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 knockdown 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 *Drosphila* is mediated by Frizzled, which activates Rac, Rho, c-Jun NH_2-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-1α/β-catenin^{SA}, 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-αPAK-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′-CTGTGGATAACACCTCTGTTT-3′ and 5′-AAA-AACAGAGGTGTTATCCAC-3′, were used. Scrambled siRNA, 5′-CAGTCGC-GTTTGCGACTGG-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% H_2O_2-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-μm thick, 8-μm pores; Transwell, Costar, Cambridge, MA) as described (27). The lower surface of filters was coated with 10 μg/mL collagen. MKN-1, MKN-7, MKN-74, and MKN-45 cells (2.5×10^4 cells in 100 μL) 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 μg/mL) was added to the medium.

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

	Wnt-5a expression		*P*
	Positive, *n* (%)	Negative, *n*	
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: T_1, tumor invades lamina propria or submucosa; T_2, tumor invades muscularis propria or subserosa; T_3, tumor penetrates serosa without invasion of adjacent structures; and T_4 tumor invades adjacent structures. N_0, no regional lymph node metastasis; N_1, metastasis in 1 to 6 regional lymph nodes; N_2, metastasis in 7 to 15 regional lymph nodes; and N_3, metastasis in >15 regional lymph nodes.

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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′-CTTCGCCCAGGTTG-TAATTGAAGC-3′ and 5′-CTGCCAAAAACAGAGGTGTTATCC-3′; Wnt-5b, 5′-AGGGAACCCTACTCTGG-3′ and 5′-ACATCTCGGGTCTCTG-3′; and ribosomal high basic 23-kDa protein (RPL), GACCGTCTCAAGGTGT and CTT-CCGGTAGTGGATCT. 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. 1*A*). 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. 1*A*).

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. 1*B*). 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. 1*C, 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. 1*C, 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. 1*C, 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. 1*C, 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. 1*D, 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. 2*A, 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. 2*A, b*). However, expression of Wnt-5a in MKN-45 cells did not affect cell growth (Fig. 2*A, 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. 2*B, 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. 2*B, b*), and knockdown of Wnt-5a by RNA interference in MKN-1 cells suppressed the migration (Fig. 2*B, c*). Furthermore, whereas control MKN-1 cells invaded the Matrigel, Wnt-5a knockdown MKN-1 cells were less invasive (Fig. 2*B, 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. 2*C*). Furthermore, anti-Wnt-5a antibody suppressed the migration of MKN-1 cells in scratch-wound cultures (Fig. 2*D, a* and *b*). The inhibitory activity of this antibody for Wnt-5a was confirmed by

