Crosstalk between Hedgehog and Wnt Pathways in Gastric Cancer Cell Differentiation

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Crosstalk between Hedgehog and Wnt Pathways in Gastric Cancer Cell Differentiation

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Jie-Hyun Kim

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

<ABSTRACT>

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Gastric cancer has different biological behaviors to the histological type. Thus, the different histological growth patterns of gastric cancers may involve the activation of distinct signaling pathways necessary for the growth and survival of cancer cells. This study investigated the differentiation-specific signal changes between hedgehog (Hh) and Wnt signaling pathways in AGS and MKN-45 gastric cancer cells, and HT-29 colon cancer cells were used as positive controls for differentiation *in vitro*. Differentiation was induced by sodium butyrate and all-*trans* retinoid acid. Sonic hedgehog (SHh), patched (Ptc)-1, and glioma-associated oncogene (Gli)-1 were analyzed for Hh signaling, and nuclear β-catenin was analyzed for Wnt signaling. Paraffin-embedded tissues from human gastric cancers were used to evaluate the expression of SHh, Gli-1, and β-catenin *in vivo*. Secreted frizzled-related protein (sFRP)-1

was analyzed as a regulator between Hh and Wnt signaling. As results, expression of Hh signaling was increased during differentiation. In contrast, the expression of Wnt signaling was decreased during differentiation. Ectopic expression of Gli-1 increased the level of the sFRP-1 transcript, whereas the inhibition of Gli-1 reduced the level of the sFRP-1 transcript. Chromatin immunoprecipitation assay indicated that Gli-1 was involved in the transcriptional regulation of sFRP-1. Ectopic expression of Gli-1 decreased the expression of nuclear β-catenin, and the inhibition of Gli-1 recovered the level of nuclear β-catenin. SHh- and Gli-1-positive immunoexpression was higher in well differentiated than in poorly differentiated tissues. However, nuclear β-catenin-positive immunoexpression was lower in well differentiated compared to poorly differentiated tissues. In conclusion, the activation of the Hh pathway and suppression of the Wnt pathway by Hh signaling occurred during gastric cancer cell differentiation. It is suggested that the analysis of differentiation-specific signal changes, combined with signals related to the process of tumor progression or migration, may be a clue in discovering the molecular heterogeneity of gastric cancers.

Key words: Differentiation, Hedgehog, Wnt, carcinogenesis, crosstalk

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I. INTRODUCTION

Recent evidences suggest that the accumulation of genetic and/or epigenetic alterations in multipotent adult stem cells and/or their early progeny may contribute to their oncogenic transformation into tumorigenic and migrating cancer progenitor cells.[1-5] These cancer progenitor cells, also known as cancer stem cells or cancer-initiating cells, can give rise to more differentiated cancer cell phenotypes *in vitro* and *in vivo*, and appear to play key roles in tumor formation, progression, and metastasis to distant sites.[5] According to this theory, the activation of specific oncogenic cascades in cancer-initiating cells during cancer initiation and progression could result in either highly or weakly invasive cancer subtypes.[5]

Generally, gastric cancers are believed to have different biologic behaviors according to their histological type. For example, histologically poorly differentiated growth type cancers, according to the World Health Organization (WHO) classification, have more aggressive behaviors than histologically well and moderately differentiated growth types. Thus, the histological type is one of the determining factors in the clinical therapeutic approach. Based on cancer-initiating cell theory, the different histological growth types of gastric cancer may involve the activation of distinct signaling pathway(s) necessary for the growth and survival of cancer cells.

