction may result in chromosomal instability and telomerase reactivation. The colorectal adenomas and adenocarcinomas share shorter telomeres than those in normal tissues. We have found that more than 90% of colorectal adenocarcinomas express extremely high levels of telomerase activity regardless of tumor staging and histological differentiation. All the adenomas also exhibit considerable levels of telomerase activity. Telomerase activity and stabilization of telomeres occur concomitantly with the acquisition of

immortality, contributing to an early stage of colorectal carcinogenesis.

Beside the major genetic pathway (adenoma–carcinoma sequence), an alternative genetic pathway exists for colorectal carcinogenesis. The so-called *de novo* carcinogenesis exhibits no adenoma–carcinoma sequence but develops directly from the colorectal epithelial cells that share p53 inactivation, followed by APC inactivation. K-ras mutation is not detected in *de novo* carcinogenesis. This type of colorectal carcinogenesis is frequently found in Japanese patients.

Tab. 4 Differences between MSI positive and MSI negative colorectal cancers.

	MSI +	*MSI –*
Location	Proximal	Distal
Ploidy	Near diploid	Aneuploid
Chromosomal instability	Rare	Common
Mutation Frequency		
P53	Low	High
APC	Low	High
TGFβ RII	High	Low
BAX	High	Low
Methylation	High	Low
Survival	Better	Worse
Hereditary syndrome	HNPCC	FAP

4.2 Factors Associated with Increased Incidence of Colorectal Cancer

Genetic and epigenetic alterations in DNA mismatch repair genes including hMHL1 and hMSH2 raise MSI that has implications for predisposition to colorectal cancer. The MSI occurs in 10 to 15% of sporadic colorectal cancer. These sporadic, mostly right-side colon cancers with MSI are associated with hypermethylation of the CpG islands in the hMLH1 promoter, resulting in loss of hMLH1, loss of function of other genes such as *p16*, defective mismatch repair, and widespread MSI. Hereditary nonpolyposis colorectal cancer (HNPCC), caused by inherited germline mutations in hMLH1 and hMLH2, accounts for 3 to 10% of colorectal cancer. Genes coding for TGFβ type II receptor, insulin-like growth factor (IGF)2 receptor, proapoptotic protein BAX, cell cycle regulator E2F-4, and mismatch repair proteins MSH3 and MSH6 are mutated in HNPCC or MSI sporadic colon cancer.

Some differences between MSI positive and MSI negative colorectal cancers are presented in Table 4. Tumor location, ploidy, mutation frequency, methylation, and survival are different between MSI positive and MSI negative tumors, although there are overlaps between the two.

In addition, sporadic colon cancer with MSI is also associated with altered expression of IGF2, namely, loss of imprinting (LOI) of IGF2. Importantly, the normal colonic mucosa exhibits aberrant hypermethylation and LOI of IGF2 as a sign of a field defect. However, Feinberg et al. have recently reported that hypomethylation of H19 and IGF2 is a mechanism for LOI and is found in both colorectal cancers and normal mucosa from the same patients. Moreover, they reported that LOI of IGF2 provides a potential heritable biomarker for colon cancer predisposition.

NF-κB activation is also associated with chronic inflammatory bowel diseases (IBD) and colorectal cancer. As mentioned in Sect. 3.2, NF-κB activation leads to production of enzymes such as iNOS and COX2 and enhanced expression of growth factors and cytokines. IBD including ulcerative colitis and Crohn's disease

induce persistent NF-κB activation in tissue macrophages and epithelial cells of the colonic mucosa. Both inflammatory bowel diseases are well known to increase the risk of colorectal cancer. The link between COX2 and colorectal cancer is supported strongly by epidemiological and experimental evidence. COX2 is overexpressed in adenomas and carcinomas of the colon. COX2-null mice are resistant to colorectal carcinogenesis. Long-term consumption of aspirin or other COX inhibitors has been reported to reduce the relative risk of colorectal cancer. These results indicate that COX2 contributes to colorectal tumorigenesis.

4.3 Abnormal Growth Factor/Cytokine Network in Colorectal Cancer

Colorectal carcinomas express multiple growth factors, such as EGF and TGFα and their receptors thus creating autocrine loops. TGFα and EGF are overexpressed in colorectal adenomas and the majority of colorectal carcinomas. Coexpression of TGFα, EGF or both, and EGFR is well correlated with high grade of malignancy and metastasis.

As described in gastric cancer, NRP-1 induced by EGF is expressed in all of colorectal cancer tissues and cell lines but not in the adjacent nonmalignant colonic mucosa. A recent study of NRP-1 in colon cancer suggests that NRP-1 may contribute to colon cancer angiogenesis and that EGF and mitogen-activated protein kinase signaling may play an important role in NRP-1 regulation in colon cancer cells.