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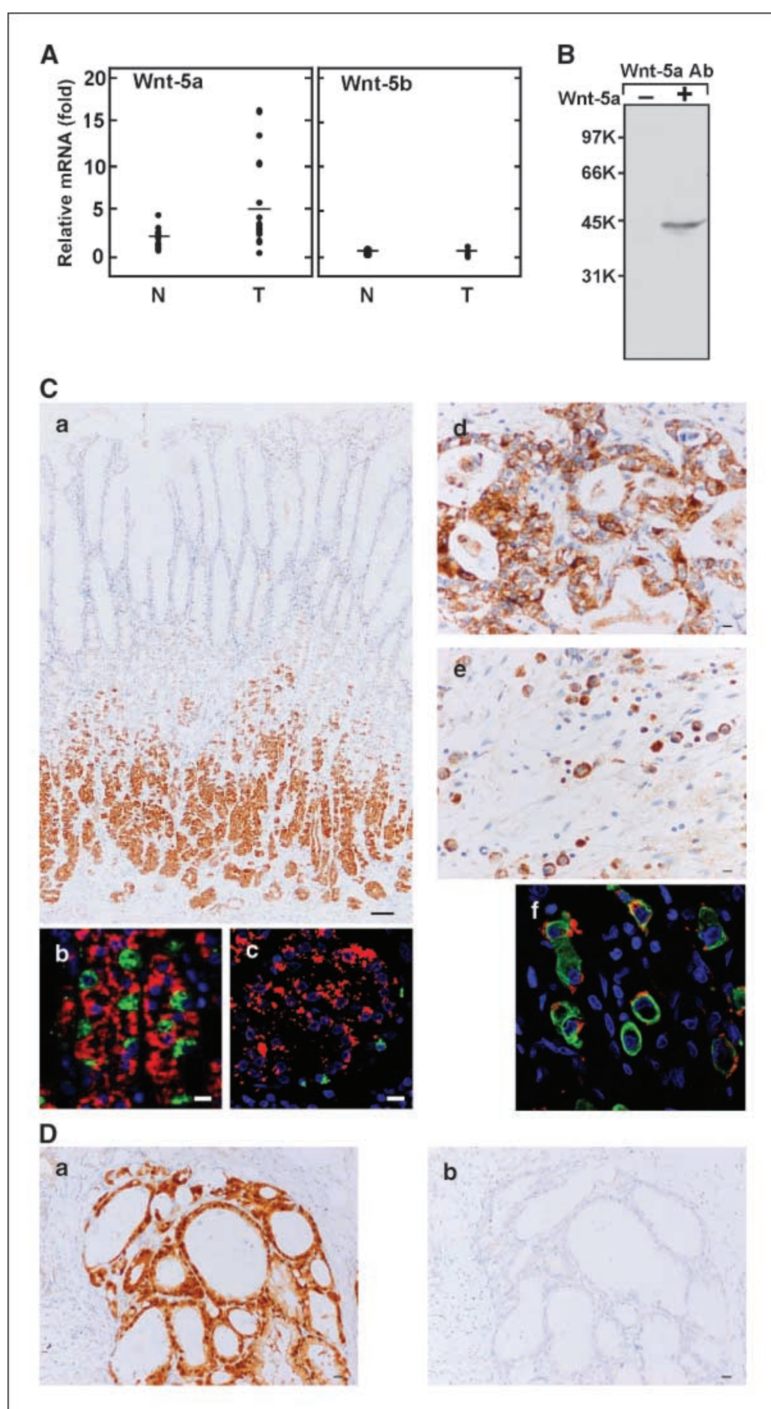

Figure 1. Expression of mRNA and proteins of Wnt-5a in nontumor and tumor regions of human stomach. *A,* expression of mRNA of Wnt-5a and Wnt-5b in normal stomach and gastric cancer. RT-PCR was used to quantitatively amplify mRNA of Wnt-5a, Wnt-5b, and RPL from tumor (*T*) and adjacent nontumor (*N*) 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 μm. *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 μm. *d,* intestinal-type gastric cancer was immunostained with anti-Wnt-5a antibody. Bar, 20 μm. *e,* diffuse-scattered-type gastric cancer was immunostained with anti-Wnt-5a antibody. Bar, 20 μm. *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 μm. *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 μm.

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. 2*D, 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. 3*A*), 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 Tyr^{397}, 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. 3*B*). Addition of purified Wnt-5a protein to MKN-45 cells activated FAK and Rac within 5 minutes (Fig. 3*C*). 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. 3*D*). 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. 4*A*). Ruffling and

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phalloidin staining were observed at the cell front along the leading edge of control migrating cells (Fig. 4*B*). 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. 4*B*). 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. 4*B*). 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. 4*C*). The rate constants of assembly and disassembly of GFP-paxillin at adhesion sites were decreased in Wnt-5a knockdown cells (Fig. 4*D*). 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 (T_2, T_3, and T_4) 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. 5*A, 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