The Hedgehog (Hh) and Wnt pathways are representative positive regulators for cancer-initiating cells. In Hh signaling, secreted Hh molecules [three mammalian hh genes: Sonic, Indian, and Desert hedgehog (SHh, IHh, and DHh, respectively)] bind to patched (Ptc; Ptc-1 and Ptc-2) receptors, alleviating Ptc-mediated suppression of smoothened (Smo), a putative seven-transmembrane protein. Smo signaling triggers a cascade of intracellular events, leading to activation of a pathway through glioma-associated oncogene (Gli)-dependent transcription.[6] In Wnt signaling, the signal-transducing components of the Wnt receptor are members of the low-density lipoprotein receptor-related protein (LRP) and Frizzled protein families.[6] In the absence of signal stimulation, β-catenin protein is destabilized by a cytoplasmic complex

containing the proteins Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK-3β).[6] Wnt signaling stabilizes β-catenin, which acts as a transcriptional co-activator by associating with the TCF/LEF family of transcription factors.[6]

These signaling pathways are necessary for the growth and survival of cancer cells. Activation of Hh or Wnt signaling has been implicated in the development of gastric cancer in many studies.[7-17] However, the association between the activation of these signals and the histological growth types of gastric cancer has been inconsistent.[16, 18-20] The discrepancy is probably due to the different proportion of cancer stages in each study and no consideration of signal interaction(s). Because the histological type or differentiation of gastric cancer can be altered by tumor invasion, morphological and phenotypic shifts could occur in the process of tumor progression.[21, 22] Furthermore, a molecular link between Hh and Wnt signaling has been reported.[18, 23-25] Thus, an *in vitro* differentiation model by human gastric cancer cell lines was used to investigate differentiation-specific signaling changes between Hh and Wnt signaling in gastric cancer cells. The analysis of changes in differentiation-specific signals in gastric cancer may help in understanding the heterogeneity of gastric cancers.

II. MATERIALS AND METHODS

1. Tumor samples and immunohistochemistry

Stored surgical specimens obtained from 20 patients with gastric cancer were used. All cases were provided by the Gastrointestinal Tumor Working Group Tissue Bank, Yonsei University Medical Center, Seoul, Korea, between December 1996 and December 2004. Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Yonsei University Health System. The 10 specimens were well differentiated adenocarcinoma, and the others were poorly differentiated adenocarcinoma.

Tissue sections in microslides were deparaffinized with xylene, dehydrated in serial dilutions of alcohol, and immersed in 3% H_2O_2. Following antigen retrieval in citrate buffer (pH 6.0), the tissue sections were incubated with protein blocking agent (Immunotech, Coulter, Inc., Marseille, France) to block nonspecific antibody binding for 20 minutes at room temperature and then incubated overnight at 4°C with respective primary polyclonal goat antibodies against human SHh (clone H-160) (Santa Cruz Biotechnology, Santa Cruz, California, USA), Gli-1 (clone H-300) (Santa Cruz), and β-catenin (clone E-5) (Santa Cruz). After washing with phosphate buffered saline (PBS) three times, the sections were incubated with a biotinylated secondary antibody (goat anti-

rabbit IgG, Immunotech) and streptavidin conjugated to horseradish peroxidase (Immunotech) for 20 minutes at room temperature, followed by a PBS wash. The chromogen was developed for five minutes with liquid 3, 3′-diaminobenzidine (Immunotech). Next, slides were counterstained with Meyer's hematoxylin. Expression status was quantified by scoring both the intensity and proportion of SHh, Gli-1, and β-catenin staining. The intensity of cytoplasmic SHh staining was scored as 0, negative (weak or similar to background); 1, weak (less intense than normal cells); 2, moderate (similar intensity to normal cells); and 3, strong (stronger than normal cells).[26] The intensity of Gli-1 and β-catenin staining was scored as 0, no detectable nuclear staining; 1, weak nuclear staining; and 2, strong nuclear staining.[27] The proportion of expression in each tissue was determined by counting positively stained cancer cells in relation to a total of three hundred cells, and expressing the result as a percentage.