The cripto gene was originally identified in undifferentiated human embryonal carcinoma cells and encodes a 37 amino acid region that shares structural homology with other members of the EGF family. However, cripto does not bind to the EGFR and its receptor has not been identified. Strong expression of mRNA and protein for cripto is found in 60 to 80% of colorectal cancers but not in normal colorectal mucosa. It is detected in 40% of tubular adenomas and 86% of tubulovillous adenomas, respectively. These findings suggest that cripto expression may be involved in the early stages of malignant transformation. Amphiregulin (AR) is another member of the EGF family that utilizes EGFR. About half of colorectal carcinomas as well as 60% of adenomas express AR. It has been confirmed that cripto and AR act as autocrine growth stimulators for colorectal cancer cell lines. Because AR is also expressed in normal colorectal epithelium, AR may participate in the regulation of growth of normal as well as colorectal cancer cells.

TGFβ-1 is expressed in over half of colorectal cancers. Interestingly, high levels of TGFβ-1 expression in tumor cells and elevated plasma levels of mRNA are associated with advanced Dukes' stage, suggesting that there is a correlation between TGFβ overexpression and tumor progression. Moreover, circulating TGFβ-1 may serve as a predictor of liver metastasis after resection of colorectal cancer. In addition, TGFβ produced by cancer cells stimulates angiogenesis by inducing thymidine phosphorylase, regulation of extracellular matrix adhesion molecules such as carcinoembryonic antigen (CEA), and by the enhanced secretion of gelatinase B, a matrix degrading enzyme.

VEGF and bFGF are also expressed strongly in colorectal cancer in contrast to normal colorectal epithelium and adenoma. The expression of bFGF is higher in Dukes stage D than in Dukes stage B colorectal cancer. Moreover, bFGF and

VEGR are elevated in the serum of patients with aggressive advanced colorectal cancer. These circulating growth factors as well as TGFβ may be useful biomarkers for understanding angiogenesis and malignancy. On the other hand, the expression of VEGF-C and VEGF-D correlates with lymph-node metastasis in colorectal carcinoma, and these expressions are heterogeneous and elevated at the invasive edge of tumors.

Activation of the pp60src protein kinase activity occurs during colorectal tumorigenesis. The kinase activity of pp60src is highly regulated and is induced by many growth factors. Recently, pp60src has been reported to be essential for the induction of VEGF by hypoxia. The specific activity of pp60src is higher in colorectal polyp than in normal mucosa, and is further increased in colorectal carcinoma and in metastatic colon tumor in liver. Further study is needed to clarify the mechanism for increased pp60src activity during colorectal carcinogenesis.

The abnormal transcripts of the *CD44* gene are also expressed in all of colorectal cancers. As mentioned in gastric cancer, the *CD44* gene consists of at least 20 exons, of which 10 are alternatively spliced to make up variants. Among several *CD44* variants, aberrant transcripts with retention of intron 9 are best for distinguishing carcinoma tissues from normal tissues in the colorectum. However, the variants do not correlate with nodal or distant metastasis.

A candidate suppressor gene of tumor metastasis, *nm23*, encodes nucleotide diphosphate kinase and c-myc transcription factor (PuF). Although most of colorectal cancers express nm23 at higher levels than the corresponding normal mucosa, an inverse correlation is observed between nm23 expression and tumor staging. Moreover, reduced expression of nm23 is associated with distant metastasis.

Another candidate for a molecular marker that indicates metastatic potential is cell surface carbohydrate, sialyl-dimeric Le antigens. Both sialyl Lex (SLX) and sialyl Lea (SLA or Ca 19-9) as ligands bind to E-selectin, also known as ELAM-1, one of the adhesion molecules on activated endothelial cells. SLX and SLA may participate in distant metastasis through interaction between cancer cells and endothelial cells. The expression of SLX in colorectal carcinomas shows significant correlation with liver metastasis and poor prognosis.

5 Conclusion

A large number of molecular events are involved in the development and progression of gastrointestinal carcinomas. Among them, common and distinct events of genetic and epigenetic alterations are observed in esophageal, gastric, and colorectal cancers. MSI confers the initial step of gastric and colorectal carcinomas, while it is less involved in esophageal SCC. Chromosomal instability (telomere reduction) and telomerase activation participate commonly in the very early stage of gastrointestinal carcinogenesis. p53 inactivation and RUNX3 loss by promoter hypermethylation are also common events, although RUNX3 loss is less in colorectal cancer. APC LOH and DCC LOH are commonly detected in the majority of the three gastrointestinal cancers, although APC mutations occur mainly in colorectal cancer. K-ras mutation is often found in colorectal cancer, whereas it is extremely rare in esophageal SCC and gastric cancer. Amplification of the cyclin D1 gene

is preferentially found in esophageal SCC, while the gene amplification of cycline E is frequently associated with both gastric and colorectal adenocarcinomas. Reduced expression of the CDK inhibitors such as p16 and p27 is often found in gastrointestinal cancers. In gastric cancer, the pattern of genetic and epigenetic alterations also differs depending on the two histological types, intestinal or well-differentiated type and diffuse or poorly differentiated type. The amplification of *c-met* and *K-sam* genes and the mutation/loss of the E-cadherin gene as well as *RARβ2* loss occur preferentially in diffuse type, whereas the amplification of *c-erbB2* gene, pS2 reduction, p16 loss and hMLH1 loss as well as APC mutation is predominantly found in intestinal type.