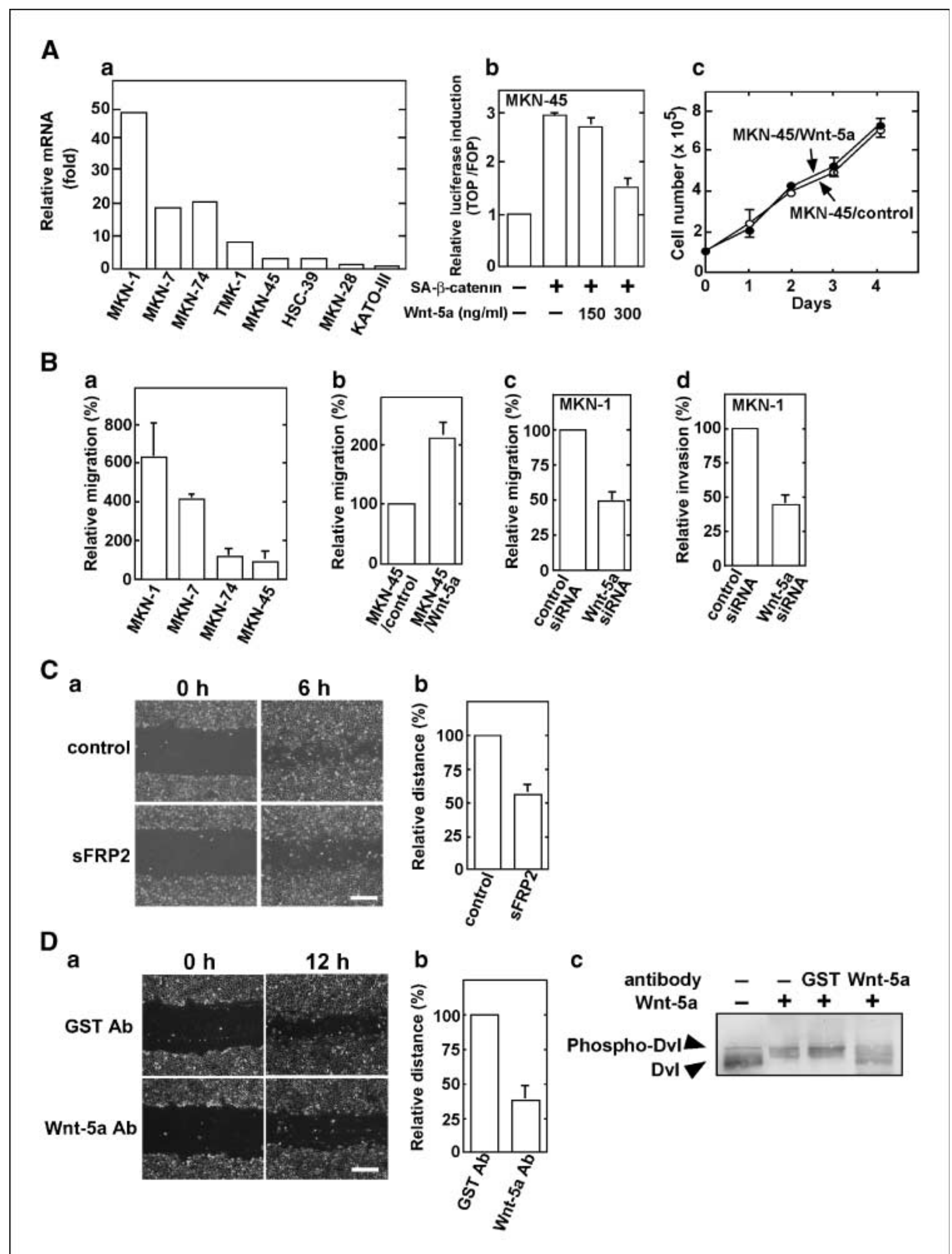

Figure 2. Actions of Wnt-5a on gastric cancer cells. *A,* inhibition of the β-catenin pathway by Wnt-5a. *a,* the level of expression of Wnt-5a mRNA in the gastric cancer cells was quantified by quantitative RT-PCR. *b,* after MKN-45 cells were transfected with pEF-BOS/hTcf-4E (0.1 μg), pUC/EF-1α/β-catenin^{SA} (0.5 μg), and TOP-*fos*-Luc (0.5 μg) or FOP-*fos*-Luc (0.5 μg), 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 μm. *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 μg/mL) were wounded. The culture was continued for 12 hours. Bar, 200 μm. *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.