2. Cell culture and induction of differentiation

AGS (ATCC CRL 1739, poorly differentiation), MKN45 (KCLB 80103, poorly differentiation) for gastric cancer cells and HT-29 (ATCC HTB38) for colon cancer cells were maintained in RPMI 1640 medium (Gibco, Grand Island. NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin sulfate (Gibco). All cultures were maintained in a 37°C incubator supplemented with 5% CO_2. Exponentially growing cells were

trypsinized and seeded into 10 cm^2 cell culture Petri dishes at a density of $5x10^6$cells/cm^2 for AGS, and MKN-45, and HT-29 cells were as positive control for induction of cell differentiation. When cells reached 50-70% confluency as determined by microscopic examination, medium was renewed and drugs were added from concentrated stock solutions. For drug-induced cell differentiation, sodium butyrate (NaBU) (Sigma, Munich, Germany) and all-*trans* retinoic acid (Sigma) was used at a final concentration of 1-3 μM for 48hrs.

Alkaline phosphatase (ALP) activity was determined as markers for differentiation. For ALP activity, cell lysates were assayed using 7 μM *p*-nitrophenylphosphate as substrate and 2-amino-2-methylpropan-1-ol as solvent. To determine ALP activity, the product (*p*-nitrophenol) produced per minute was measured and normalized for cellular protein.[28] Carcinoembryonic antigen (CEA) was also detected by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting as a marker for differentiation.[22, 29] Furthermore, the expression of Brahma (Brm), which is lost in poorly differentiated gastric cancer *in vivo*,[30] was analyzed by RT-PCR and western blot analysis in gastric cancer cells after NaBU treatment.

3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells using an RNeasy mini kit (Qiagen, Tokyo, Japan). The RNA was reverse transcribed using oligo (dT)

primers and Superscript^{TM} Ⅱ reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was performed with a PCR Maxi kit (iNtRON, Sungnam, Korea) according to the manufacturer's instructions. Amplification conditions included denaturation at 95°C for 5 min, followed by 30 cycles of 30 sec each at 95°C, 57°C, and 72°C for SHh, 32 cycles of 45sec each at 95°C, 55°C, and 72°C for CEA, 32cycles of 45 sec each at 95°C, 58°C, and 72°C for Brm, 30 cycles of 30 sec each at 95°C, 55°C, and 72°C for sFRP-1, and 18 cycles of 30 sec each at 95°C, 60°C, and 72°C for β-actin. PCR products were separated in 1.5% agarose gels. The oligonucleotide primers used for RT-PCR were as follows: human SHh, 5'-GAG ATG TCT GCT GCT AGT CC-3' and 5'-GTT TCT GGA GAT CTT CCC TT-3'; CEA, 5'-CCA GAA CGT CAC CCA GAA TG-3' and 5'-GGT TCA GAT TTT CCC CTG GA-3'; Brm, 5'-CTG CAA GAG CGG GAA TAC AGA CTT CAG GCC CG-3' and 5'-GGC TGC CTG GGC TTG CTT GTG CTC CCA AAC C-3'; sFRP-1, 5'-TCA TGC AGT TCT TCG GCT TC-3' and 5'-CCA ACT TCA GGG GCT TCT TC-3'; β-actin, 5'-TTG CCG ACA GGA TGC AGA AGA-3' and 5'-AGG TGG ACA GCG AGG CCA GGA T-3'.

4. Western blot analysis

Prepared cells were harvested after washing with PBS. Collected cells were lysed with buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor

cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA)). Fractionation was performed by sequential extraction of cytosolic and nuclear proteins in non-ionic detergent for analysis of β-catenin. The same amount of protein was boiled at 95°C after adding SDS sample buffer (62.5 mM Tris-Cl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, β-mercaptoethanol, and 0.002% bromophenol blue). Samples were loaded in 12% SDS-PAGE gels for SHh, sFRP-1, 10% SDS-PAGE gels for CEA, β-catenin, 8% SDS PAGE gels for Ptc-1, Gli-1, and 6% SDS-PAGE gels for Brm, and then transferred to PVDF membranes (Amersham Biosciences, Pisctaway, NJ, USA).