In addition to these events, gastrointestinal cancer cells express a broad spectrum of the growth factor/cytokine receptor systems that organize complex interactions between cancer cells and stromal cells, which confer cell growth, apoptosis, morphogenesis, angiogenesis, progression and metastasis. However, these abnormal growth factor/cytokine networks are also evidently different among esophageal, gastric, and colorectal cancers, respectively. Importantly, NF-κB activation induced by inflammation may act as a key player for induction of growth factor/cytokine networks in gastrointestinal cancers.

Overall, the observations on the molecular events involving growth factors and oncogenes in gastrointestinal cancers will no doubt provide a deeper understanding of prevention, molecular diagnosis, and therapeutics of these cancers. In fact, by applying these molecular events of gastrointestinal cancers to routine clinical practice, we have implemented molecular pathological diagnosis of gastrointestinal cancer in collaboration with Hiroshima City Medical Clinical Laboratory since 1993. We have analyzed more than 10 000 cases of gastrointestinal biopsy and surgery and then obtained additional information on differential diagnosis, biological malignancy, and tumor multiplicity. We believe this approach will better serve science, but more importantly, patient care.

See also Growth Factors; Oncology, Molecular.

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Harvesting Chemical Energy: *see* Metabolic Basis of Cellular Energy

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Gene Expression Profile of Gastric Carcinoma: Identification of Genes and Tags Potentially Involved in Invasion, Metastasis, and Carcinogenesis by Serial Analysis of Gene Expression

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ABSTRACT

Gastric carcinoma (GC) is one of the most common malignancies worldwide. To better understand the genetic basis of this disease, we performed serial analysis of gene expression (SAGE) on four primary GC samples and one associated lymph node metastasis. We obtained a total of 137,706 expressed tags (Gene Expression Omnibus accession number GSE 545, SAGE Hiroshima gastric cancer tissue), including 38,903 that were unique. Comparing tags from our GC libraries containing different stages and different histologies, we found several genes and tags that are potentially involved in invasion, metastasis, and carcinogenesis. Among these, we selected 27 genes and measured mRNA expression levels in an additional 46 GC samples by quantitative reverse transcription-PCR. Frequently overexpressed genes (tumor/normal ratio > 2) were *COL1A1* (percentage of cases with overexpression, 78.3%), *CDH17* (73.9%), *APOC1* (67.4%), *COL1A2* (58.7%), *YF13H12* (52.2%), *CEACAM6* (50.0%), *APOE* (50.0%), *REGIV* (47.8%), *S100A11* (41.3%), and *FUS* (41.3%). Among these genes, mRNA expression levels of *CDH17* and *APOE* were associated with depth of tumor invasion ($P = 0.0060$ and $P = 0.0139$, respectively), and those of *FUS* and *APOE* were associated with degree of lymph node metastasis ($P = 0.0416$ and $P = 0.0006$, respectively). In addition, mRNA expression levels of *FUS*, *COL1A1*, *COL1A2*, and *APOE* were associated with stage ($P = 0.0414$, $P = 0.0156$, $P = 0.0395$, and $P = 0.0125$, respectively). Quantitative reverse transcription-PCR analysis also showed a high level of *REGIV* expression (>100 arbitrary units) in 14 of 46 GC samples (30.4%) but not in noncancerous tissues. We detected V5-tagged RegIV protein in the culture media of cells transfected with pcDNA-RegIV-V5 by Western blot. Our results provide a list of candidate genes that are potentially involved in invasion, metastasis, and carcinogenesis of GC. *REGIV* may serve as a specific biomarker for GC.

INTRODUCTION

Gastric carcinoma (GC) is one of the most common human cancers. Despite improvements in cancer therapy, ~650,000 patients with GC die/year (1). A variety of genetic and epigenetic alterations are associated with GC (2–4). However, the underlying mechanism of gastric carcinogenesis is still poorly understood. To identify potential molecular markers for GC and to better understand the development of GC at the molecular level, comprehensive gene expression analysis may be useful. Although several large-scale gene expression studies with cDNA or oligonucleotide arrays have been performed in GC (5–8), they have used different platforms that varied in the number and identity of genes printed on them. On the other hand, serial analysis of gene expression (SAGE) analyzes 14-bp tags derived from defined positions of cDNAs without *a priori* knowledge of the sequence of the genes expressed (9). Thus, SAGE offers an unbiased, comprehensive gene expression profiling approach. Recently, three SAGE studies of GC were reported, and several up-regulated and down-regulated genes were identified (10–12). However, only one (10) or two samples (11, 12) were examined, and the relation to invasion and metastasis was not analyzed. In the present study, we performed SAGE analysis on four samples of GC of different stages and different histologies. In addition, we performed SAGE analysis on one lymph node metastasis of GC. We report here the identification of several genes and tags potentially involved in invasion, metastasis, and carcinogenesis of GC. Among these, we focused on the *REGIV* gene because this gene is frequently overexpressed in GC, and *REGIV* expression is narrowly restricted in noncancerous tissues. In addition, the amino acid sequence of the RegIV protein suggests that it may be secreted.