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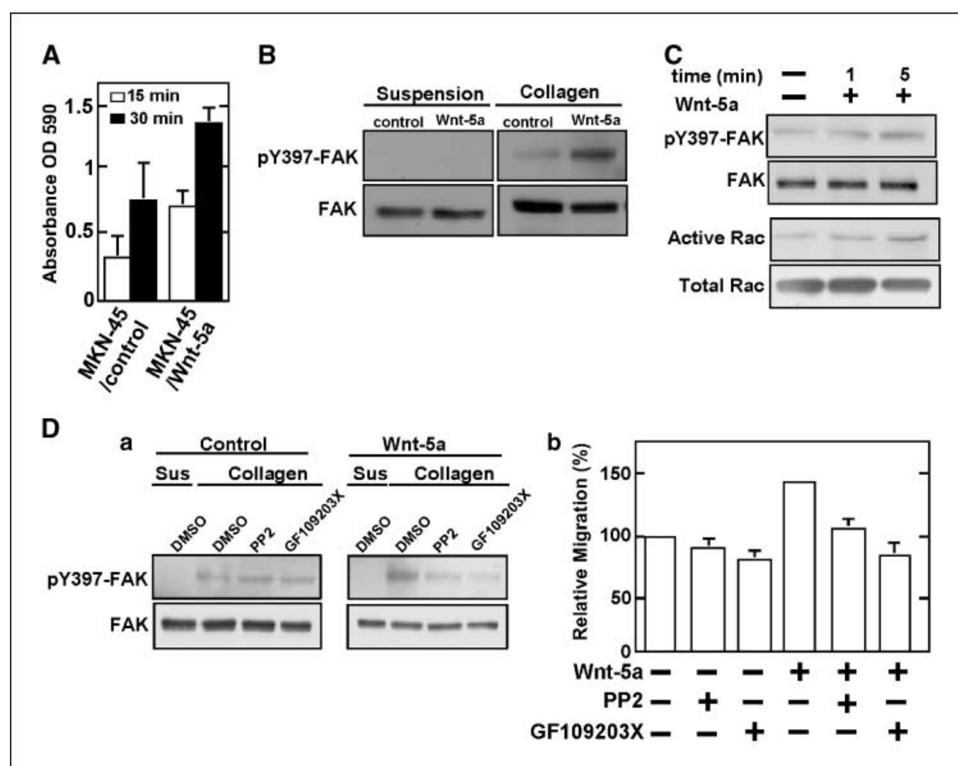

Figure 3. Molecular mechanism by which Wnt-5a stimulates cell migration. *A,* enhancement of cell adhesion by Wnt-5a. MKN-45/control or MKN-45/Wnt-5a cells were subjected to the adhesion assay for the indicated time. *B,* enhancement of collagen-induced activation of FAK by Wnt-5a. MNK-45/control and 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 pTyr^{397} (*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. 5*A, 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. 5*B, 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. 5*B, 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. 5*C, 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. 5*C, 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

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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

