

important role in inactivation of the *RIZ1* gene. Previous studies have demonstrated that CpG island hypermethylation in the region examined was well correlated with epigenetic silencing of the *RIZ1* gene.^(17,18) In the present study, DNA methylation of the *RIZ1* gene was not associated with clinicopathological features. Because DNA methylation of *RIZ1* is a rare event in non-malignant prostate tissues,⁽¹⁹⁾ these results suggest that DNA methylation of the *RIZ1* gene may be associated with prostate carcinogenesis.

It has been reported that *p53* is mutated in late-stage PCa.⁽²¹⁾ In the present study, nuclear accumulation of *p53* was observed only in stage III PCa, and all PCa tissues with nuclear accumulation of *p53* showed DNA methylation of *RIZ1*. In the present study, of the four PCa specimens with nuclear accumulation of *p53*, only two exhibited a *p53* mutation. These inconsistent results between immunohistochemistry and PCR-SSCP may be due to methodological differences. Because only exons 5–8 were examined by PCR-SSCP in the present study, the mutation of *p53* may be in the remaining exons. It is also possible that the sensitivity of PCR-SSCP may not be sufficient to detect a small number of mutant alleles in a background of wild-type alleles from stromal cells and normal epithelial cells. It has been reported that the correlation between nuclear accumulation of *p53* and the presence of *p53* gene mutations can vary.⁽³⁰⁾

RIZ1 has HMT activity.⁽⁹⁾ HMT activity is thought to be important to the tumor suppression function of *RIZ1*, because this activity is reduced by *RIZ1* mutations found in human cancers.⁽⁹⁾ Because histone modification is thought to affect chromatin structure directly,⁽³¹⁾ aberrant chromatin structure may induce mutation of the *p53* gene. In fact, *Suv39h* (an HMT)-deficient mice display severely impaired viability and chromosomal instabilities.⁽³²⁾ A subset of PCa cells with mutations in *p53* may arise from PCa cells with DNA methylation of *RIZ1*. Therefore, DNA methylation of *RIZ1* may predict development

of *p53* mutant PCa cells. In the PCa cell lines, *p53* mutation status has been described previously (PC3, mutant-type *p53*; LNCaP, wild-type *p53*; DU145, mutant-type *p53*).⁽³³⁾ Because DNA hypermethylation of *RIZ1* was detected in PC3 cells, mutation of the *p53* gene may be induced by inactivation of *RIZ1*. In contrast, DNA hypermethylation of *RIZ1* was not detected in DU145 cells despite the presence of *p53* mutation. Whether silencing of *RIZ1* induces *p53* mutation should be verified experimentally in the near future.

Treatment options for the early stages of PCa have been limited to local treatment. Treatments for more advanced disease rely on suppression of testosterone production, primarily with hormonal therapy. Until the introduction of luteinizing hormone-releasing hormone agonist therapy, estrogen therapy was often used for hormonal manipulation of PCa.⁽³⁴⁾ Because estradiol treatment produces a selective decrease in *RIZ1* expression,⁽³⁵⁾ it is interesting to investigate the *RIZ1* expression in PCa tissues from patients who received preoperative hormone treatment.

In conclusion, our results suggest that genetic alterations are associated with epigenetic alterations and that these alterations are not random events in PCa. To better understand the development of PCa at the molecular level, molecular classification of PCa based on genetic and epigenetic alterations may be useful.

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MOLECULAR PATHOBIOLOGY OF GASTRIC CANCER

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ABSTRACT

Gastric carcinogenesis is a multistep process, during which numerous genetic and epigenetic alterations accumulate: there are abnormalities of growth factors/receptors, angiogenic factors, cell cycle regulators, DNA mismatch repair genes etc. These abnormalities define, at the same time, the biological character of the cancer cell and may thus serve as therapeutic targets. Genetic instability may cause accumulation of genetic abnormalities. The most important epigenetic alterations are DNA methylation, histone modification and chromatin remodeling. Some of these changes are common in gastric cancer, regardless of subtype, and some differ by histological type or (gastric or intestinal) mucin phenotype. Genetic polymorphism is a crucial endogenous cause and fundamental aspect of cancer risk. Importantly, genetic polymorphisms are also associated with the therapeutic efficacy and toxicity of anti-cancer drugs. Genomic science and technology such as Serial Analysis of Gene Expression (SAGE) allows the identification of novel genes and molecules specifically up-regulated or down-regulated in gastric cancer, e.g., RegIV and claudin-18 can be identified. Advances in our understanding of the genetic and molecular bases lead to improved diagnosis, personalised medicine and prevention of gastric cancer.

Key words: Gastric cancer; molecular pathology; cell cycle regulator; angiogenic factor; genetic instability; epigenetics; serial analysis of gene expression

INTRODUCTION

Gastric cancer is one of the most common human cancers worldwide. Although recent diagnostic and therapeutic advances have provided excellent survival for patients with early gastric cancer, the prognosis of patients with advanced cancer is still poor. The pathogenesis of gastric cancer involves *Helicobacter pylori* (*H. pylori*) infection, chronic active or atrophic gastritis and intestinal metaplasia. Integrated research in molecular pathology has shown that

gastric cancer is a chronic proliferative disease characterised by multiple genetic and epigenetic alterations, i.e., a disease of altered gene expression (1-5). Genetic polymorphism is an important endogenous cause and a modulator of the risk of a person of developing gastric cancer. Advances of genomic science, including global analysis of gene expression, have uncovered detailed molecular mechanisms of gastric carcinogenesis (5, 6). A better understanding of the molecular bases of gastric cancer may lead to new diagnostic, therapeutic and preventive approaches to the disease. Recently identified molecules that participate in carcinogenesis appear to be useful targets for cancer therapy.

This review describes an outline of the molecular pathway of stomach carcinogenesis, of the importance of epigenetic alterations, of the significance of genetic polymorphism, and of the biological and clinical roles of recently identified genes involved in gastric cancer.

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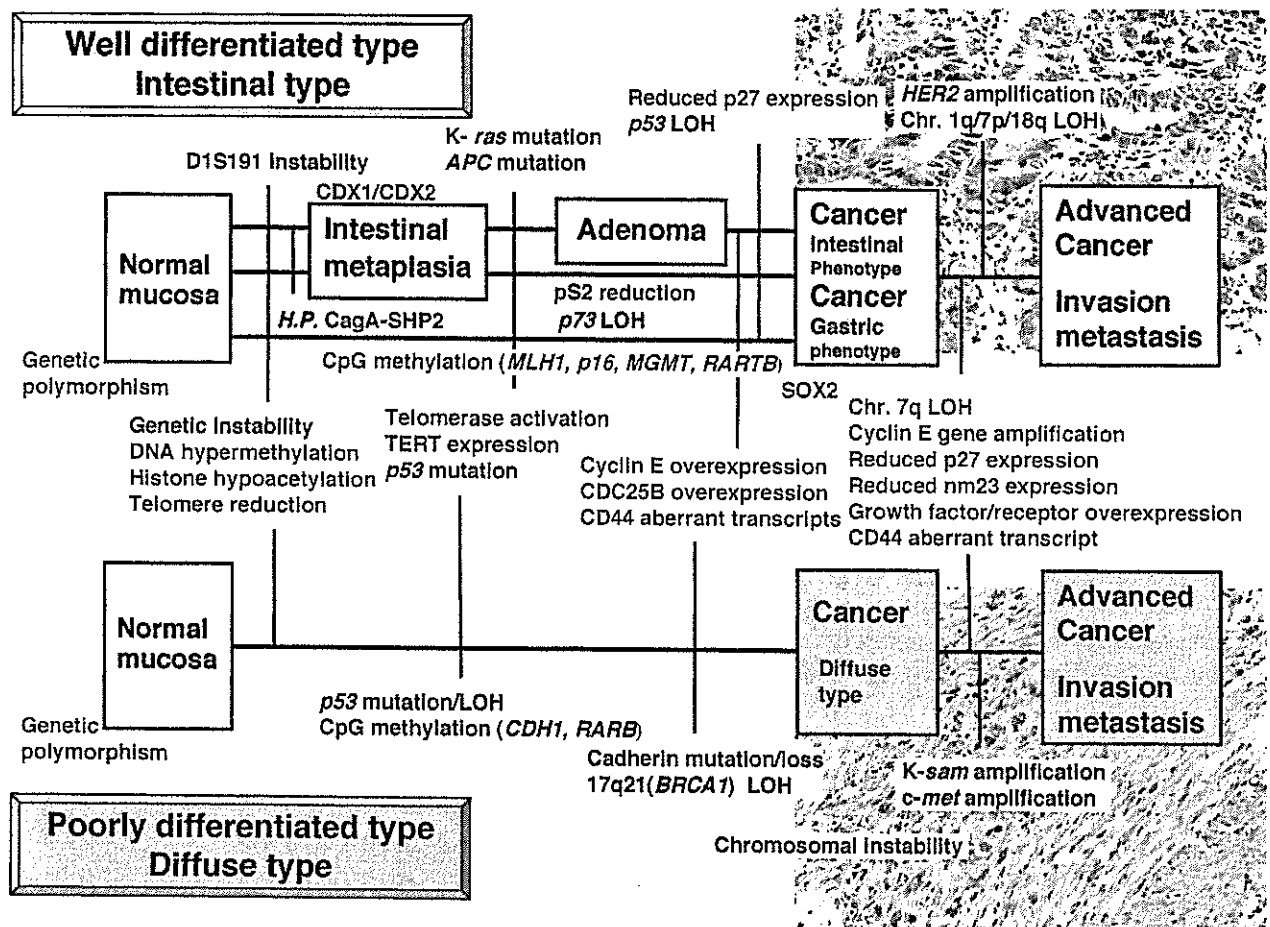


Fig. 1. Multiple genetic and epigenetic alterations during gastric carcinogenesis. Texts written in dark-blue stand for genetic alterations and those in green stand for epigenetic alterations.

OUTLINE OF MOLECULAR CARCINOGENESIS

An accumulation of genetic and molecular abnormalities occurs during gastric carcinogenesis, which is a multistep process (Fig. 1) (2, 4). The steps include activation of oncogenes; overexpression of growth factors/receptors and matrix metalloproteinases; inactivation of tumour suppressor genes, DNA repair genes and cell adhesion molecules; and abnormalities of cell cycle regulators that define biological characteristics of cancer cells (and could thus serve as therapeutic targets). Common genetic alterations in gastric cancer include loss of heterozygosity and point mutations of tumour suppressor genes, while common epigenetic changes are gene silencing of tumour suppressors by CpG island methylation and overexpression of tumour promoting genes at the transcriptional level. Genetic polymorphisms predispose to cancer and alter the person's susceptibility to cancer. Genetic instability, DNA methylation, telomerase activation and *p53* mutations participate in the early stage of gastric carcinogenesis. Increased hTERT expression and strong telomerase activity confer cellular immortality in a majority of gastric cancers. Am-

plification and overexpression of the *c-met*, the EGF receptor (EGFR) and the cyclin E genes are associated with the late stage. Overexpression of growth factors and angiogenic factors lead to cancer progression and metastasis. Some of these changes occur often in well differentiated and in poorly differentiated types of cancers and some differ by histological type or (gastric or intestinal) mucin phenotype. Intestinal metaplasia and adenomas share abnormalities similar to those in the well-differentiated type of gastric cancer, such as the *APC* and *K-ras* mutation albeit at low frequency.