MATERIALS AND METHODS

Tissue Samples. For SAGE analysis, four primary GC samples and 1 associated lymph node metastasis were used (Table 1). We confirmed microscopically that the tumor specimens consisted mainly (>80%) of carcinoma tissue with the exception of S219T. For quantitative reverse transcription-PCR (RT-PCR), 46 GC samples and corresponding nonneoplastic mucosa samples were used. Of the 46 GC samples, lymph node metastasis samples were available for 9. The samples were obtained at surgery at Hiroshima University Hospital and affiliated hospitals. Noncancerous samples of the heart, aorta, lung, tongue, esophagus, stomach, duodenum, ileum, colon, liver, gallbladder, pancreas, kidney, urinary bladder, thyroid gland, adrenal gland, spleen, skin, endometrium, and lymph node were obtained at autopsy from a 28-year-old woman diagnosed with multiple sclerosis. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. Histological classification of GC was performed according to the Lauren classification system (13). In addition, diffuse-type GC samples were additionally classified into diffuse-adherent and diffuse-scattered subtypes (14). Tumor staging was carried out according to the Tumor-Node-Metastasis Stage Grouping (15).

SAGE. SAGE was performed 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 (The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine). Clinicopathological details of the 5 samples are shown in Table 1. To identify genes involved in tumor progression, we analyzed 2 GC samples (W226T and W246T). Both samples were classified as intestinal type GC. However, W226T was early, and W246T was advanced. Early GC is limited to the mucosa or the mucosa and submucosa, regardless of nodal status (16). We confirmed microscopically that these 2 samples showed similar histological features (Fig. 1). To identify genes involved in tumor metastasis, we analyzed 1 GC sample (P208T) and its lymph node metastasis (P208L). Histologically, these samples were classified as diffuse-adherent type, and we confirmed microscopically that both the primary tumor (P208T) and the metastatic tumor (P208L) contained few stromal cells and lymphocytes (Fig. 1). Scirrhous-type GC belongs to the diffuse-scattered type, often occurs in young women, and is characterized by extensive fibrous stroma, infiltrative and rapid growth, and poor prognosis (17). Sample S219T was a scirrhous-type GC showing scattering growth in an abundant fibrous stroma (Fig. 1). To

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Table 1 *Clinicopathological details of the 5 samples analyzed by serial analysis of gene expression*

Sample name	Sample type	Sex	Age (yrs)	Tumor[a]	Node[a]	Metastasis[a]	Stage[a]	Histological classification[b]	Total number of tags	Number of unique tags in each sample
W226T	Primary	Male	59	1	0	0	IA	Intestinal	43,908	16,082
W246T	Primary	Male	44	2	2	0	IIIA	Intestinal	32,174	12,792
S219T	Primary	Female	29	3	3	0	IV	Diffuse-scattered	34,660	14,576
P208T	Primary	Male	60	4	3	0	IV	Diffuse-adherent	11,582	6,135
P208L	Lymph node metastasis of P208T								15,382	7,425
									137,706[c]	38,903[d]

[a] Tumor staging of gastric carcinoma (GC) were done according to the Tumor-Node-Metastasis Stage Grouping (15).
[b] Histological classification of GC was done according to the Lauren classification system (13). In addition, diffuse-type GC were additionally classified into diffuse-adherent and diffuse-scattered subtypes (14).
[c] Total number of tags of 5 GC samples.
[d] Total number of unique tags among 5 GC samples.

permit direct comparison, each library was normalized to a total of 1,000,000 tags.

Cluster Analysis. The Cluster and TreeView computer programs were obtained from online resources.[1] We compared SAGE tags from 4 primary GC samples with those from samples of normal gastric epithelia [GSM784, SAGE normal gastric body epithelial (10)], available from SAGEmap (18).[2] We also compared SAGE tags from 2 primary GC samples, also available from SAGEmap [GSM757, SAGE gastric cancer-G234 (10) and GSM2385, SAGE gastric cancer-G189] with those from normal gastric epithelia (GSM784) and obtained the 20 most up-regulated and 20 most down-regulated tags. This produced a dataset of 128 tags. These data were imported into the Cluster program and were log-transformed, and complete linkage clustering was performed.