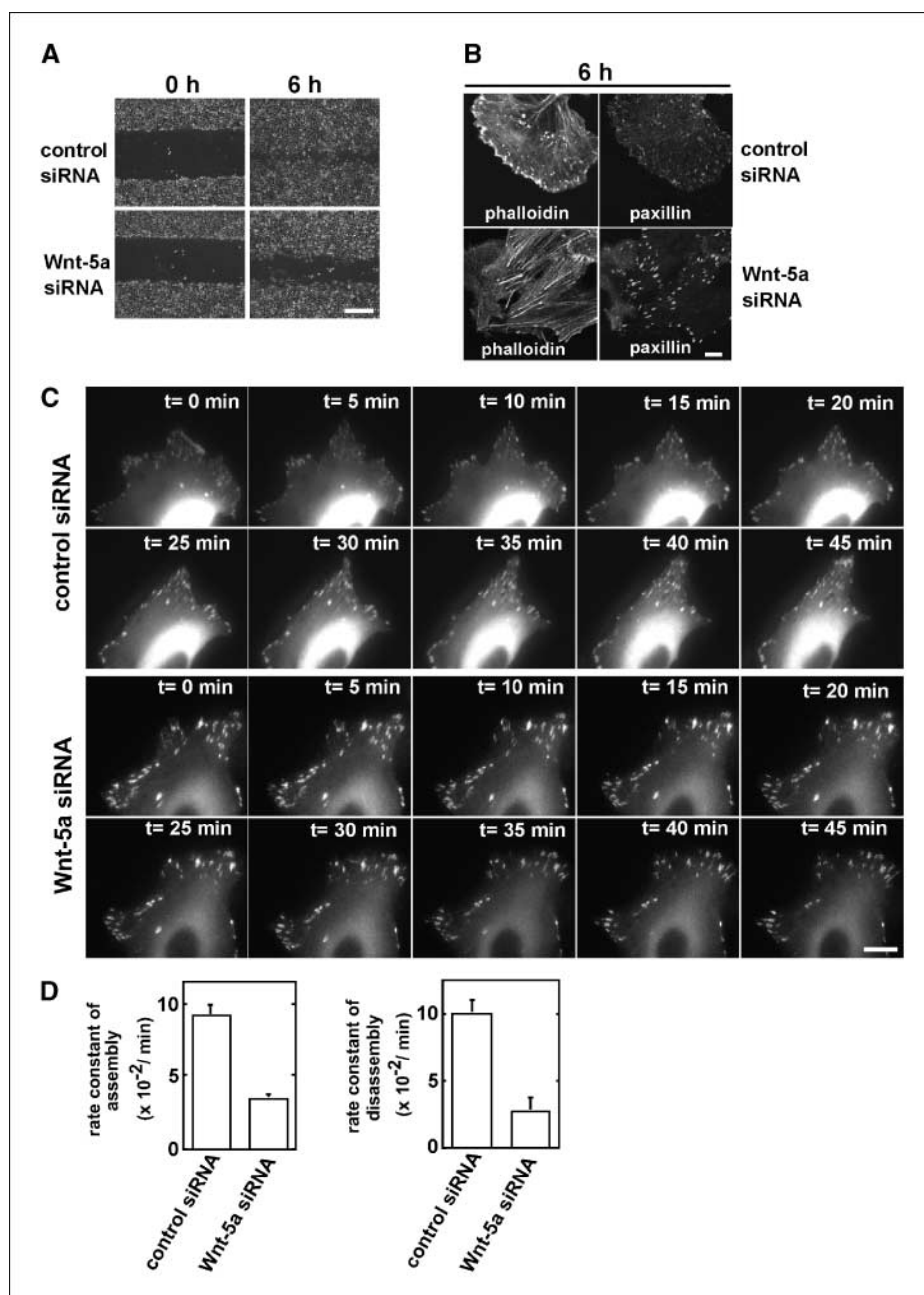

Figure 4. Effects of Wnt-5a on dynamics of focal adhesions. *A,* MKN-1 cells transfected with the control or Wnt-5a siRNA for 6 hours were wounded. Bar, 200 μm. *B,* MKN-1 cells transfected with the control