GROWTH FACTORS, CYTOKINES, ANGIOGENIC FACTORS

Gastric cancer cells produce various growth factors and express their receptors to bring about biological events, including cancer-stromal interactions (2, 4). These factors induce not only cell growth but also extracellular matrix degradation and angiogenesis which facilitate tumour invasion and metastasis. Simultaneous expression of EGF/TGF- α and EGFR

is associated with cancer progression and correlates with poor patient prognosis. IL- α acts as an autocrine growth factor of gastric cancer and it interacts with the EGFR system. Gene amplification of the *c-met* (hepatocyte growth factor [HGF]) receptor frequently occurs in the scirrhous type of gastric cancer. HGF produced by stromal cells stimulates cancer cell motility. Gene amplification and overexpression of *HER-2/c-erbB2* and *K-sam* are preferentially found in well differentiated and poorly differentiated gastric cancer, respectively.

Angiogenesis is regulated by several angiogenic factors produced by cancer cells and stromal cells (4). Neovascularisation stimulates the growth of the primary tumour and provides an avenue for haematogenous metastasis. In gastric cancer, there is a positive association between microvessel count, metastatic potential and poor prognosis. Among the best known angiogenic factors of gastric cancer cells are vascular endothelial growth factor (VEGF), interleukin (IL-8), basic fibroblast growth factor (bFGF) and platelet-derived endothelial cell growth factor (PD-ECGF). Most gastric cancers express IL-8/receptor systems, and IL-8 levels correlate closely with neovascularity. Well differentiated gastric cancer expresses VEGF at high levels, while poorly differentiated scirrhous cancer expresses strongly bFGF.

CELL CYCLE REGULATORS

Cell cycle checkpoints are regulatory pathway that control cell cycle transitions, and ensure DNA replication and chromosome segregation with high fidelity. Any dysbalance in the actions of cell cycle regulators results in genomic instability and unbridled cell proliferation and will contribute to tumour development (2, 4). Several abnormalities of positive and negative regulators of the cell cycle are associated with gastric cancer. Gene amplification of cyclin E is present in 15-20% of gastric cancer samples and an increased expression of cyclin E correlates with tumour progression. p53 is multifunctional and participates in cell cycle regulation in part through p21 induction. Inactivation of p53 by loss of heterozygosity (LOH) and through mutations occurs in over 60% of all gastric cancers. Expression of p27 is reduced in gastric adenoma, and further reduction is associated with metastasis, indicating participation in development as well as in progression of gastric cancer. Aberrant (reduced or increased) expression of p16 is frequently found in gastric cancer, but the biological role is unknown. An important downstream target for cyclins/cyclin-dependent kinases (CDKs) at the G1/S checkpoint is the retinoblastoma (RB) protein. The expression of RB is weaker in nodal metastases of primary gastric cancers. Checkpoint kinase 1 (Chk1) and Chk2 are DNA damage-activated kinases involved at the G2/M checkpoint. Both Chk1 and Chk2 are overexpressed in more than 70% of gastric cancers associated with the p53 mutation. Chk1 and Chk2 may play a role in the checkpoint function of gastric cancers with the p53 mutation.

GENETIC INSTABILITY

Genomic instability is classified into microsatellite instability (MSI) and chromosome instability. Dysfunction of DNA mismatch repair causes MSI. The target genes for MSI include *TGFBRII*, *IGFIIR*, *BAX*, *hMSH3*, *hMSH6* and *MBD4* (2, 4). MSI or genetic instability leads to accumulation of genetic alterations and contributes to the pathogenesis not only of hereditary non-polyposis colorectal cancer but also of sporadic gastric cancer. CpG island hypermethylation of *hMLH1* and loss of expression are the main mechanisms of MSI in sporadic gastric cancer. The frequency of MSI is especially high in well differentiated gastric cancer of the foveolar phenotype with a papillary structure. There is a tight association between MSI and tumour multiplicity; the frequency of MSI is apparently high in patients with multiple primary cancers. Therefore, detection of MSI serves as a good predictor of another cancer in a patient with gastric cancer. Many reports have, on the other hand, indicated that MSI is associated with less aggressive behaviour of the tumour and a more favourable prognosis.

EPIGENETIC ALTERATIONS

Among the epigenetic alterations that occur in cancer, abnormalities of the transcription step are important; post-translational modifications (such as ubiquitin-dependent degradation) are also involved. For regulation of gene expression on a transcriptional level, DNA methylation, histone modification and chromatin remodeling function as an on-off switch, while the transcription factors act as a volume switch. The abnormal methylation of CpG islands – usually associated with tumour suppressor genes – leads to transcriptional silencing, inactivation of gene function and, ultimately, to carcinogenesis (3, 4). In gastric cancer, aberrant methylation occurs in several tumour suppressor genes, such as *p16*, *CDH1* (E-cadherin), *hMLH1*, *RAR-beta*, *RUNX3*, *MGMT* (O⁶-methylguanine methyltransferase), *TSP1* (thrombospondin-1), *HLTF* (helicase-like transcription factor), and *RIZ1* (retinoblastoma protein-interacting zinc finger gene-1), with the incidence of methylation ranging from 10% to 70%. When DNA methylation associated with 12 tumour-related genes (*hMLH1*, *MGMT*, *p16*, *CDH1*, *RAR-beta*, *HLTF*, *RIZ1*, *TM*, *FLNs*, *LOX*, *HRASLS*, *HAND1*) was analysed, a high methylation rate (5 or more methylated genes) was observed more frequently in stage III/IV cancers than in stage I/II cancers. Thus it indicates that DNA methylation of tumour-related genes accumulates as the tumour progresses (7). On the other hand, some gene promoters become hypermethylated in non-neoplastic conditions, like aging. The incidence of promoter hypermethylation of *hMLH1* and *p16* is higher in non-neoplastic gastric mucosa of gastric cancer patients than in the gastric mucosa of healthy individuals.

The amino terminals of histones are subjected to acetylation and methylation, which recruit downstream regulatory factors, influence chromatin struc-

ture, and are critical determinants of transcription (3). A tight chromatin structure by histone deacetylation causes transcriptional silencing. Gastric cancer exhibits global histone hypoacetylation and, interestingly, the degree of hypoacetylation is associated with the depth of tumour invasion and with nodal metastases. Hypoacetylation of histones H3 and H4 in the *p21^{WAF1/Cip1}* promoter region takes place in more than a half of gastric cancers. Hypoacetylation of histone H3 in this promoter is associated with reduced expression of *p21*, regardless of *p53* gene status (8). Up-regulation of histone acetylation in gastric cancer cells by treatment with trichostatin A (a histone deacetylase [HDAC] inhibitor) induces growth arrest, causes apoptosis and suppresses invasion, as the expression of *p21*, CBP and Bak is induced. In addition to acetylation, phosphorylation and methylation may enhance or inhibit transcription, depending on the residues affected and on the molecules that interact. Histone modification may be a therapeutic target.

MOLECULAR BASES OF GASTRIC AND INTESTINAL PHENOTYPES

In addition to classification by histology (well differentiated and poorly differentiated; intestinal and diffuse), gastric cancers may also be classified into four phenotypes by the mucin expression profile: G-type (gastric or foveolar phenotype), I-type (intestinal phenotype), GI-type (intestinal and gastric mixed phenotype) and N-type (neither gastric nor intestinal phenotype) (4, 9). The G-type is considered to behave more aggressively than the I-type. Mutations of *p53* and LOH of the adenomatous polyposis coli (*APC*) gene occur more frequently in patients with the I-type than with the G-type, while MSI and alterations in the *p73* gene are more common in the G-type than in the I-type. MSI in the G-type is usually associated with inactivation of hMLH1 following promoter hypermethylation. The Caudal-type homeoboxes (*CDX*) 1 and *CDX2* act as intestine-specific transcription factors and are expressed in I-type gastric cancers at high levels. *CDX2* upregulates the expression of goblet-specific *MUC2* and liver-intestine (LI) cadherin, also known as cadherin 17 (*CDH17*). LI-cadherin expression is more frequently detected in I-type than G-type and is associated with tumour invasion and a poor patient prognosis. On the other hand, the expression of *SOX2*, a member of transcription factor family containing an Sry-like high-mobility group box, is well preserved in the G-type and is down-regulated in the I-type.

MOLECULAR EVENTS AND *HELICOBACTER* INFECTION

Recent studies have shed much light on the molecular mechanisms of how *H. pylori* infection is involved in the development of cancer in the stomach (10, 11). Strains of *H. pylori* carrying the cytotoxin-associated antigen A (*cagA*) gene are associated with the devel-

opment of gastric cancer. The *cagA* gene product, CagA, is delivered into gastric epithelial cells by the bacterial type IV secretion system; this induces morphological transformations and cell motility increases. CagA is injected and undergoes tyrosine phosphorylation by Src family kinases, after which it binds to SHP-2 and activates SHP-2 phosphatase. Focal adhesion kinase (FAK) is a tyrosine kinase that plays a critical role in focal adhesion turnover. Activated SHP-2 dephosphorylates and inhibits FAK activity.

Impaired cell adhesion and increased motility by CagA may be involved in the development of gastric lesions associated with *H. pylori* infection. More recently, it has been shown that CagA interacts with cytoplasmic domain of E-cadherin, and competes with beta-catenin binding; this results in accumulation of beta-catenin in both cytoplasm and nucleus. One of the important downstream targets of beta-catenin signaling induced by CagA is *CDX1*. Forced expression of CagA in gastric epithelial cells induces *MUC2* expression. Through this mechanism, CagA may cause intestinal metaplasia which predisposes to gastric cancer, especially of I-type.

GENETIC POLYMORPHISM AND CANCER RISK

An individual's cancer risk varies in line with genetic polymorphisms in the normal population since this connects directly with the person's cancer prevention. Most genetic polymorphism between individuals is due to single nucleotide polymorphisms (SNPs). Genetic polymorphism is crucial for various processes related to gastric carcinogenesis, including 1) the mucosal protection and inflammatory response against *H. pylori* infection; 2) the functioning of carcinogen detoxification and antioxidant protection; 3) the intrinsic variability of DNA repair processes; and 4) cell proliferation activity (4, 12). Variants of *IL1beta* (*IL1B*) and *IL-1* receptor antagonist (*IL1RN*) genes, *IL1B* (-31 T genotype) and *IL1RN* IVS 86bp VNTR (2/2 genotype) increase *IL-1beta* production and inhibit gastric acid secretion. This is associated with a risk of a chronic hypochlorhydric response to *H. pylori* infection and an increased gastric cancer risk. *NAT1* is responsible for N-acetyltransferase activity which catalyses acetylation and modification of aromatic and heterocyclic amine carcinogens. A significantly increased risk of gastric cancer is associated with certain genotypes of *NAT1* (1088 T>A, 1095 C>A).