Quantitative RT-PCR Analysis. Total RNA was extracted with an RNeasy Mini kit (Qiagen, Hilden, Germany), and 1 μg of total RNA was converted to cDNA with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). PCR was performed with a SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously (19). The sequences primer are listed in Supplementary Table 1. We calculated the ratio of target gene mRNA expression levels between GC tissue (T) and corresponding nonneoplastic mucosa (N). T/N ratios > 2-fold were considered to represent overexpression. Genes with T/N ratios > 2 in >40% of the samples examined were defined as frequently up-regulated genes.

Cell Lines, Expression Vector, and Western Blot. Two cell lines derived from human GC were used. MKN-28 was kindly provided by Dr. Toshimitsu Suzuki. HSC-39 was kindly provided by Dr. Kazuyoshi Yanagihara (20). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For constitutive expression of the *RegIV* gene, cDNA was PCR amplified and subcloned into pcDNA 3.1 (Invitrogen Corp., Carlsbad, CA) in-frame with a COOH-terminal V5 epitope tag. MKN-28 cells were transfected transiently with *REGIV* cDNA using FuGene6 Transfection Reagent (Roche Diagnostics Co., Indianapolis, IN) according to the manufacturer's instructions. For Western blot analysis, cells and culture media from MKN-28 cells transfected with pcDNA 3.1 or pcDNA-RegIV-V5 were lysed as described previously (21). The culture media was concentrated with a PROTEIN concentrate kit (Takara Bio, Inc., Shiga, Japan). The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then were subjected to 15% SDS-PAGE followed by electrotransfer onto a nitrocellulose filter. Anti-V5 monoclonal antibody was purchased from Invitrogen Corp. Peroxidase-conjugated antimouse IgG was used as the secondary antibody. The immune complex was visualized with an ECL Western blot detection system (Amersham Pharmacia Biotech).

Statistical Methods. Statistical analyses were performed with the Mann-Whitney *U* test. *P* of <0.05 was regarded as statistically significant.

[1] Internet address: http://www.microarrays.org/software.html.
[2] Internet address: http://www.ncbi.nlm.nih.gov/SAGE/.

RESULTS

Generation of SAGE Data. A total of 137,706 tags was generated, including 38,903 that were unique. The numbers of tags and unique tags are shown in Table 1. Sequence data from our SAGE libraries are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue).

Comparison of Expression Patterns in GCs and Normal Stomach. We compared SAGE tags from 4 primary GC samples with those from normal gastric epithelia (GSM784). The 20 most up-regulated tags and 20 most down-regulated tags in each GC are shown in Supplementary Table 2. Among the up-regulated tags, 12 were commonly up-regulated in both W226T (intestinal type) and W246T (intestinal type). These tags included *lysozyme* (*LYZ*), *trefoil factor 3* (*TFF3*), *aldolase A* (*ALDOA*), and *S100 calcium-binding protein*, which may participate in the genesis of intestinal type GC. P208T (diffuse-adherent type) and S219T (diffuse-scattered type) showed many different tags from those of W226T and W246T. The down-regulated tags were similar in all 4 GC samples and included *lipase* (*LIPF*), *pepsinogen* (*PGA5*), and *antrum mucosa protein* (*AMP18*), which are expressed physiologically in normal gastric glands.

The SAGE data were also analyzed by a clustering algorithm to delineate patterns in the expression of 128 tags among all four libraries (our four GC libraries, our one lymph node metastasis library, and three libraries available from SAGEmap[2]; Fig. 2). These tags were selected as described in "Materials and Methods." Clusters of coexpressed tags suggested that the two intestinal type GC libraries (W226T and W246T), despite being derived from 2 different patients at different stages, were the most similar to each other. The primary GC (P208T) and its lymph node metastasis (P208L) appeared not to be similar to each other. To identify ideal biomarkers for GC, we focused on a cluster of 14 tags, the expression of which was up-regulated in 6 GC samples (our four GC libraries plus two GC libraries available from SAGEmap) and 1 lymph node metastasis. Because some genes share the same SAGE tag, these 14 tags represented 22 genes (Fig. 2 and Table 2). To validate the SAGE data, the expression of 12 known genes was analyzed by quantitative RT-PCR of an additional 46 GC samples and corresponding nonneoplastic mucosa samples. Frequently overexpressed genes were *APOC1* (percentage of samples with overexpression: T/N ratio > 2; 67.4%), *YF13H12* (52.2%), and *CEACAM6* (50.0%; Fig. 3*A*, see also Supplementary Fig. 1). Other genes were less frequently overexpressed. The expression levels of all 12 genes were not associated with T grade (depth of tumor invasion), N grade (degree of lymph node metastasis), or tumor stage.