Rabbit anti-SHh (Santa Cruz), anti-Gli1 (Santa Cruz), anti-Ptc-1 (Santa Cruz), anti-Brm (Santa Cruz), anti-sFRP1 (Santa Cruz), anti-CEA (Upstate Biotechnology, Lake Placid, NY, USA) and anti-β-catenin (Santa Cruz) were used as the primary labeling antibodies and the appropriate horseradish peroxidase–conjugated antibodies (Santa Cruz) were used as secondary antibodies. An enhanced chemiluminescence detection system (ECL-Plus, iNtRON, Seoul, Korea) was used for detection according to the manufacturer's protocol.

5. Vectors

pcDNA3.1/SRα-Gli1 and pcDNA3.1 were kindly provided by Dr. Ishii (Tsukuba Life Science Center, Ibaraki, Japan). pSG5-HA/β-catenin and pSG5-

HA were kindly provided by Prof. Kim (Department of Biochemistry and Molecular Biology, Yonsei university college of medicine, Seoul, Korea). pTopflash and pFopflash were also kindly provided by Prof. Ryu (National Research Laboratory of Tumor Virology and Department of Biochemistry, Yonsei university, Seoul, Korea). Cells were plated onto 6-well plates 24hrs before transfection and transfected with 2 μg of plasmid using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Cells were treated by NaBU for 24hrs, and then transfected. Forty-eight hours after transfection, cells were harvested and subjected to further examination.

6. RNA interference

Small interfering RNA (SiRNA) against Gli-1 and negative-control siRNA was kindly provided by Dr. Ishii (Tsukuba Life Science Center, Japan). Cells were transfected with 100nM siRNA using TransIT-TKO transfection Reagent (Mirus, Madison, WI, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested and subjected to further evaluations.

7. Luciferase reporter assay

Cells grown in 6-well tissue-culture plates were transfected with 2 μg of pTopflash and pFopflash, 2 μg of pcDNA3.1 (internal control), 2 or 3 μg of gene expression plasmid, and 50 μg of Renilla TK-plasmid. Luciferase assays

were performed 48 hours after transfection using a Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions.

8. Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was done using ChIP assay kit (Upstate Biotechnology, Inc.) according to the manufacturer's instruction. AGS cells were used, and immunoprecipitation was done overnight at 4°C with 10 μl of the sample used as the "input," or 1 μg of Gli-1 or the negative control mouse IgG, positive control anti-RNA polymerase beads. After reverse crosslinking, DNA was purified with QIAquick PCR purification kit (Qiagen, Valencia, California, USA) from the immunoprecipitates. PCR was performed with 35 cycles of 96 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s using the following primers flanking the putative Gli-binding sites in the human sFRP-1 promoter: sFRP-1 sense, 5`-GTTGGAGCTGTTTGCTGTGA- 3`; sFRP-1 anti-sense, 5`- ATG TTTTGGCTTTCCACACC-3`.

9. Statistical analysis

Statistical analyses were conducted using Student *t*-test for luciferase assay and Pearson's chi-square test for immunohistochemistry. A two-sided *P*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (SPSS 12.0, Chicago, IL, USA).

III. RESULTS

1. Hh and Wnt signaling in human tissues

To investigate Hh and Wnt signal expression in human gastric cancer tissues, immunohistochemical staining was performed for 20 paraffin-embedded surgical specimens: 10 well differentiated and 10 poorly differentiated adenocarcinomas. All specimens were obtained from patients with American Joint Committee on Cancer (AJCC) TNM stage I or II.

The samples of well differentiated adenocarcinomas displayed higher SHh and Gli-1 staining than those of poorly differentiated adenocarcinomas ($P < 0.05$; Table 1, 2, Fig. 1A, B). In contrast, the percentage of cells with nuclear staining for β-catenin was higher in poorly differentiated than in well differentiated adenocarcinomas ($P < 0.05$; Table 3, Fig. 1C). These findings suggest that Hh signaling components are expressed in well differentiated adenocarcinomas at a higher level than in poorly differentiated adenocarcinomas. The Wnt signaling pathway showed the reverse result.