SNPs are present in the transmembrane domain of *HER-2/c-erbB2* (655 Ile>Val, A>G) and in the promoter regions of *EGF* (61 A/G) and *MMP-9* (-1562 C/T). SNPs of *HER-2* and *EGF* significantly affect the risk of gastric cancer, while the genotype of *MMP-9* does not; it is, however, associated with tumour invasion, metastasis and tumour stage (5). Importantly, genetic polymorphisms are also associated with therapeutic efficacy and the toxicity of anti-cancer drugs. VNTR polymorphism in the promoter region of the thymidylate synthase gene influences the response to 5-fluorouracil (5-FU). The number of TA repeats in the promoter region of the UDP-glucuronosyltrans-

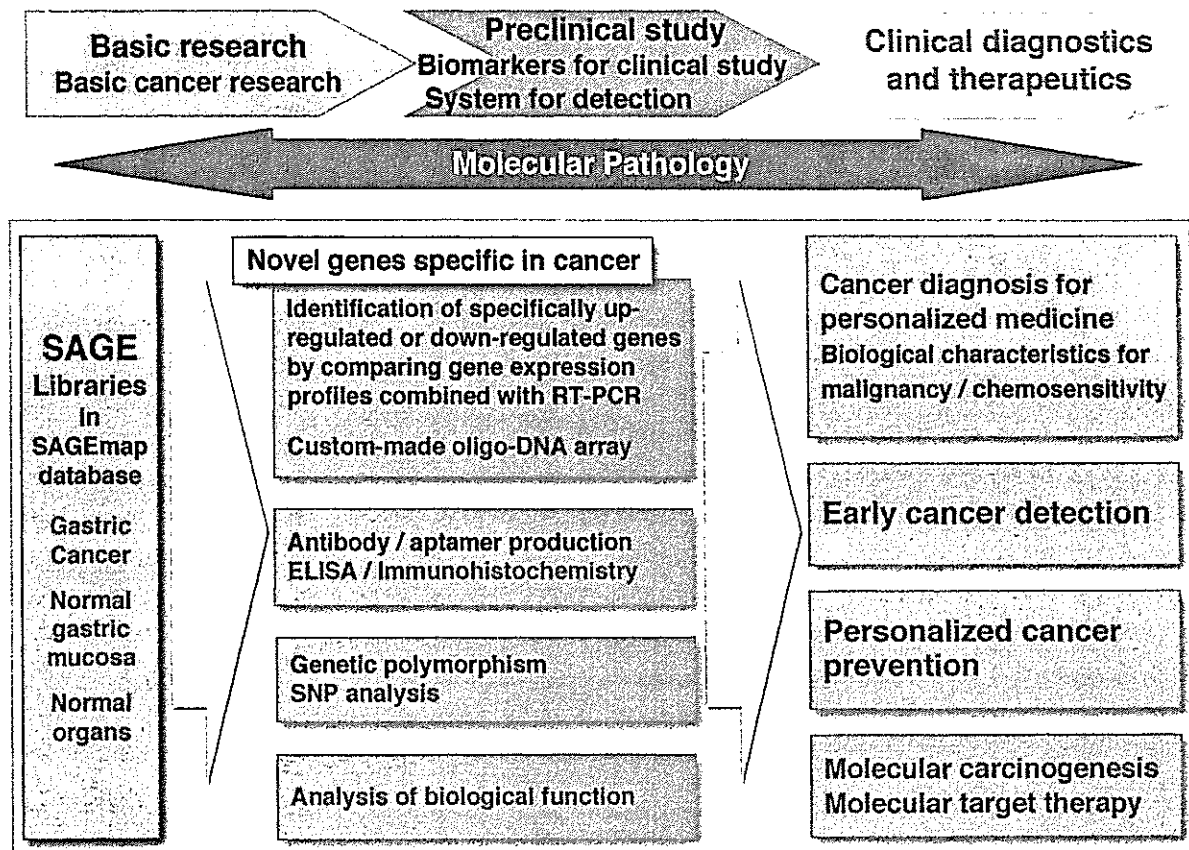


Fig. 2. Strategy to search for novel genes of gastric cancer through gene expression profiles; clinical implications.

ferase 1A1 gene affects the degree of toxicity during irinotecan therapy.

SEARCH FOR NOVEL GENES INVOLVED IN GASTRIC CARCINOGENESIS

Thus, gastric cancer is accompanied by several genetic and epigenetic alterations: mutations, gene amplifications, LOH, gene silencings by DNA methylation and losses of imprinting. All of these alterations modify gene expression. Therefore, genome-wide studies of gene expression are extremely important for studying the precise mechanisms of cancer emergence and progression. In addition to the microarray technique, the Serial Analysis of Gene Expression (SAGE) technique is a powerful tool to allow genome-wide, quantitative analysis of gene expression with high reproducibility (6). Fig. 2 shows a strategy of clinical applications of SAGE with regard to diagnostics, treatment and prevention.

We have created the largest SAGE library of gastric cancer in the world. It contains a total of 137,706 expressed tags including 38,903 unique tags (GEO accession number GSE 545) (Internet address: <http://www.ncbi.nlm.nih.gov/SAGE/>) (4, 13). The library is built on 5 samples of gastric cancer with different histology and stage. We have found that *APOC1*, *CEACAM6*,

and *YF13H12* are often overexpressed in gastric cancer, while *FUS*, *CDH17*, *COL1A1*, *COL1A2*, and *APOE* are associated with tumour progression according to studies where we have compared SAGE libraries between gastric cancers and healthy gastric mucosa in combination with quantitative RT-PCR. Tumours that are LI-cadherin (*CDH17*)-positive by immunostaining are often diagnosed only at a late stage, and the prognosis of patients with LI-cadherin-positive tumours is worse than with negative tumours.

In a search for gastric cancer-specific genes, we compared gastric cancer SAGE libraries with various healthy tissues of crucial organs in the SAGE-map database, and identified 54 candidate genes (14). Quantitative RT-PCR analysis revealed that *APIN*, *TRAG3*, *CYP2W1*, *MIA*, *MMP-10*, *DKK4*, *GW112*, *REGIV*, and *HORMAD1* are expressed much more strongly in gastric cancers than in healthy samples of 14 crucial organs. Immunostaining of *MIA* (melanoma inhibitory activity) and *MMP-10* correlated with a poor prognosis in patients who had advanced gastric cancer. According to enzyme-linked immunosorbent assay (ELISA) there were high levels of *MMP-10* in most serum samples (95%) from patients with gastric cancer. *MIA*-transfected gastric cancer cells were up to three times more invasive than cells transfected with empty vector. Therefore, *MMP-10* is a good serum marker for gastric cancer, while *MIA* and *MMP-*

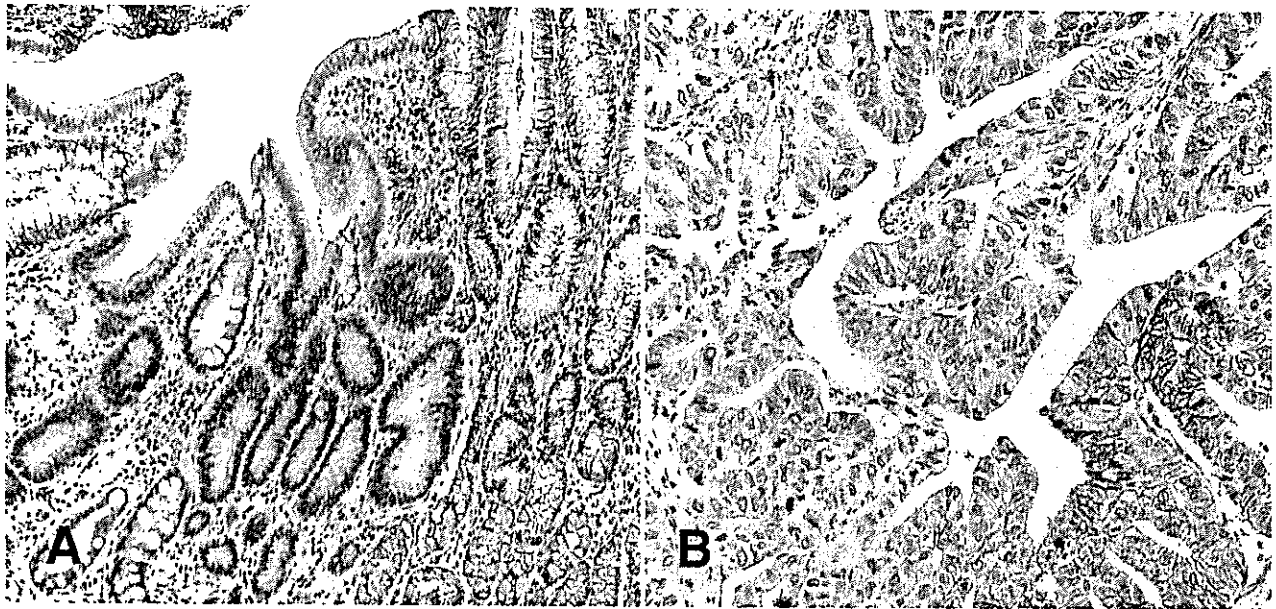


Fig. 3. Reduced expression of claudin-18 in intestinal metaplasia, adenoma and adenocarcinoma of the stomach. Normal gastric epithelial cells express uniformly claudin-18 along with the cell membrane, but there is an obvious reduction of claudin-18 expression in adenoma and adjacent intestinal metaplasia (A) and adenocarcinoma (B) of the stomach.

10 are prognostic factors for gastric cancer patients. Since expression of MIA and MMP-10 is so closely restricted in cancer, these two molecules might also turn out to be good and nontoxic therapeutic targets.

Regenerating islet-derived family member 4 (RegIV) belongs to a superfamily of calcium-dependent lectins. RegIV-positive tissue is associated with the intestinal mucin phenotype as well as with neuroendocrine differentiation of gastric cancer (15). RegIV is also expressed in colorectal carcinomas, carcinoids and pancreatic cancers, but not in lung and breast cancers. High and specific levels of RegIV are detected in 36% of serum samples by ELISA from gastric cancer patients. *In vitro* studies with RegIV-transfected cells have shown that RegIV inhibits 5-FU induced apoptosis through induction of EGFR phosphorylation and inhibition of caspase-3 and -9. Expression of RegIV is strongly associated with resistance to combination chemotherapy with 5-FU and cisplatin. These findings suggest that RegIV may serve as a novel biomarker and an indicator of chemoresistance of gastric cancer.

In an effort to search for novel tumour suppressor genes in gastric cancer, genes with a decreased expression in gastric cancer tissue were screened by SAGE data analysis and RT PCR; *CLDN18* (encoding claudin-18) was down-regulated in gastric cancer (16). The claudin protein family comprises 24 members (claudin-1 to claudin-24) that are important components of tight junction strands in polarised epithelial cells, including gastric epithelia. Expression of claudin-18 is reduced in some instances of intestinal metaplasia, and many gastric adenomas and carcinomas (Fig. 3). Downregulation of claudin-18 was cor-

related with poor survival in advanced gastric cancers. Down-regulation of claudin-18 was observed in over 70% of gastric cancers with intestinal mucin phenotype. Downregulation of claudin-18 may be an early event in gastric carcinogenesis and constitutes also a good marker of poor survival of gastric cancer patients. SAGE-based analyses thus provide a list of candidate genes that are involved in gastric carcinogenesis and may serve as novel diagnostic markers and therapeutic targets in our strive to combat gastric cancer.

CONCLUSION

Molecular events during gastric carcinogenesis and a search for novel genes through global analysis of gene expression were described. Advances in our understanding the genetic and molecular bases of cancer lead to new diagnosis and therapy. Decision of cancer treatment may be guided by the molecular attributes of the individual patient. It is said that cancer treatment gets personal (17).

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Expression of Wnt-5a Is Correlated with Aggressiveness of Gastric Cancer by Stimulating Cell Migration and Invasion

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Abstract

Wnt-5a is a representative ligand that activates a β -catenin-independent pathway in the Wnt signaling. Although abnormal activation of β -catenin-dependent pathway is often observed in human cancer, the relationship between β -catenin-independent pathway and tumorigenesis is not clear. We sought to clarify how Wnt-5a is involved in aggressiveness of gastric cancer. Abnormal expression of Wnt-5a was observed in 71 of 237 gastric cancer cases by means of immunohistochemistry. The positivity of Wnt-5a expression was correlated with advanced stages and poor prognosis of gastric cancer. Wnt-5a had the abilities to stimulate cell migration and invasion in gastric cancer cells. Wnt-5a activated focal adhesion kinase and small GTP-binding protein Rac, both of which are known to play a role in cell migration. Cell migration, membrane ruffling, and turnover of paxillin were suppressed in Wnt-5a knock-down cells. Furthermore, anti-Wnt-5a antibody suppressed gastric cancer cell migration. These results suggest that Wnt-5a stimulates cell migration by regulating focal adhesion complexes and that Wnt-5a is not only a prognostic factor but also a good therapeutic target for gastric cancer. (Cancer Res 2006; 66(21): 10439-48)

Introduction

Wnt proteins are a large family of cysteine-rich secreted molecules (1). At least 19 Wnt members are present in mammals to date. The members exhibit unique expression patterns and distinct functions in development. The Wnt family members can be divided into three distinct types based on their ability to induce transformation of the mouse mammary epithelial cell line C57MG (2, 3). The highly transforming members include Wnt-1, Wnt-3a, and Wnt-7a. The intermediately transforming members include Wnt-2, Wnt-5b, and Wnt-7b and nontransforming members are Wnt-4, Wnt-5a, Wnt-6, and Wnt-11.