Comparison of Expression Patterns in Early and Advanced GC. To identify genes involved in tumor progression, we compared tags from early GC (W226T) and advanced GC (W246T). The 10

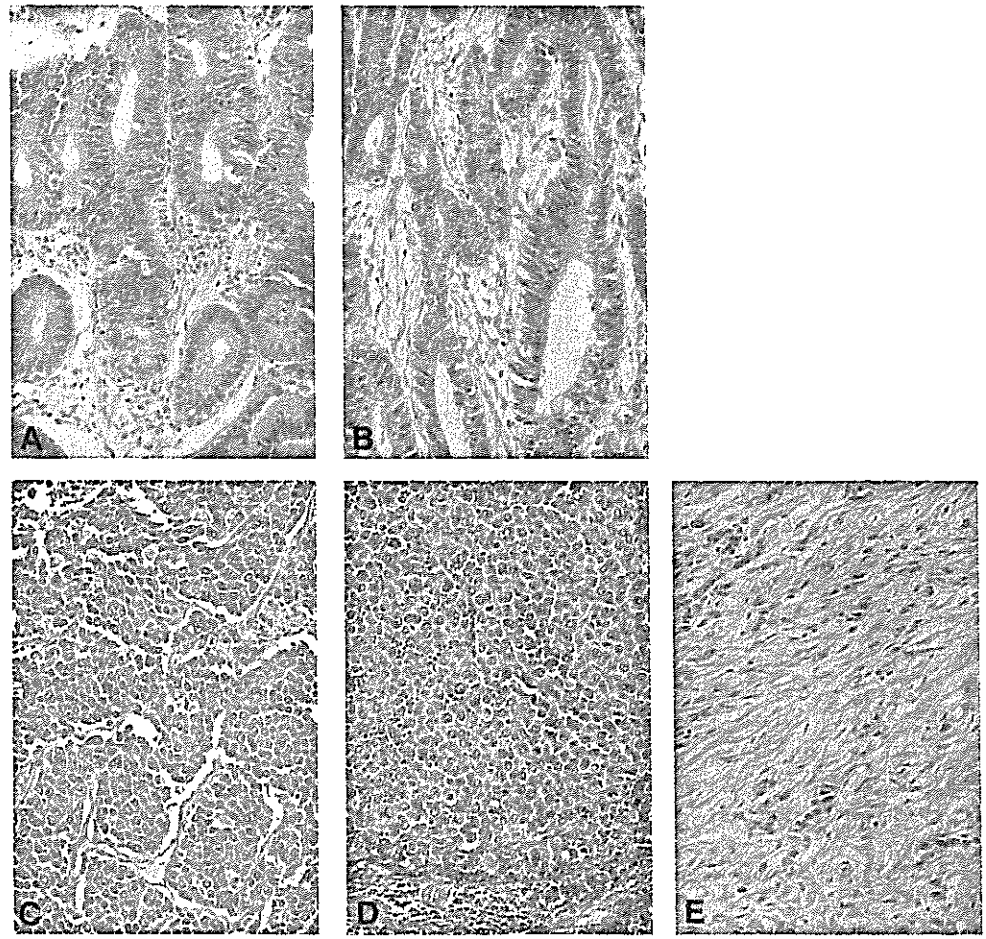

Fig. 1. Histological features of gastric carcinoma (GC) samples analyzed by serial analysis of gene expression. Formalin-fixed, paraffin-embedded sections were stained with H&E. Both W226T (*A*) and W246T (*B*) were to intestinal type GC, and histological features were similar. Both P208T (*C*) and P208L (*D*) were diffuse-adherent type GC. P208L was a lymph-node metastasis of P208T. S219T (*E*) was diffuse-scattered type GC. Obvious histological heterogeneity was not seen in all specimens (original magnification, ×100).

most up-regulated tags are shown in Table 3, and the 10 most down-regulated tags are shown in Supplementary Table 3. Because some genes share the same SAGE tag, these up-regulated 10 tags represented 12 genes (Table 3). To validate the SAGE data, the expression of 9 known genes was analyzed by quantitative RT-PCR of an additional 46 GC samples and corresponding nonneoplastic mucosa samples. Genes frequently overexpressed in GC compared with nonneoplastic mucosa were *COL1A1* (78.3%), *CDH17* (73.9%), *COL1A2* (58.7%), and *FUS* (41.3%; Fig. 3*B*, see also Supplementary Fig. 2). Other genes were less frequently overexpressed. The mRNA expression levels of *CDH17* were associated with T grade ($P = 0.0060$). The mRNA expression levels of *FUS* were associated with N grade ($P = 0.0416$). The mRNA expression levels of *FUS*, *COL1A1*, and *COL1A2* were associated with tumor stage ($P = 0.0414$, $P = 0.0156$, and $P = 0.0395$, respectively; Table 4).