Table 1. Relationship between sonic hedgehog expression[1] and differentiation in gastric cancer

	Expression intensity of SHh (%)[2]				
	0	**1**	**2**	**3**	***P* value***
WD	15.5	6.9	32.2	45.4	<0.05
PD	10.0	26.8	44.5	18.7	

SHh, sonic hedgehog; WD, well-differentiated; PD, poorly-differentiated

* Pearson's chi-square test

[1]The proportion of expression in each tissue was determined by counting positively stained cancer cells in relation to a total of three hundred cells, and expressing the result as a percentage.

[2]The intensity of cytoplasmic SHh staining was scored as 0, negative (weak or similar to background); 1, weak (less intense than normal cells); 2, moderate (similar intensity to normal cells); and 3, strong (stronger than normal cells).

Table 2. Relationship between glioma-associated oncogene expression[1] and differentiation in gastric cancer

	Expression intensity of Gli-1 (%)[2]			
	0	**1**	**2**	***P* value***
WD	41.1	39.4	19.5	<0.05
PD	55.2	32.8	12.0	

Gli-1, glioma-associated oncogene; WD, well-differentiated; PD, poorly-differentiated

* Pearson's chi-square test

[1]The proportion of expression in each tissue was determined by counting positively stained cancer cells in relation to a total of three hundred cells, and expressing the result as a percentage.

[2]The intensity of Gli-1 staining was scored as 0, no detectable nuclear staining; 1, weak nuclear staining; and 2, strong nuclear staining.

Table 3. Relationship between nuclear β-catenin expression[1] and differentiation in gastric cancer

	Expression intensity of β-catenin (%)[2]			
	0	**1**	**2**	***P* value***
WD	49.1	38.2	12.7	<0.05
PD	27.3	55.0	17.7	

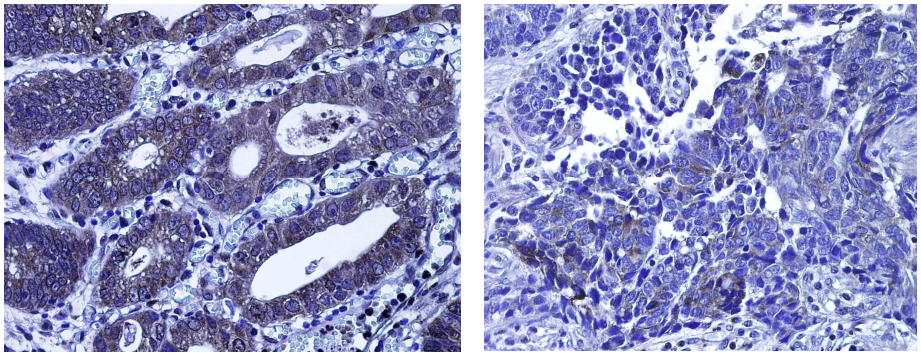
WD, well-differentiated; PD, poorly-differentiated

* Pearson's chi-square test

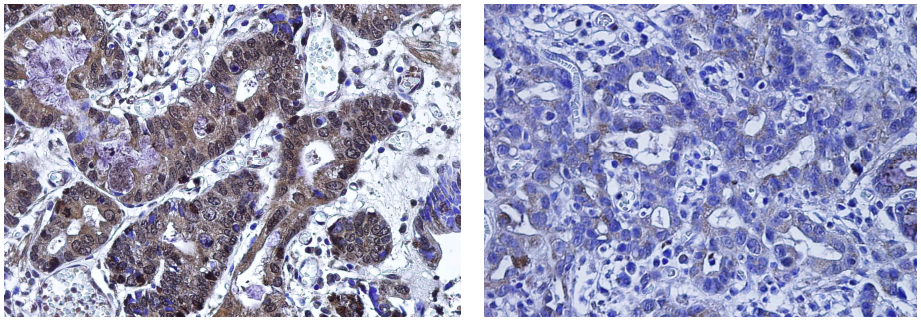
[1]The proportion of expression in each tissue was determined by counting positively stained cancer cells in relation to a total of three hundred cells, and expressing the result as a percentage.