The intracellular signaling pathway activated by Wnt proteins was originally identified as a β -catenin-dependent signaling pathway that is highly conserved among species (1). According to the most widely accepted current model of the β -catenin pathway, in the absence of Wnt, β -catenin is phosphorylated and ubiquitinated in the Axin complex, resulting in the degradation of

β -catenin by the proteasome (4–7). Consequently, the cytoplasmic β -catenin level is low. When Wnt acts on its cell surface receptor consisting of Frizzled and lipoprotein receptor-related protein 5/6, β -catenin escapes from degradation in the Axin complex (8). The accumulated β -catenin is translocated to the nucleus, where it binds to the transcription factor T-cell factor (Tcf)/lymphoid enhancer factor (Lef) and thereby stimulates the expression of various genes (9). It is thought that the Wnt proteins showing the high transforming activity in C57MG cells activate the β -catenin pathway.

Some Wnt proteins activate a β -catenin-independent pathway that primarily modulates cell movement and polarity (3, 10). There are at least three mechanisms, which overlap with other signaling pathways. First, specific Wnt and Frizzled can activate calcium/calmodulin-dependent protein kinase II and protein kinase C (PKC). Second, some Frizzled receptors act through heterotrimeric G proteins to activate phospholipase C and phosphodiesterase. Lastly, the planar cell polarity pathway in *Drosophila* is mediated by Frizzled, which activates Rac, Rho, c-Jun NH₂-terminal kinase (JNK), and Rho-associated kinase. It is generally believed that the intermediately transforming and nontransforming Wnt proteins activate the β -catenin-independent pathway (3).

Wnt-5a is a representative of Wnt proteins that activate the β -catenin-independent pathway. Some reports indicate that Wnt-5a acts as a tumor suppressor because Wnt-5a has an ability to inhibit the β -catenin pathway. For instance, Wnt-5a has been shown to induce the down-regulation of β -catenin through Siah2 (11) or to inhibit the transcriptional activity of Tcf/Lef (12, 13). Antisense Wnt-5a mimics Wnt-1-mediated C57MG cell transformation (14). Wnt-5a negatively regulates B-cell proliferation, and Wnt-5a heterozygous mice develop B-cell lymphoma (15). Furthermore, Wnt-5a inhibits proliferation, migration, and invasiveness in thyroid tumor and colorectal cancer cell lines (16, 17). In contrast to these observations, it has also been suggested that Wnt-5a has oncogenic properties based on the findings that the Wnt-5a mRNA level is up-regulated in lung cancers, prostate cancers, and breast cancers (18). There is a correlation between Wnt-5a expression and increased cell motility and invasiveness in melanoma cells and breast cancer cells with tumor-associated macrophages (19, 20). Thus, the functions of Wnt-5a in human cancers are controversial and still unclear.

According to the WHO, gastric cancer is the fourth most common malignancy worldwide, with ~870,000 new cases occurring yearly (21). However, the relationship between the expression of Wnt-5a and aggressiveness of gastric cancer is not known. In this study, we found that the Wnt-5a protein is highly expressed in advanced stages of gastric cancer and that its expression is correlated with poor prognosis. Furthermore, we showed that Wnt-5a stimulates cell migration and invasion of gastric cancer cells through regulating focal adhesion complexes.

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Materials and Methods

Materials and chemicals. TOP-*fos*-Luc, pUC/EF-1 α / β -catenin^{5A}, and pCMV-sFRP2-Flag were provided by Drs. H. Clevers (University Medical Center, Utrecht, the Netherlands), A. Nagafuchi (Kumamoto University, Kumamoto, Japan), and T. Akiyama (Tokyo University, Tokyo, Japan), respectively. pEGFP-Paxillin and pGEX- α PAK-CRIB were provided by Drs. H. Sabe (Osaka Bioscience Institute, Osaka, Japan) and K. Kaibuchi (Nagoya University, Nagoya, Japan), respectively. Two anti-Wnt-5a antibodies were generated in rabbits by immunization with synthetic peptides corresponding to residues 165 to 181 and residues 275 to 290 of human Wnt-5a. The former antibody recognized both Wnt-5a and Wnt-5b, whereas the latter reacted with Wnt-5a only. Wnt-5a was purified to near homogeneity by the similar procedures to the purification of Wnt-3a (22). The details of the purification of Wnt-5a will be described elsewhere. Secreted Frizzled-related protein 2 (sFRP2) conditioned medium was prepared from culture medium of HEK293T cells stably expressing sFRP2. The cells were seeded at a density of 1×10^6 /mL in DMEM/Ham's F-12 supplemented with 10% fetal bovine serum (FBS). At 24 hours after plating, the medium was replaced with Opti-MEM medium (Life Technologies, Minneapolis, MN), and then 3 days after, sFRP2 conditioned medium was collected. Anti-Wnt-3a antibody was prepared as described (22). MKN-45 and MKN-1 cells were grown in RPMI 1640 supplemented with 10% FBS. MKN-45 cells stably expressing mouse Wnt-5a were generated by selection with G418. MKN-1 cells stably expressing green fluorescent protein (GFP)-paxillin were generated by selection with puromycin. In analyses with small interfering RNA (siRNA) for Wnt-5a, the human Wnt-5a mRNA target sequences, 5'-CTGTGGATAACCTCTGTTT-3' and 5'-AAA-AACAGAGGTGTTATCCAC-3', were used. Scrambled siRNA, 5'-CAGTCGC-GTTTGCAGCTGG-3', was used as a control. Anti-Wnt-4, anti-HK-ATPase, and anti-chromogranin A antibodies were obtained from R&D Systems (Minneapolis, MN), Affinity Bioreagents (Golden, CO), and Novocastra (Newcastle, United Kingdom), respectively. Other materials were obtained from commercial sources.

Tissue samples. In all, 250 primary tumors were collected from patients diagnosed with gastric cancer. Patients were treated at the Hiroshima University Hospital (Hiroshima, Japan) or an affiliated hospital. For quantitative reverse transcription-PCR (RT-PCR), 13 gastric cancer samples and corresponding nonneoplastic mucosa samples were used. For immunohistochemical analysis, archival formalin-fixed, paraffin-embedded tissues from 237 patients who had undergone surgical excision for gastric cancer were used. Histologic classification (intestinal, diffuse-adherent, and diffuse-scattered types) was made according to the Lauren classification system (23, 24). Tumor staging was according to the tumor-node-metastasis staging system. The procedure to protect privacy was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Immunohistochemistry and immunocytochemistry. The samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then immunohistochemically stained. For immunostaining of Wnt-5a, a DAKO CSA kit (DAKO, Carpinteria, CA) was used according to the manufacturer's recommendations. In brief, sections were pretreated by microwaving in citrate buffer for 30 minutes to retrieve antigenicity. After peroxidase activity was blocked with 3% H₂O₂-methanol for 10 minutes, sections were incubated with normal goat serum (DAKO) for 20 minutes to block nonspecific antibody binding sites. Anti-Wnt-5a antibody was incubated with tissue samples for 15 minutes at room temperature and detected by incubating for 15 minutes with biotinylated goat anti-rabbit immunoglobulins, and the signal was amplified and visualized by the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The staining for Wnt-5a, Wnt-3a, or Wnt-4 was classified according to the percentage of stained cancer cells. Expression was considered to be "negative" if <50% (0, 0-10%; 1+, 10-50%) of cancer cells were stained. When at least 50% (2+, 50-80%; 3+, >80%) of cancer cells were stained, the immunostaining was considered "positive."

The immunostaining of β -catenin, HK-ATPase, chromogranin A, epidermal growth factor receptor (EGFR), melanoma inhibitory activity

(MIA), and matrix metalloproteinase-10 (MMP-10) was basically done as described previously (25). The immunocytochemical analyses of the cultured cells were done as described (26, 27).

Statistical analyses. Statistical analyses for Table 1 were carried out with Fisher's exact test. Kaplan-Meier survival curves were constructed for patients positive and negative with Wnt-5a, β -catenin, EGFR, MIA, or MMP-10. Differences between survival curves in Fig. 5 were tested for statistical significance by log-rank test. $P < 0.05$ was considered statistically significant.

Cell migration and invasion assays. To measure the cell migration activity, Transwell assays were done using a modified Boyden chamber (tissue culture treated, 6.5 mm in diameter, 10- μ m thick, 8- μ m pores; Transwell, Costar, Cambridge, MA) as described (27). The lower surface of filters was coated with 10 μ g/mL collagen. MKN-1, MKN-7, MKN-74, and MKN-45 cells (2.5×10^4 cells in 100 μ L) suspended in serum-free RPMI 1640 containing 0.1% bovine serum albumin were applied to the upper chamber. The same medium was added to the lower chamber. When necessary, 600 ng/mL Wnt-5a was added to the lower chamber. After the cells were incubated at 37°C for 4 to 8 hours, the number of cells that migrated to the lower side of the upper chamber was counted. The invasive potential of the cells was analyzed using a Matrigel-coated modified Boyden chamber (Becton Dickinson, Bedford, MA). RPMI 1640 containing 10% FBS was added to the lower chamber. After incubation at 37°C for 24 hours, the number of cells that migrated to the lower side of the upper chamber was counted.

To carry out the wound healing assay, the cells were plated onto collagen-coated coverslips. The monolayer MKN-1 cells were then scratched manually with a plastic pipette tip, and after being washed with PBS, the wounded monolayers of the cells were allowed to heal for 6 to 12 hours in RPMI 1640 containing 10% FBS (27). When necessary, conditioned medium containing sFRP2 or anti-Wnt-5a antibody (50 μ g/mL) was added to the medium.

Table 1. Relationship between Wnt-5a expression and clinicopathologic characteristics in gastric cancer

	Wnt-5a expression		<i>P</i>
	Positive, <i>n</i> (%)	Negative, <i>n</i>	
T grade			
T ₁	5 (7.6)	61	<0.0001
T ₂ /T ₃ /T ₄	66 (38.6)	105	
N grade			
N ₀	15 (14.2)	91	<0.0001
N ₁ /N ₂ /N ₃	56 (42.7)	75	
Stage			
I/II	18 (13.8)	112	<0.0001
III/IV	53 (49.5)	54	
Histologic type			
Diffuse-scattered	32 (59.3)	22	<0.0001
Other	39 (21.3)	144	
β -Catenin nuclear/cytoplasmic accumulation			
Positive	9 (18.0)	41	0.0386
Negative	62 (33.2)	125	

NOTE: T₁, tumor invades lamina propria or submucosa; T₂, tumor invades muscularis propria or subserosa; T₃, tumor penetrates serosa without invasion of adjacent structures; and T₄ tumor invades adjacent structures. N₀, no regional lymph node metastasis; N₁, metastasis in 1 to 6 regional lymph nodes; N₂, metastasis in 7 to 15 regional lymph nodes; and N₃, metastasis in >15 regional lymph nodes.

Cell proliferation assay. Cells were seeded at a density of 1.0×10^5 /mL. At 12 hours after plating, the medium was replaced with 2% serum medium. The cell number was counted every 24 hours for 4 days.

Live imaging of focal adhesion. The dynamics of GFP-paxillin of the scattered monolayer cells were quantitated as described previously (27). Fluorescence intensities of individual adhesions from background-subtracted images were measured over time using MetaMorph software (Universal Imaging Corp., Downingtown, PA).