Comparison of Expression Patterns in Primary GC and Associated Lymph Node Metastasis. To identify genes involved in tumor metastasis, we compared tags from primary GC (P208T) and its lymph node metastasis (P208L). The 10 most up-regulated tags are shown in Table 5, and the 10 most down-regulated tags are shown in Supplementary Table 4. The up-regulated tags represented 12 genes (Table 5). To validate the SAGE data, the expression of 5 known genes was analyzed by quantitative RT-PCR of an additional 46 GC samples and their lymph node metastases in 9 samples. A frequently overexpressed gene in lymph node metastasis compared with primary GC was not found (Fig. 3*C*, see also Supplementary Fig. 3). *APOE* mRNA expression in lymph node metastasis tended to be higher than that in primary GC. Other genes were less frequently overexpressed. Genes frequently overexpressed in GC compared with nonneoplastic

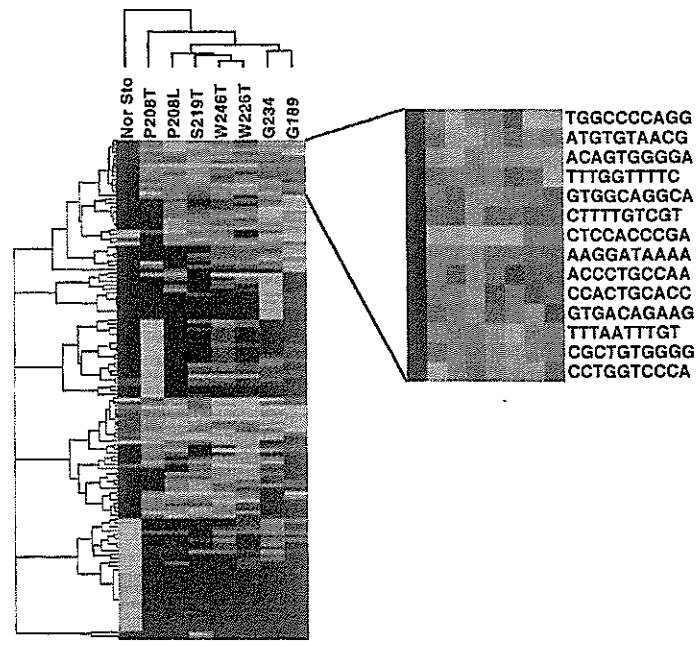

Fig. 2. Cluster analysis of 128 tags form eight serial analysis of gene expression (SAGE) libraries and dendrogram showing similarities in expression patterns among libraries. Tags were selected as described in the "Materials and Methods." *Brackets* indicate the cluster of tags commonly up-regulated in gastric carcinoma, which is expanded in size on the right for visualization. On the dendrogram, two intestinal type gastric carcinoma samples cluster together, indicating their high degree of similarity. Each row represents a tag, whereas each column corresponds to a SAGE library sample. The absolute abundance of the SAGE tag in the library (SAGE tag number) correlates with the intensity of the red color (*black*, not present; *intense red*, highly abundant).

Table 2 *Genes and tags commonly up-regulated in gastric carcinoma obtained by serial analysis of gene expression*

Tag sequence	UniGene ID	Symbol	Description
TGGCCCCAGG	Hs.268571	*APOC1*	Apolipoprotein C-I
ATGTGTAACG	Hs.81256	*S100A4*	S100 calcium binding protein A4 (calcium protein, Calvasculin, metastasin, murine placental homologue)
	Hs.173611	*NDUFS2*	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49 kDa (NADH-coenzyme Q reductase)
ACAGTGGGGA	Hs.278270	*TEBP*	Unactive progesterone receptor, 23 kDa
	Hs.288443		Homosapiens transcribed sequences
	Hs.355693		Homosapiens transcribed sequence with strong similarity to protein pir:A56211 (H. sapiens) A56211 progesterone receptor-related protein P23-human
TTTGGTTTTC	Hs.179573	*COL1A2*	Collagen, type I, α2
	Hs.21431	*SUFU*	Suppressor of fused homologue (Drosophila)
GTGGCAGGCA	Hs.47334	*SYAP1*	Synapse associated protein 1, SAP47 homologue (Drosophila)
	Hs.13255	*KIAA0930*	KIAA0930 protein
CTTTTGTCGT	Hs.19597	*KIAA1694*	KIAA1694 protein
CTCCACCCGA	Hs.82961	*TFF3*	Trefoil factor 3 (intestinal)
AAGGATAAAA	Hs.73848	*CEACAM6*	Carcinoembryonic antigen-related cell adhesion molecule 6 (nonspecific cross reacting antigen)
ACCCTGCCAA	Hs.405871	*FLJ20249*	Hypothetical protein FLJ20249
CCACTGCACC	Hs.6853	*FLJ22167*	Hypothetical protein FLJ22167
	Hs.146844		na similar to hypothetical protein FLJ10891
GTGACAGAAG	Hs.356129	*EIF4A1*	Eukaryotic translation initiation factor 4A, isoform 1
TTTAATTTGT	Hs.182793	*GOLPH2*	Golgi phosphoprotein 2
	Hs.220689	*G3BP*	Ras-GTPase-activating protein SH3-domain-binding protein
CGCTGTGGGG	Hs.7486	*YF13H12*	Protein expressed in thyroid
CCTGGTCCCA	Hs.23881	*KRT7*	Keratin 7
	Hs.167679	*SH3BP2*	SH3-domain binding protein 2
TGGAAATGAC	Hs.172928	*COL1A1*	Collagen, type I, α1
	Hs.20506	*LOC284371*	Hypothetical protein LOC284371