[2]The intensity of β-catenin staining was scored as 0, no detectable nuclear staining; 1, weak nuclear staining; and 2, strong nuclear staining.

A. (a) SHh, WD (b) SHh, PD



B. (a) Gli-1, WD (b) Gli-1, PD



C. (a) β-catenin, WD (b) β-catenin, PD

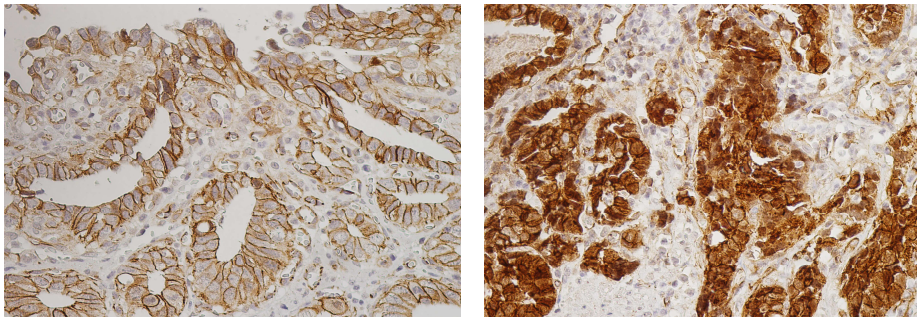


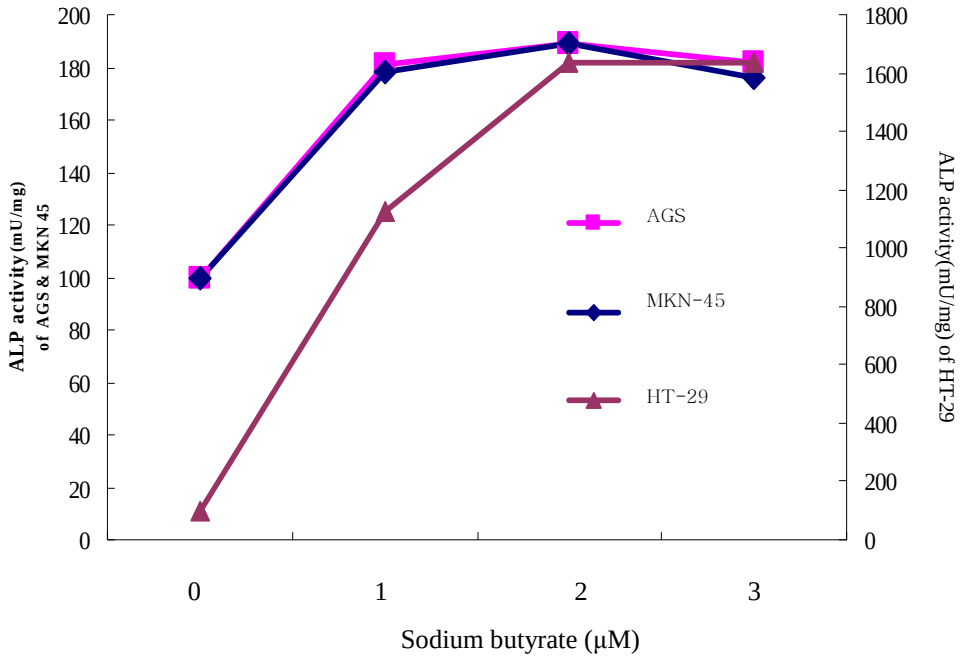
Fig. 1. Immunohistochemical staining for sonic hedgehog (SHh), glioma-associated oncogene-1 (Gli-1), and β-catenin in well-differentiated (WD) and

poorly-differentiated (PD) gastric carcinoma tissues (x400). Cytoplasmic SHh and nuclear Gli-1 staining revealed stronger expression in WD than PD tissues. On the contrary, the expression of nuclear β-catenin was stronger in PD than WD tissues.