Others. Semiquantitative RT-PCR was done as described (28). Forward and reverse primers were as follows: Wnt-5a, 5'-CTTCCGCCAGGTTG-TAATTGAAGC-3' and 5'-CTGCCAAAACAGAGGTGTATCC-3'; Wnt-5b, 5'-AGGGAACCCTACTCTGG-3' and 5'-ACATCTCGGGTCTCTG-3'; and ribosomal high basic 23-kDa protein (RPL), GACCGTCTCAAGGTGT and CTTCCGGTAGTGGATCT. Tcf-4 transcriptional activity was measured as described (26, 29). Cell adhesion assay was done by placing the cells on collagen-precoated dishes (27). Activation of Rac was assayed using glutathione *S*-transferase (GST)-CRIB (27).

Results

Expression of Wnt-5a in gastric cancer. There are two Wnt-5 family members, Wnt-5a and Wnt-5b, and they share 90% amino acid identity. The medium mRNA levels of Wnt-5a in the nontumor and tumor regions in the stomach of 13 gastric cancer patients were 3.6- and 11.6-fold higher than those of Wnt-5b (Fig. 1A). The median mRNA level of Wnt-5a in gastric cancer samples was up-regulated 2.6-fold ($P < 0.05$, Wilcoxon test) compared with that in corresponding nontumor mucosa samples, whereas the mRNA level of Wnt-5b showed no significant differences between gastric cancer and corresponding nontumor mucosa of individuals (Fig. 1A).

To evaluate where Wnt-5a protein is expressed in the normal gastric mucosa and which type of gastric cancer exhibits Wnt-5a expression, we did immunohistochemistry. Anti-Wnt-5a antibody specifically recognized Wnt-5a in the lysates of L cells expressing Wnt-5a (Fig. 1B). This anti-Wnt-5a antibody did not recognize Wnt-3a, Wnt-4, Wnt-7a, or Wnt-11 (data not shown). Immunohistochemical staining revealed the presence of Wnt-5a at the base of the normal gastric corpus mucosa (Fig. 1C, a). Weak or no staining of Wnt-5a was observed in foveolar epithelium and stromal cells. Wnt-5a-positive cells were negative for HK-ATPase (a marker of parietal cells) and chromogranin A (a marker of endocrine cells), indicating that Wnt-5a-positive cells were the Chief cells (Fig. 1C, b and c). This staining was not observed when anti-Wnt-5a antibody was preincubated with a peptide corresponding to a portion of Wnt-5a (data not shown).

Next, we did immunohistochemical staining of Wnt-5a in 237 human gastric cancer cases. Representative results of Wnt-5a immunostaining of gastric cancer are shown in Fig. 1C, d and e. Wnt-5a was detected in cancer cells in both intestinal-type and diffuse-type (diffuse-adherent and diffuse-scattered) gastric cancer. Although it is difficult to distinguish diffuse-scattered-type gastric cancer cells from stromal cells, CAM5.2 (a marker for epithelial cell)-positive cells were positive for Wnt-5a (Fig. 1C, f). These results indicate that Wnt-5a is expressed in epithelial-derived cancer cells.

Among 237 gastric cancer cases, expression of Wnt-5a was observed in 71 (30.0%) cases. Positivity for Wnt-5a was associated with advanced T grade (depth of invasion) and N grade (degree of lymph node metastasis; $P < 0.0001$ and $P < 0.0001$, respectively; Table 1). Moreover, Wnt-5a staining was observed more frequently in stage III/IV cases [53 of 107 (49.5%) cases] than in stage I/II cases [18 of 130 (13.8%) cases; $P < 0.0001$; Table 1]. When all the cases

were classified into diffuse-scattered type and other types (intestinal type and diffuse-adherent type), Wnt-5a was more frequently expressed in diffuse-scattered-type gastric cancer [32 of 54 (59.3%) cases] than in other types of gastric cancer [39 of 183 (21.3%) cases; $P < 0.0001$; Table 1]. We generated two antibodies against Wnt-5a, and analyses with the different antibodies produced almost the same results. In contrast, the immunohistochemical analyses using anti-Wnt-3a or anti-Wnt-4 antibody revealed that these gastric cancer cases show heterogeneity of weak staining for Wnt-3a or Wnt-4 and that the positivity of the expression of these Wnt proteins does not associate with aggressiveness of gastric cancer (data not shown). These findings suggest that highly expressed Wnt-5a could be generally related to tumor progression of gastric cancer.

It has been reported that adenomatous polyposis coli (APC) or β -catenin gene mutations are detected in gastric cancer (30). Cytosomal or nuclear accumulation of β -catenin was observed in 50 (21.1%) cases of 237 gastric cancer cases. Among the 50 gastric cancer cases with cytosomal or nuclear accumulation of β -catenin, only 9 gastric cancer cases showed Wnt-5a expression (Table 1). However, Wnt-5a and β -catenin were rarely expressed in the same gastric cancer cells. In the nine gastric cancer cases with positive Wnt-5a expression, none of β -catenin-positive cancer cells showed the abnormal expression of Wnt-5a (Fig. 1D, a and b), except for one gastric cancer case. These findings suggest that β -catenin and Wnt-5a are expressed exclusively in gastric cancer.

Actions of Wnt-5a on cell growth and migration of gastric cancer cells. Comparison of various gastric cancer cell lines revealed that the expression level of Wnt-5a mRNA in MKN-1, MKN-7, and MKN-74 cells is higher than that of TMK-1, MKN-28, MKN-45, HSC-39, and KATO-III cells (Fig. 2A, a). The genes of APC and β -catenin in these cells are not mutated, except for MKN-28 and HSC-39 cells (31). It has been reported that Wnt-5a suppresses the transcriptional activity of Tcf/Lef by acting downstream of β -catenin (12, 13) or is involved in cell migration (19, 20). Ectopic expression of β -catenin in MKN-45 cells activated Tcf-4, and this β -catenin-dependent transcriptional activity was indeed decreased by purified Wnt-5a protein (Fig. 2A, b). However, expression of Wnt-5a in MKN-45 cells did not affect cell growth (Fig. 2A, c). These results suggest that Wnt-5a does not have an influence on cell proliferation in gastric cancer cells where β -catenin is not abnormally expressed. Therefore, we asked whether Wnt-5a is involved in migration activity of gastric cancer.

The migration activities in MKN-1 and MKN-7 cells were higher than those of MKN-74 and MKN-45 cells (Fig. 2B, a). Because it seemed that the migration activity in the cell lines has a tendency to be dependent on the expression levels of Wnt-5a, we used MKN-1 and MKN-45 cells in the following experiments. Expression of Wnt-5a in MKN-45 cells stimulated the cell migration (Fig. 2B, b), and knockdown of Wnt-5a by RNA interference in MKN-1 cells suppressed the migration (Fig. 2B, c). Furthermore, whereas control MKN-1 cells invaded the Matrigel, Wnt-5a knockdown MKN-1 cells were less invasive (Fig. 2B, d).

sFRP2 binds to Wnt proteins and acts as a negative regulator of Wnt signaling (32). MKN-1 cells were allowed to migrate in scratch-wound cultures, resulting in wound closure after 6 hours, and the migration of MKN-1 cells in scratch-wound cultures was inhibited by the addition of sFRP2 conditioned medium (Fig. 2C). Furthermore, anti-Wnt-5a antibody suppressed the migration of MKN-1 cells in scratch-wound cultures (Fig. 2D, a and b). The inhibitory activity of this antibody for Wnt-5a was confirmed by

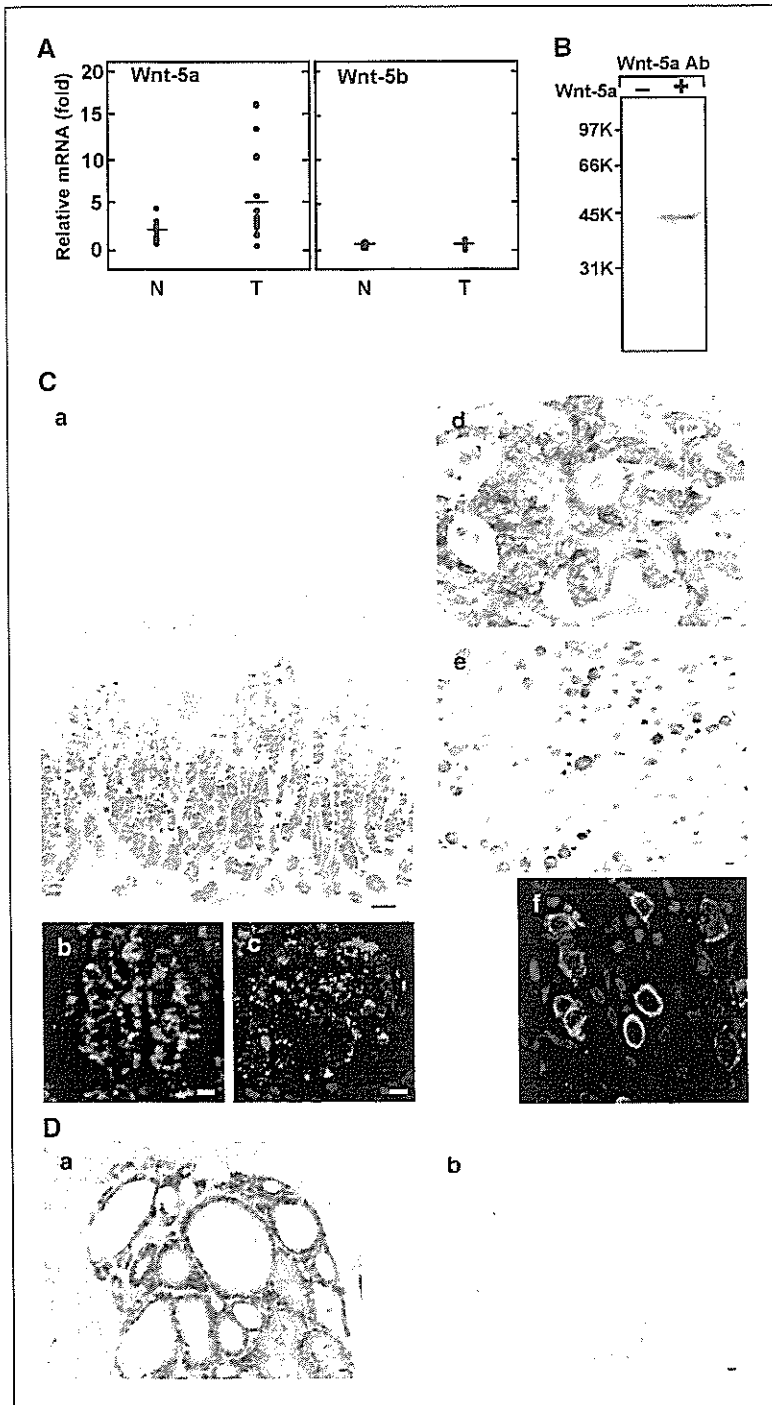


Figure 1. Expression of mRNA and proteins of Wnt-5a in nontumor and tumor regions of human stomach. **A**, expression of mRNA of Wnt-5a and Wnt-5b in normal stomach and gastric cancer. RT-PCR was used to quantitatively amplify mRNA of Wnt-5a, Wnt-5b, and RPL from tumor (T) and adjacent nontumor (N) regions in 13 individuals. The mRNA levels of Wnt-5a (left) and Wnt-5b (right) are presented as arbitrary units for the mRNA levels of RPL. **B**, specificity of anti-Wnt-5a antibody. The lysates of control L cells and L cells expressing Wnt-5a were probed with anti-Wnt-5a antibody. **C**, expression of Wnt-5a in gastric cancer. **a**, normal human stomach was immunostained with anti-Wnt-5a antibody (brown). Bar, 100 μ m. **b** and **c**, the samples was stained with anti-Wnt-5a antibody (red) and anti-HK-ATPase (b) or chromogranin A (c) antibody (green). Nuclei were shown by 4',6-diamidino-2-phenylindole (DAPI; blue). Bar, 10 μ m. **d**, intestinal-type gastric cancer was immunostained with anti-Wnt-5a antibody. Bar, 20 μ m. **e**, diffuse-scattered-type gastric cancer was immunostained with anti-Wnt-5a antibody. Bar, 20 μ m. **f**, diffuse-scattered-type gastric cancer was stained with anti-Wnt 5a antibody (red), anti-CAM5.2 (green; a marker for epithelial cell) antibody, and DAPI (blue). Bar, 10 μ m. **D**, expression of β -catenin in gastric cancer. A sample of gastric cancer was stained with anti- β -catenin (a) and anti-Wnt-5a (b) antibodies. Bar, 40 μ m.

another way. Wnt-5a has been shown to induce the phosphorylation of Dvl (33). Preincubation of Wnt-5a and anti-Wnt-5a antibody inhibited Wnt-5a-dependent phosphorylation of Dvl (Fig. 2D, c). These results indicate that Wnt-5a stimulates cell migration and invasion in the gastric cancer cells.