mucosa were *APOE* (50.0%) and *S100A11* (41.3%; Fig. 3*D*). The mRNA expression levels of *APOE* were associated with T grade ($P = 0.0139$), N grade ($P = 0.0006$), and tumor stage ($P = 0.0125$; Table 4).

REGIV Overexpression in GC. Among the 20 up-regulated tags in each GC sample (Supplementary Table 2), we focused on *REGIV* because *REGIV* expression was narrowly restricted by Virtual Northern analysis by SAGEmap (Fig. 4*A*). Besides GCs, *REGIV* was detected at low levels in only eight libraries, including one colon cancer and two normal colon libraries. Quantitative RT-PCR analysis showed overexpression of the *REGIV* gene in 22 samples of the 46 GC samples (47.8%; Fig. 4*B*). When we focused on *REGIV* gene expression in GC, high levels of *REGIV* expression (>100, arbitrary units) were found in 14 of 46 samples (30.4%; Fig. 4*C*). Among various normal tissues obtained from an autopsy, obvious *REGIV* expression was found in noncancerous stomach, duodenum, ileum, colon, and pancreas, as reported elsewhere (22). However, the levels of *REGIV* expression were low (<60 arbitrary units).

Analysis of the amino acid sequence of the RegIV protein suggests that it may be secreted. To investigate whether RegIV is a secreted protein, we performed Western blot analysis of cell extracts and culture media of MKN-28 cells transiently transfected with pcDNA 3.1 or pcDNA-RegIV-V5. With an anti-V5 antibody, we detected an approximate M_r 20,000 band corresponding to V5-tagged RegIV protein in cell extracts and culture media from RegIV-V5-expressing MKN-28 cells but not in control cells (Fig. 4*D*).

DISCUSSION

To identify potential molecular markers for GC and to better understand the development of GC at the molecular level, we performed SAGE on 5 GC samples from 4 patients that showed distinct histological types and tumor stages. We analyzed with respect to (*a*) commonly up-regulated genes in GC compared with normal stomach, (*b*) up-regulated genes in advanced compared with early GC (genes potentially involved in tumor progression), (*c*) up-regulated genes in GC lymph node metastasis compared with primary GC (genes potentially involved in tumor metastasis), and (*d*) genes specifically ex-

Table 3 *The 10 most up-regulated tags in advanced gastric carcinoma in comparison with early gastric carcinoma*

Tag sequence	Tags/million W226T	Tags/million W246T	UniGene ID	Symbol	Description
TCCCCGTAAA	22[a] (1)[b]	559 (18)			No match
TCCCGTACAT	0 (0)	279 (9)			No match
AAAAGAGTGG	0 (0)	217 (7)	Hs.89436	*CDH17*	Cadherin 17, LI cadherin (liver-intestine)
			Hs.99969	*FUS*	Fusion, derived from t(12;16) malignant liposarcoma
CCAGAGAACT	0 (0)	217 (7)	Hs.356442	*PRO1073*	PRO1073 protein
AACCTCCCCA	0 (0)	186 (6)	Hs.137396		Sapiens cDNA FLJ36926 fis, clone BRACE2005196
			Hs.232092		Sapiens cDNA FLJ30146 fis, clone BRACE2000256
AATACTTTTG	0 (0)	186 (6)	Hs.356427	*PAI-RBP1*	PAI-1 mRNA-binding protein
			Hs.179573	*COL1A2*	Collagen, type I, α2
TCCTATTAAG	22 (1)	372 (12)			No match
TGGAAATGAC	0 (0)	186 (6)	Hs.172928	*COL1A1*	Collagen, type I, α1
			Hs.193076	*GRAP2*	GRB2-related adaptor protein 2
TCCCCGTACA	227 (10)	3325 (107)	Hs.2730	*HNRPL*	Heterogeneous nuclear ribonucleoprotein L
			Hs.151734	*NUTF2*	Nuclear transport factor 2
AAGTGAAACA	0 (0)	155 (5)	Hs.93659	*ERP70*	Protein disulfide isomerase-related protein (calcium-binding protein, intestinal-related)

[a] The absolute tag counts are normalized to 1,000,000 total tags/sample.
[b] Number in parentheses indicates the absolute tag counts.