or Wnt-5a siRNA were stained with FITC-labeled phalloidin for visualization of F-actin at 6 hours 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.

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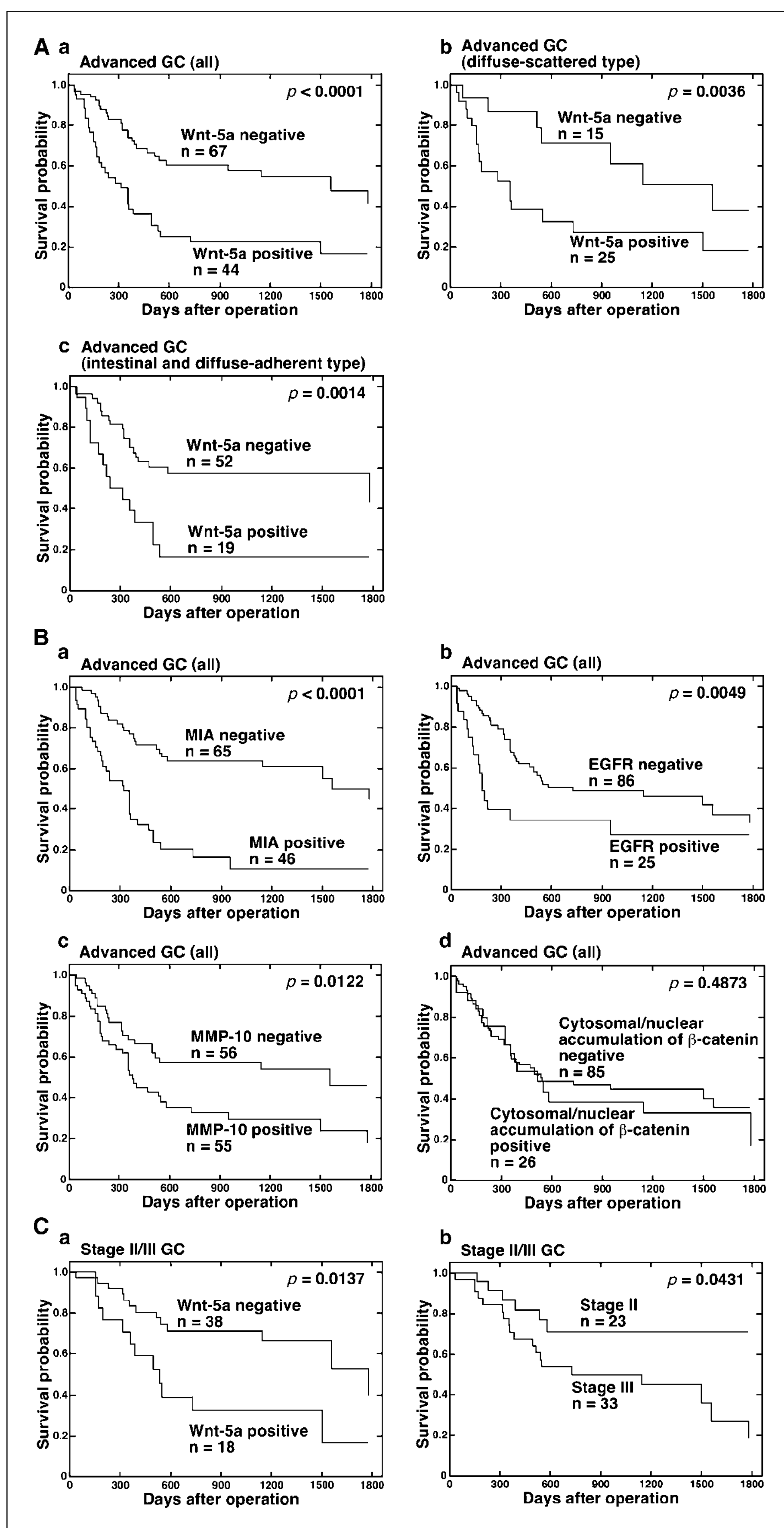