Molecular mechanism by which Wnt-5a stimulates cell migration. Expression of Wnt-5a in MKN-45 cells increased cell adhesiveness (Fig. 3A), suggesting that Wnt-5a enhances signaling from focal adhesions. Activation of focal adhesion kinase (FAK) and small GTP-binding protein Rac is necessary for cell migration (34). FAK is activated via autophosphorylation at Tyr³⁹⁷, which is

initiated by integrin engagement with its ligand (34). Adhesion-dependent FAK activation was enhanced by expression of Wnt-5a in MKN-45 cells (Fig. 3B). Addition of purified Wnt-5a protein to MKN-45 cells activated FAK and Rac within 5 minutes (Fig. 3C). Treatment of the cells with PP2, a Src inhibitor, and GF109203X, a PKC inhibitor, suppressed Wnt-5a-dependent FAK activation as well as cell migration (Fig. 3D). Therefore, Wnt-5a could stimulate cell migration by regulating the activities of FAK and Rac, probably through PKC and Src.

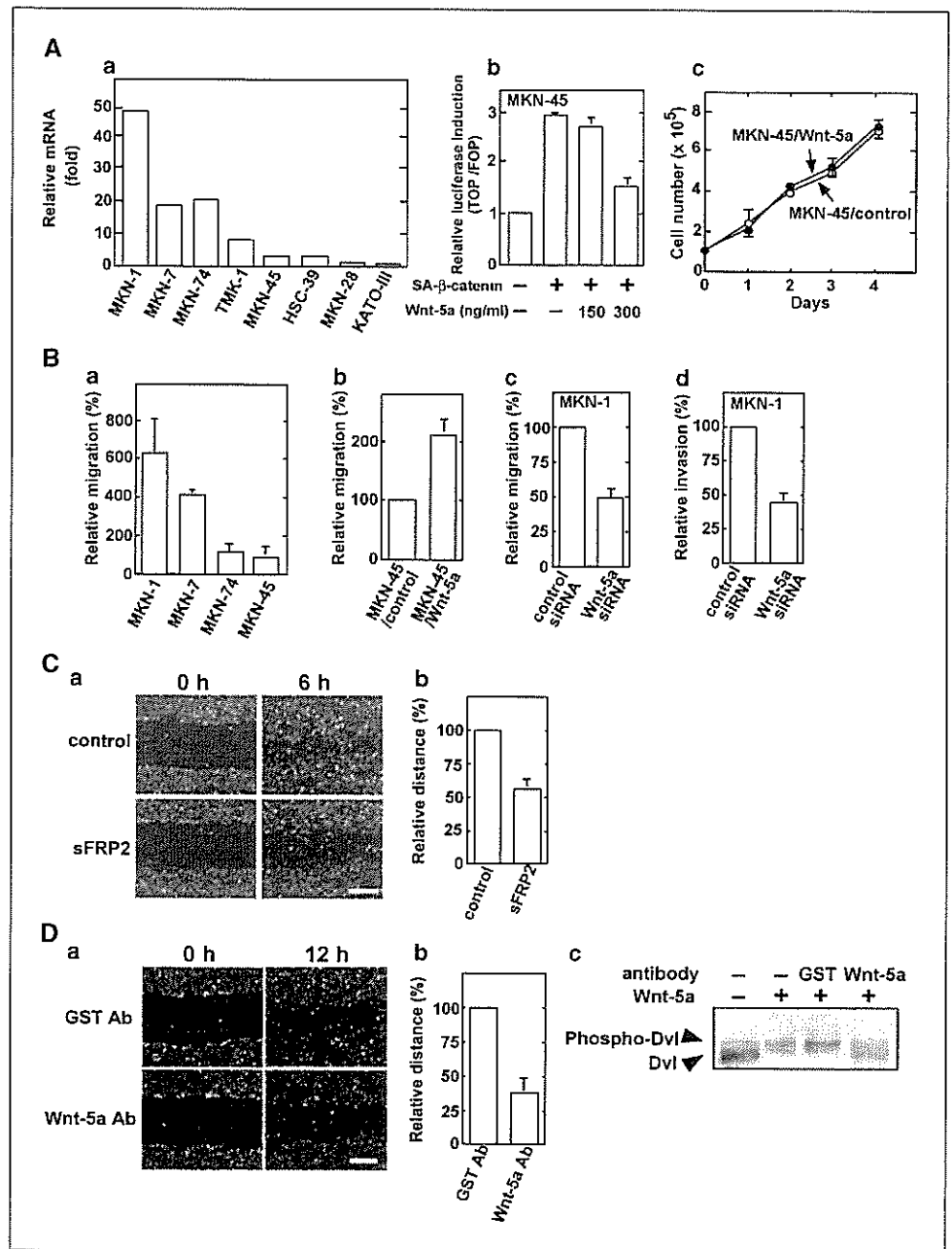
The migration of MKN-1 cells in scratch-wound cultures was suppressed by knockdown of Wnt-5a (Fig. 4A). Ruffling and

phalloidin staining were observed at the cell front along the leading edge of control migrating cells (Fig. 4B). Paxillin is a focal adhesion protein (35). When the control MKN-1 cells were stained with anti-paxillin antibody, focal adhesions, including paxillin, were detected faintly (Fig. 4B). Knockdown of Wnt-5a obviously suppressed phalloidin staining at the leading edge, whereas it instead strengthened the formation of stress fibers and enlarged the size of focal adhesions, including paxillin (Fig. 4B). Because it has been suggested that the formation of large focal adhesions is due to the reduced turnover of adhesions (36), the effects of Wnt-5a on the dynamics of focal adhesions were examined. To this end, we expressed GFP-paxillin in MKN-1 cells and analyzed the turnover of dynamics by live fluorescence imaging. At the cell front in control cells, paxillin-containing adhesions disassembled as new adhesions were formed near the leading edge, whereas the turnover of

paxillin-containing adhesions was suppressed in Wnt-5a knock-down cells (Fig. 4C). The rate constants of assembly and disassembly of GFP-paxillin at adhesion sites were decreased in Wnt-5a knockdown cells (Fig. 4D). These results suggest that Wnt-5a regulates the turnover of focal adhesion complexes, thereby stimulating cell migration.

Survival of gastric cancer patients with Wnt-5a expression. Finally, we examined the relationship between the expression of Wnt-5a and survival in advanced stages (T₂, T₃, and T₄) of gastric cancer (*n* = 111). The 5-year survival was 50% in patients with Wnt-5a-negative gastric cancer, whereas it was 20% in patients with Wnt-5a-positive gastric cancer (*P* < 0.0001; Fig. 5A, a). Furthermore, irrespective of histologic classification, the 5-year survival rate was significantly lower in patients with Wnt-5a-positive gastric cancer than in those with Wnt-5a-negative gastric cancer [*P* = 0.0036 for

Figure 2. Actions of Wnt-5a on gastric cancer cells. **A**, inhibition of the β -catenin pathway by Wnt-5a. **a**, the level of expression of Wnt-5a mRNA in the gastric cancer cells was quantified by quantitative RT-PCR. **b**, after MKN-45 cells were transfected with pEF-BOS/hTcf-4E (0.1 μ g), pUC/EF-1 α / β -catenin^{SA} (0.5 μ g), and TOP-*fos*-Luc (0.5 μ g) or FOP-*fos*-Luc (0.5 μ g), the cells were incubated with the indicated amounts of Wnt-5a for 8 hours. The luciferase activity was measured and expressed as the ratio of the activity in the cells transfected with TOP-*fos*-Luc to that in the cells transfected with FOP-*fos*-Luc. **c**, MKN-45 cells containing empty vector (MKN-45/control cells) or stably expressing Wnt-5a (MKN-45/Wnt-5a cells) were cultured in the presence of 2% serum for the indicated number of days, and then cell numbers were counted. **B**, stimulation of cell migration by Wnt-5a. **a**, the indicated gastric cells were placed in Transwell chamber for the migration assay. **b**, MKN-45/control cells or MKN-45/Wnt-5a cells were placed in Transwell chambers for the migration assay. **c** and **d**, MKN-1 cells transfected with the control or Wnt-5a siRNA were placed in noncoated (**c**) or Matrigel-coated (**d**) Transwell chambers for the migration or invasion assay, respectively. **C**, inhibition of cell migration by sFRP2. **a**, control or sFRP2 conditioned medium was added to MKN-1 cells, and then the cells were wounded. The culture was continued for 6 hours. Bar, 200 μ m. **b**, migrating distances in (**a**) were measured. **D**, inhibition of cell migration by anti-Wnt-5a. **a**, MKN-1 cells incubated with anti-GST (as control) or anti-Wnt-5a antibody (50 μ g/mL) were wounded. The culture was continued for 12 hours. Bar, 200 μ m. **b**, migrating distances in (**a**) were measured. **Columns**, mean of three independent experiments; **bars**, SE. **c**, after Wnt-5a had been preincubated with anti-GST or anti-Wnt-5a antibody for 1 hour at 4°C, NIH3T3 cells were treated with 80 ng/mL Wnt-5a for 1 hour, and then cells were lysed and probed with anti-Dvl antibody.