2. Differentiation of gastric cancer cells

Poorly differentiated gastric cancer cell lines (AGS and MKN-45) were treated with NaBU and RA, well-known differentiation-inducing agents. HT-29 colon cancer cells were also differentiated with NaBU as a positive control. ALP, CEA, and Brm were used as differentiation markers. ALP is a well characterized marker of cell differentiation. In this study, increased ALP activity was noted in NaBU-treated AGS and MKN-45 cells, in a pattern similar to that in HT-29 cells (Fig. 2A). Maximal ALP activity was found in gastric cancer cells treated with 2 μM NaBU, consistent with a previous study.[32] ALP activity also increased in gastric cancer cell line after treatment with another differentiation-inducing agent, RA (Fig. 2B). The expression of CEA and Brm increased in gastric cancer cells after NaBU treatment (Figs. 3, 4). These findings suggest that gastric cancer cells can be differentiated by differentiation-inducing agents (NaBU, RA). Furthermore, differentiated gastric cancer cells over-expressed Brm, a valid marker for well differentiated gastric cancer in human tissues.

A.



B.

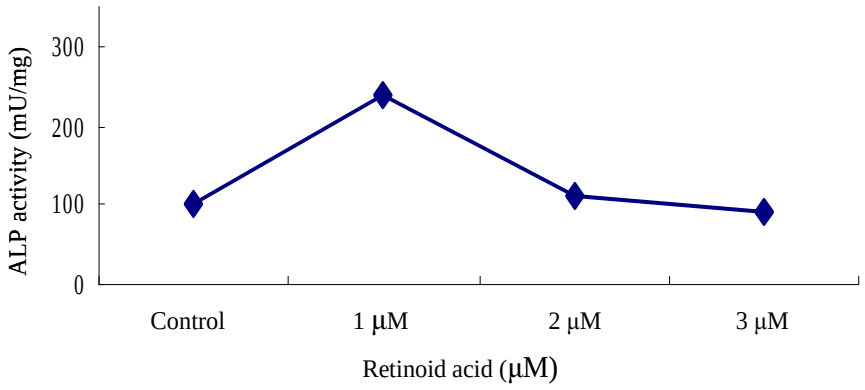


Fig. 2. Increased alkaline phosphatase activity by differentiaion.

A. Sub-confluent AGS, MKN-45 and HT-29 cells were treated with sodium butyrate (0-3 μM) for 48 hours. ALP activity was increased in AGS and MKN-45 cells as similar pattern of the HT-29 cells.

B. Sub-confluent AGS cells were treated with all-*trans* retinoic acid (0-3 μM) for 48 hours. ALP activity was increased after 1 μM retinoid acid treatment.

ALP, alkaline phosphatase; RA, all-*trans* retinoid acid.