Figure 5. Overall survival of gastric cancer patients with Wnt-5a expression. *A,* association of expression of Wnt-5a with poor prognosis irrespective histologic type. *a,* all advanced stages of gastric cancer patients (n = 111); *b,* diffuse-scattered-type gastric cancer patients (n = 40); *c,* other 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).

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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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References

1. Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 1998;14:59–88.
2. Wong GT, Gavin BJ, McMahon AP. Differential transformation of mammary epithelial cells by wnt genes. Mol Cell Biol 1994;14:6278–86.
3. Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT. The Wnt/Ca2+ pathway: a new vertebrate wnt signaling pathway takes shape. Trends Genet 2000;16: 279–83.
4. Ikeda S, Kishida S, Yamamoto H, Murai H, Koyama S, Kikuchi A. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3b and b-catenin and promotes GSK-3b-dependent phosphorylation of b-catenin. EMBO J 1998;17:1371–84.
5. Kitagawa M, Hatakeyama S, Shirane M, et al. An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of b-catenin. EMBO J 1999;18:2401–10.
6. Kikuchi A. Roles of Axin in the Wnt signalling pathway. Cell Signal 1999;11:777–88.
7. Liu C, Li Y, Semenov M, et al. Control of b-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 2002;108:837–47.
8. He X, Semenov M, Tamai K, Zeng X. LDL receptor-related proteins 5 and 6 in Wnt/b-catenin signaling: arrows point the way. Development 2004;131: 1663–77.
9. Hurlstone A, Clevers H. T-cell factors: turn-ons and turn-offs. EMBO J 2002;21:2303–11.
10. Kohn AD, Moon RT. Wnt and calcium signaling: b-catenin-independent pathways. Cell Calcium 2005;38: 439–336.
11. Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ, Yang Y. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent b-catenin degradation. J Cell Biol 2003;162:899–908.
12. Ishitani T, Kishida S, Hyodo-Miura J, et al. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca2+ pathway to antagonize Wnt/b-catenin signaling. Mol Cell Biol 2003;23:131–9.
13. Mikels AJ, Nusse R. Purified Wnt5a protein activates or inhibits b-catenin-TCF signaling depending on receptor context. PLoS Biol 2006;4:570–82.
14. Olson DJ, Gibo DM. Antisense wnt-5a mimics wnt-1-mediated C57MG mammary epithelial cell transformation. Exp Cell Res 1998;241:134–41.
15. Liang H, Chen Q, Coles AH, et al. Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. Cancer Cell 2003;4:349–60.
16. Kremenevskaja N, von Wasielewski R, Rao AS, Schofl C, Andersson T, Brabant G. Wnt-5a has tumor suppressor activity in thyroid carcinoma. Oncogene 2005;24:2144–54.
17. Dejmek J, Dejmek A, Safholm A, Sjolander A, Andersson T. Wnt-5a protein expression in primary dukes B colon cancers identifies a subgroup of patients with good prognosis. Cancer Res 2005;65:9142–6.
18. Iozzo RV, Eichstetter I, Danielson KG. Aberrant expression of the growth factor Wnt-5A in human malignancy. Cancer Res 1995;55:3495–9.
19. Weeraratna AT, Jiang Y, Hostetter G, et al. Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell 2002;1:279–88.
20. Pukrop T, Klemm F, Hagemann T, et al. Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. Proc Natl Acad Sci U S A 2006; 103:5454–9.
21. Ohgaki H, Matsukura N. Stomach cancer. Lyon: IARC Press; 2003. p. 197.
22. Kishida S, Yamamoto H, Kikuchi A. Wnt-3a and Dvl induce neurite retraction by activating Rho-associated kinase. Mol Cell Biol 2004;24:4487–501.
23. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965;64:31–49.
24. Oue N, Motoshita J, Yokozaki H, et al. Distinct promoter hypermethylation of p16INK4a, CDH1, and RAR-β in intestinal, diffuse-adherent, and diffuse-scattered type gastric carcinomas. J Pathol 2002;198:55–9.
25. Aung PP, Oue N, Mitani Y, et al. Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene 2006;25:2546–57.
26. Yamamoto H, Ihara M, Matsuura Y, Kikuchi A. Sumoylation is involved in b-catenin-dependent activation of Tcf-4. EMBO J 2003;22:2047–59.
27. Kobayashi T, Hino S, Oue N, et al. Glycogen synthase kinase 3 and h-prune regulate cell migration by modulating focal adhesions. Mol Cell Biol 2006;26: 898–911.
28. Hino S, Tanji C, Nakayama K-I, Kikuchi A. Phosphorylation of b-catenin by cyclic AMP-dependent protein kinase stabilizes b-catenin through inhibition of its ubiquitination. Mol Cell Biol 2005;25:9063–72.
29. Ihara M, Yamamoto H, Kikuchi A. SUMO-1 modification of PIASy, an E3 ligase, is necessary for PIASy-dependent activation of Tcf-4. Mol Cell Biol 2005;25: 3506–18.
30. Zheng L, Wang L, Ajani J, Xie K. Molecular basis of gastric cancer development and progression. Gastric Cancer 2004;7:61–77.
31. Yokozaki H. Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathol Int 2000;50: 767–77.
32. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. J Cell Sci 2003;116:2627–34.
33. Gonzalez-Sancho JM, Brennan KR, Castelo-Soccio LA, Brown AM. Wnt proteins induce dishevelled

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phosphorylation via an LRP5/6- independent mechanism, irrespective of their ability to stabilize b-catenin. Mol Cell Biol 2004;24:4757–68.
34. Parsons JT. Focal adhesion kinase: the first ten years. J Cell Sci 2003;116:1409–16.
35. Turner CE. Paxillin and focal adhesion signalling. Nat Cell Biol 2000;2:E231–6.
36. Ridley AJ, Schwartz MA, Burridge K, et al. Cell migration: integrating signals from front to back. Science 2003;302:1704–9.
37. Yasui W, Oue N, Aung PP, Matsumura S, Shutoh M, Nakayama H. Molecular-pathological prognostic factors of gastric cancer. Gastric Cancer 2005;8:86–94.
38. Ushijima T, Sasako M. Focus on gastric cancer. Cancer Cell 2004;5:121–5.
39. Yamanaka H, Moriguchi T, Masuyama N, et al. JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. EMBO Rep 2002;3:69–75.
40. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002;420:629–35.
41. Huang C, Rajfur Z, Borchers C, Schaller MD, Jacobson K. JNK phosphorylates paxillin and regulates cell migration. Nature 2003;424:219–23.
42. Huguet EL, Smith K, Bicknell R, Harris AL. Regulation of Wnt5a mRNA expression in human mammary epithelial cells by cell shape, confluence, and hepatocyte growth factor. J Biol Chem 1995;270:12851–6.
43. Bui TD, Tortora G, Ciardiello F, Harris AL. Expression of Wnt5a is downregulated by extracellular matrix and mutated c-Ha-ras in the human mammary epithelial cell line MCF-10A. Biochem Biophys Res Commun 1997;239:911–7.

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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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