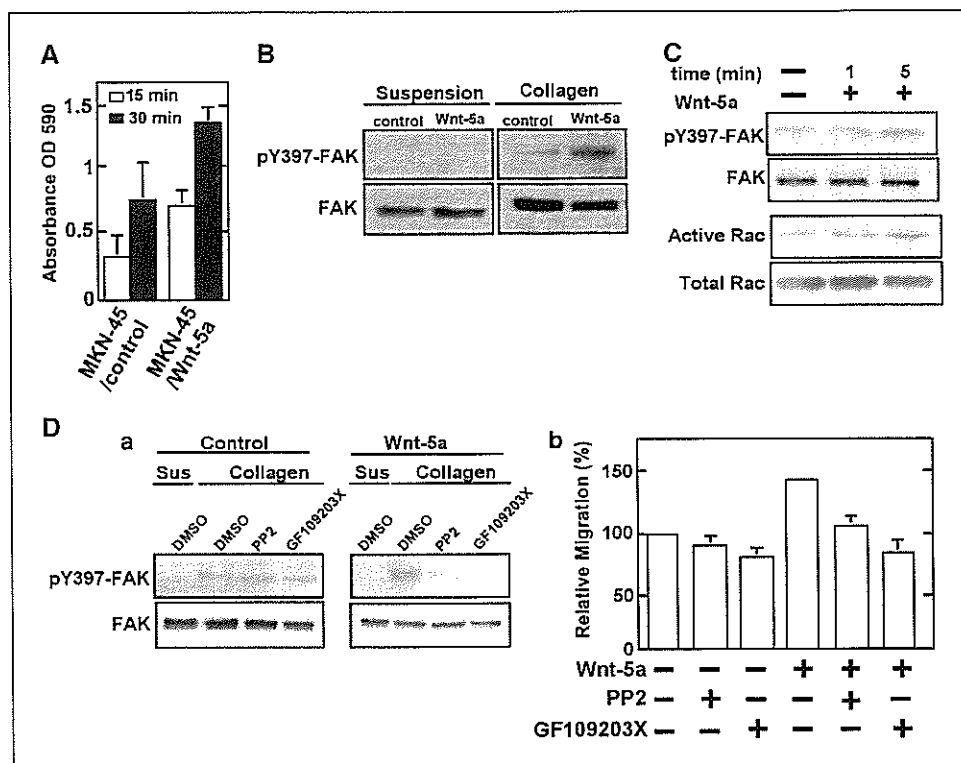


Figure 3. Molecular mechanism by which Wnt-5a stimulates cell migration. **A**, enhancement of cell adhesion by Wnt-5a. MKN-45/control or MKN-45/Wnt-5a cells were subjected to the adhesion assay for the indicated time. **B**, enhancement of collagen-induced activation of FAK by Wnt-5a. MKN-45/control and MKN-45/Wnt-5a cells were suspended in serum-free medium and kept in suspension or replated onto collagen-coated dishes. After 1 hour, the cells were lysed and probed with a phosphorylated specific antibody to FAK pY³⁹⁷ (pY397-FAK) or with anti-FAK antibody. **C**, activation of FAK and Rac by Wnt-5a. MKN-45 cells were treated with 80 ng/mL Wnt-5a for the indicated time, and then cells were lysed and probed with anti-pY397-FAK or anti-FAK antibody. The same lysates were incubated with GST-CRIB immobilized on glutathione-Sepharose. The total lysates and precipitates were probed with anti-Rac-1 antibody. **D**, involvement of PKC and Src in Wnt-5a-induced activation of FAK and cell migration. **a**, after MKN-45/control and MKN-45/Wnt-5a cells were treated with 62.5 nmol/L PP2 or GF109203X for 60 minutes, the cells were suspended in serum-free medium and kept in suspension (Sus) or replated onto collagen-coated dish. After 1 hour, the cells were lysed and probed with anti-pY397-FAK or anti-FAK antibody. **b**, after MKN-45 cells were treated with 62.5 nmol/L PP2 or GF109203X, the cells were subjected to the Transwell migration assay in the presence or absence of 600 ng/mL Wnt-5a.

diffuse-scattered type ($n = 40$); $P = 0.0014$ for other type ($n = 71$); Fig. 5A, b and c).

It has been reported that the expression of MIA, EGFR, or MMP-10 is associated with the aggressiveness of tumors, including gastric cancer (37). Although the prognosis of patients expressing MIA, EGFR, or MMP-10 was significantly worse ($P < 0.0001$, $P = 0.0049$, and $P = 0.0122$, respectively), the prognostic value of MIA was comparable with that of Wnt-5a (Fig. 5B, a-c). Cytosomal and nuclear accumulation of β -catenin did not show a significant association with the survival of gastric cancer ($P = 0.4873$; Fig. 5B, d).

It is generally known that patients with gastric cancer at stage I and stage IV show a good and poor survival, respectively, but it is difficult to predict the prognosis of patients with gastric cancer at stage II and stage III. Therefore, we analyzed the prognostic value of Wnt-5a, MIA, EGFR, or MMP-10 in the group of patient with gastric cancer at stage II and stage III ($n = 56$). The patients with Wnt-5a-positive gastric cancer had a significantly worse rate of survival than the patients with Wnt-5a-negative gastric cancer ($P = 0.0137$; Fig. 5C, a). In contrast, there was no significant association between the expression of MIA, EGFR, or MMP-10 and the survival of patients with gastric cancer at stages II and stage III (data not shown). The patients with gastric cancer at stage III indeed had the shorter survival time than those at stage II ($P = 0.0431$; Fig. 5C, b). These results clearly indicate that the 5-year

survival of gastric cancer is associated with expression of Wnt-5a in a histology-independent manner and suggest that Wnt-5a is a good prognostic indicator for gastric cancer patients.

Discussion

Gastric cancer is one of the leading causes of death due to cancer worldwide (21, 38). In this study, we showed that Wnt-5a is required for the migration and invasive ability of gastric cancer cells and that the expression of Wnt-5a is correlated with aggressiveness and poor prognosis of gastric cancer. It is notable that prognosis is not dependent on histologic type but rather is dependent on expression of Wnt-5a. Wnt-5a positivity is correlated with a poor prognosis of gastric cancer patients with stage II or stage III despite lack of statistical significance of other prognostic markers. Because it is hard to predict the prognosis of these patients, investigating the staining of Wnt-5a in gastric cancer cells may be helpful for determining the therapeutic strategy after surgical operation. Considering the results that sFRP2, anti-Wnt-5a antibody, or siRNA for Wnt-5a inhibits the cell migration of gastric cancer cells, Wnt-5a is not only a good prognostic indicator of gastric cancer but also a candidate for a molecular target of therapy for gastric cancer.

We also found that Wnt-5a is expressed in Chief cells in normal gastric mucosa. It is generally believed that stem cells are present

in the proliferative cell zone in the isthmus region of the gastric glands and that cells derived from stem cells undergo complex bipolar migration from the isthmus either upward or downward. Wnt-5a may be involved in the downward migration of cells differentiating into normal Chief cells.

Wnt-5a has been reported to be capable of activating JNK, which regulates convergent extension movement in *Xenopus* embryos and stimulates invasion of breast cancer cells (20, 39). However, the molecular mechanism by which Wnt-5a regulates cell migration is not fully understood. Cell migration is a complex cellular behavior that involves protrusion and adhesion at the cell front and contraction and detachment at the rear (36). FAK activation is best understood in the context of the engagement of integrins at the cell surface (34). Activation of FAK results in recruitment of several SH2 domain- and SH3 domain-containing proteins. Among them,

p130Cas and Crk are involved in cell migration. Small G protein Rac also plays an important role in cell migration by regulating adhesion turnover and the lamellipodium (40). Dominant-negative Rac blocks the increased migration in response to the expression of p130Cas and Crk, probably through DOCK180, which suggests that Rac acts as a downstream effector of the FAK-Cas-Crk complex.

We showed that Wnt-5a is involved in the activation of FAK and Rac, the turnover of paxillin, and the membrane ruffling. Inhibitors of PKC and Src suppressed Wnt-5a-dependent cell migration and FAK activation. It has been shown that Wnt-5a activates PKC (3) and that JNK-dependent phosphorylation of paxillin and PKC-dependent activation of FAK are important for cell migration (34, 41). These findings suggest that Wnt-5a activates PKC and JNK, thereby leading to the activation of FAK and paxillin. Taken together, these results make it conceivable that Wnt-5a and

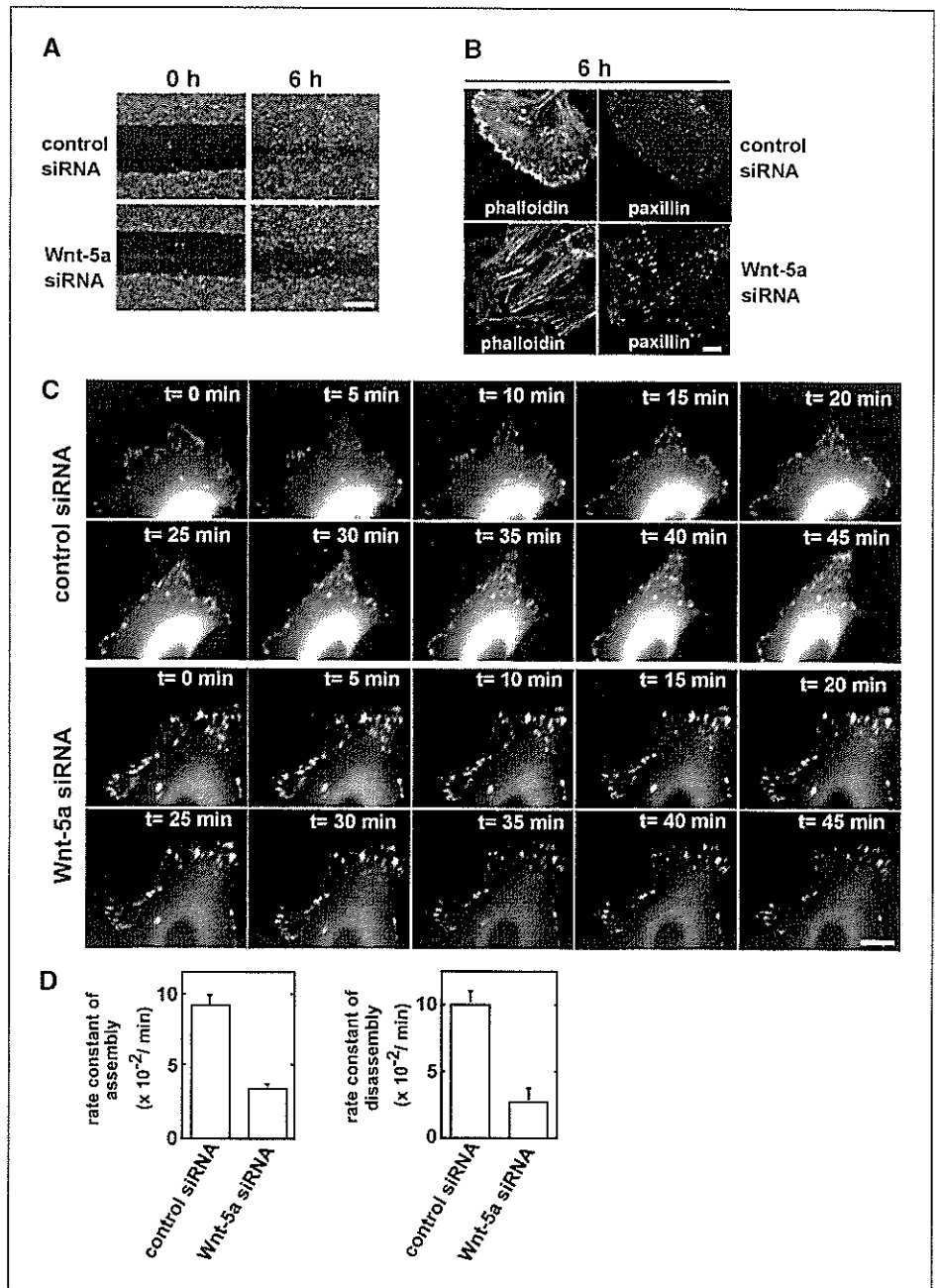


Figure 4. Effects of Wnt-5a on dynamics of focal adhesions. **A**, MKN-1 cells transfected with the control or Wnt-5a siRNA for 6 hours were wounded. Bar, 200 μm . **B**, MKN-1 cells transfected with the control or Wnt-5a siRNA were stained with FITC-labeled phalloidin for visualization of F-actin at 6 hours after wounding. The same cells were stained with anti-paxillin antibody. Bar, 10 μm . **C**, dynamics of GFP-paxillin in MKN-1 cells treated with the control or Wnt-5a siRNA visualized by time-lapse fluorescence microscopy. Bar, 5 μm . **D**, rate constants of assembly and disassembly of GFP-paxillin in (C) were calculated.

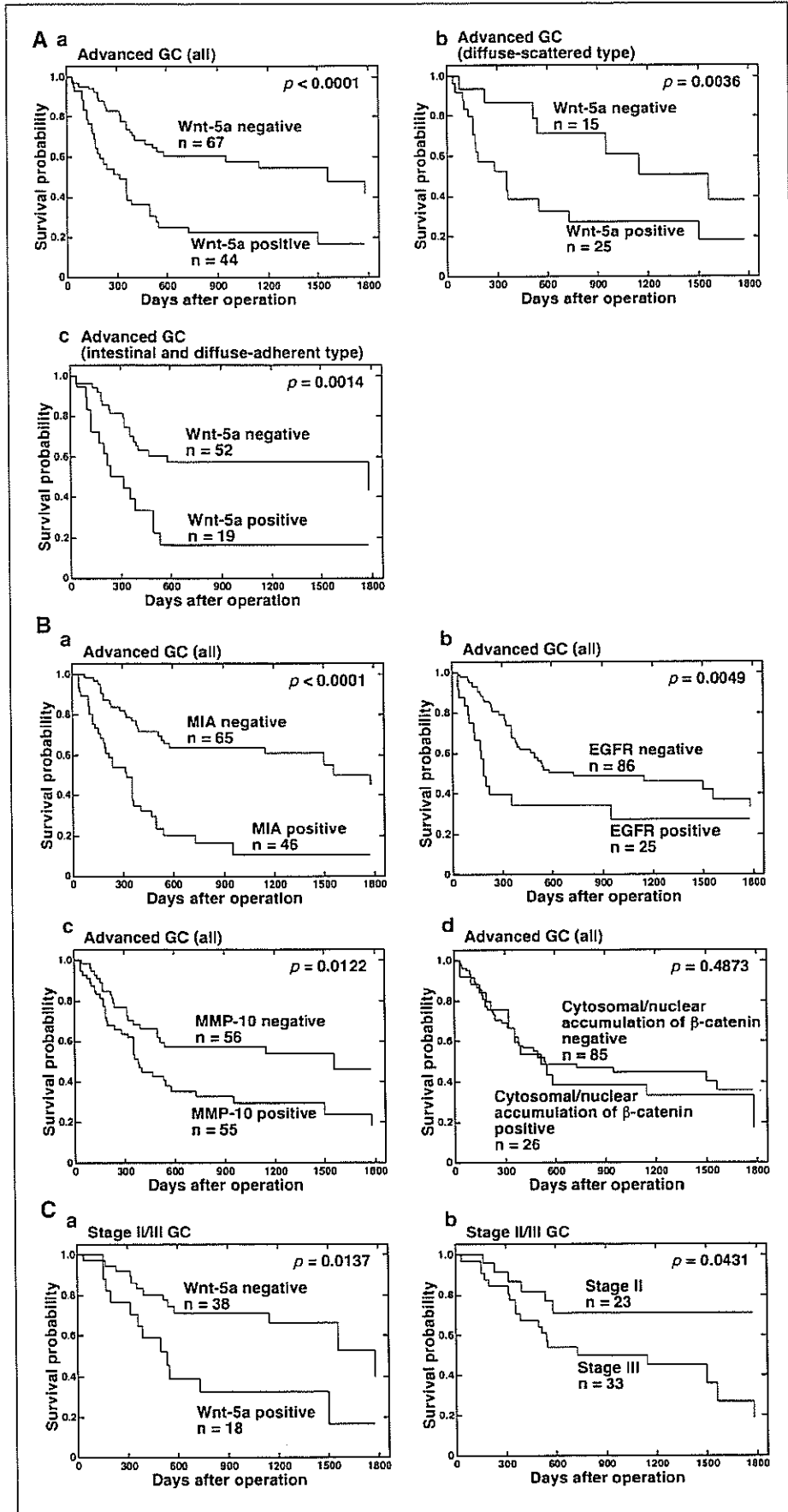


Figure 5. Overall survival of gastric cancer patients with Wnt-5a expression. **A**, association of expression of Wnt-5a with poor prognosis irrespective histologic type. **a**, all advanced stages of gastric cancer patients ($n = 111$); **b**, diffuse-scattered-type gastric cancer patients ($n = 40$); **c**, other type gastric cancer patients ($n = 71$). **B**, comparison with other prognostic factors. **a**, gastric cancer patients with or without MIA expression ($n = 111$); **b**, gastric cancer patients with or without EGFR expression ($n = 111$); **c**, gastric cancer patients with or without MMP-10 expression ($n = 111$); **d**, gastric cancer patients with or without β -catenin expression in cytoplasm and nucleus ($n = 111$). **C**, expression of Wnt-5a in stages II and III gastric cancer. **a**, stage II and III gastric cancer patients with or without Wnt-5a expression ($n = 56$); **b**, survival of stage II and III gastric cancer patients ($n = 56$).

extracellular matrix bind to Frizzled (Wnt-5a receptor) and integrin and cooperatively activate a signaling cascade to stimulate cell migration. Therefore, it is conceivable that overexpression of Wnt-5a acts as a migration activator in gastric cancer.

In addition, Wnt-5a has been suggested to act as a tumor suppressor in lymphoma, thyroid cancer, and colon cancer (15–17) because Wnt-5a inhibits the β -catenin pathway (12, 13). Our result indicated that Wnt-5a neither induces the accumulation of β -catenin nor inhibits Wnt-3a-dependent-accumulation of β -catenin in HEK293 and NIH3T3 cells (data not shown). Although Topol et al. (11) showed that Wnt-5a induces the down-regulation of β -catenin through expression of Siah2, we could not repeat this finding and our results were rather consistent with those showed by Mikels and Nusse (13). Whereas Topol et al. transfected Wnt-5a cDNA in HEK293 or SW480 cells, we added purified Wnt-5a protein to HEK293 and NIH3T3 cells. Although the reasons for the discrepancy between their and our results are not known at present, one possibility might be due to different assay conditions. Instead, we showed that Wnt-5a inhibits β -catenin-dependent Tcf activation downstream of β -catenin. It has been shown that transforming growth factor- β -activated kinase (TAK) and Nemo-like kinase (NLK) mediate Wnt-5a-dependent suppression of Tcf activity (12), but dominant negative forms of TAK or NLK did not influence it in our hands (data not shown). Therefore, the mechanism for the inhibition of the β -catenin pathway by Wnt-5a is not clear.

Among 237 gastric cancer cases, gastric cancer abnormally expressing both Wnt-5a and β -catenin were observed in only one gastric cancer case, suggesting that the expression of Wnt-5a and β -catenin is mutually exclusive. As Wnt-5a acts as a negative regulator for β -catenin-dependent cellular proliferation, the β -catenin-

positive cells may prevent the expression of Wnt-5a. Although the exact mechanism for the exclusive expression of Wnt-5a and β -catenin in gastric cancer is not known, there may be some mechanisms by which the β -catenin signaling and the Wnt-5a signaling mutually suppress the expression of Wnt-5a and β -catenin. Genetic alterations that cause abnormal expression of β -catenin in cancer cells are well understood, but how expression of Wnt-5a is regulated is totally unknown. It seems unlikely that Wnt-5a gene is amplified or rearranged in prostate cancer or melanoma (18). It has also been reported that expression of Wnt-5a mRNA is suppressed by Ras and hepatocyte growth factor (HGF; refs. 42, 43). It is intriguing to speculate that β -catenin mediates Ras- or HGF-induced suppression of Wnt-5a. Furthermore, whether mutually exclusive expression of Wnt-5a and β -catenin is specific for gastric cancer or not remains to be clarified. Therefore, immunohistochemical analyses of β -catenin are necessary for investigation of Wnt-5a in human cancers. Further studies will be necessary to understand the functions of Wnt-5a and the pathologic significance of the abnormal expression of Wnt-5a in cancer cells.

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The Neuromedin U-Growth Hormone Secretagogue Receptor 1b/Neurotensin Receptor 1 Oncogenic Signaling Pathway as a Therapeutic Target for Lung Cancer

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Abstract

Using a genome-wide cDNA microarray to search for genes that were specifically up-regulated in non-small cell lung cancers (NSCLC), we identified an abundant expression of neuromedin U (NMU) in the great majority of lung cancers. Immunohistochemical analysis showed a significant association of NMU expression with poorer prognosis of patients with NSCLC. Treatment of NSCLC cells with short interfering RNA against NMU suppressed its expression and inhibited the growth of the cells; on the other hand, the induction of exogenous expression of NMU conferred growth-promoting activity and enhanced cell mobility *in vitro*. We found that two G protein-coupled receptors, growth hormone secretagogue receptor 1b and neurotensin receptor 1, were also overexpressed in NSCLC cells, and that a heterodimer complex of these receptors functioned as an NMU receptor. The NMU-receptor interaction subsequently induced the generation of a second messenger, cyclic AMP, to activate its downstream genes including transcription factors and cell cycle regulators. Treatment of NSCLC cells with short interfering RNAs for growth hormone secretagogue receptor or neurotensin receptor 1 suppressed the expression of those genes and the growth of NSCLC cells. These data strongly implied that targeting the NMU signaling pathway would be a promising therapeutic strategy for the treatment of lung cancers. (Cancer Res 2006; 66(19): 9408-19)

Introduction

Lung cancer is one of the most common causes of cancer death worldwide, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of those cases (1). Many genetic alterations associated with the development and progression of lung cancer have been reported, but the precise molecular mechanisms remain unclear (2). Over the last decade, newly developed cytotoxic agents including paclitaxel, docetaxel, gemcitabine, and vinorelbine have emerged to offer multiple therapeutic choices for patients with

advanced NSCLC; however, each of the new regimens can provide only modest survival benefits compared with cisplatin-based therapies (3). Hence, new therapeutic strategies, such as the development of molecular-targeted agents, are eagerly awaited.

Systematic analysis of expression levels of thousands of genes on cDNA microarrays is an effective approach to identifying unknown molecules involved in pathways of carcinogenesis (4–11), and can reveal candidate targets for the development of novel anticancer drugs and tumor markers. We have been attempting to isolate novel molecular targets for the diagnosis, treatment, and prevention of NSCLC by analyzing genome-wide expression profiles of NSCLC cells on a cDNA microarray containing 23,040 genes, after pure populations of tumor cells were prepared from 37 cancer tissues by laser microdissection (4). To verify the biological and clinicopathologic significance of the respective gene products, we have been performing tumor-tissue microarray analysis of clinical lung cancer materials (7–9, 11). In the course of those studies, we observed that the gene encoding neuromedin U (NMU) was frequently overexpressed in primary NSCLCs.

NMU is a neuropeptide that was first isolated from porcine spinal cord. It has potent activity on smooth muscle (12–17), and in mammalian species, NMU is distributed predominantly in the gastrointestinal tract and central nervous system (18, 19). The peripheral activities of NMU include stimulation of smooth muscle, alternation of ion transport in the gut, and regulation of feeding (12); however, any role which NMU might have during lung carcinogenesis has not been implicated. Neuropeptides function peripherally as paracrine and autocrine factors to regulate diverse physiologic processes and act as neurotransmitters or neuro-modulators in the nervous system. In general, the receptors which mediate signaling by binding neuropeptides are members of the superfamily of G protein-coupled receptors (GPCR) having seven transmembrane-spanning domains. Two known receptors for NMU, NMU1R and NMU2R, show a high degree of homology to other neuropeptide receptors such as growth hormone secretagogue receptor (GHSR) and neurotensin receptor 1 (NTSR1), for which the corresponding known ligands are ghrelin (GHRL) and neurotensin (NTS), respectively.

In the study reported here, we identified NMU and its downstream molecules as potential targets for the development of novel therapeutic drugs and diagnostic markers. We also show that GHSR1b and NTSR1 could be a cognate heterodimerized receptor complex for NMU, and that this ligand-receptor system significantly affects the growth of lung cancer cells through the

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

K. Takahashi and C. Furukawa contributed equally to this work.

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