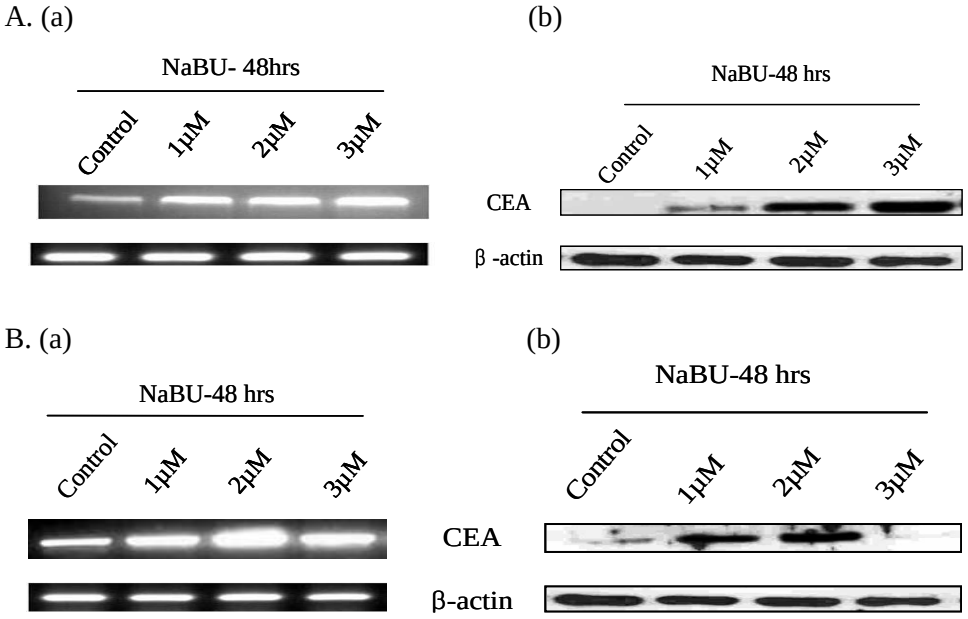


Fig. 3. Overexpression of carcinoembryonic antigen by butyrate treatment in AGS (A) and MKN-45 (B) cells.

RT-PCR (a) and Western blot (b) for CEA were performed with β-actin as internal standard. CEA, carcinoembryonic antigen.

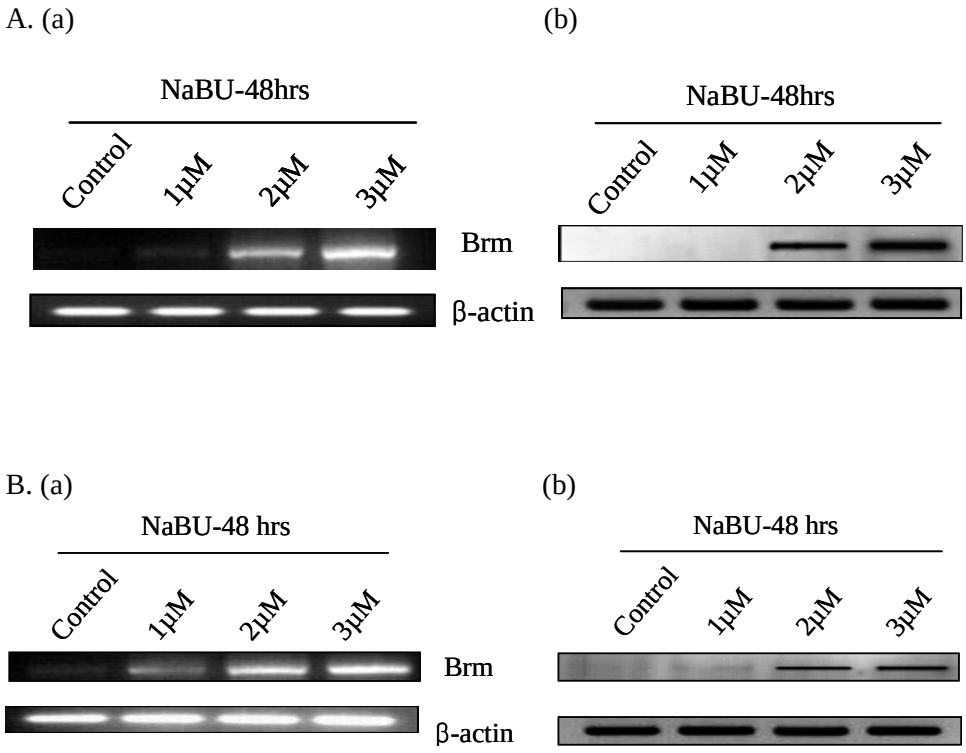


Fig. 4. Overexpression of Brm by butyrate treatment in AGS (A) and MKN-45 (B) cells. RT-PCR (a) and Western blot (b) for Brm were performed with β-actin as internal standard.

3. Hh signaling pathway in gastric cancer cell differentiation

To evaluate the Hh signaling pathway during gastric cancer cell differentiation, it was first examined whether the expression of SHh changed in gastric cancer cells after NaBU treatment using RT-PCR and Western blot analyses. NaBU was treated to 2μM, because gastric cancer cells were maximally differentiated in 2μM of NaBU in our study.

The expression of SHh increased in AGS and MKN-45 cells during differentiation by sodium butyrate (Fig. 5). It was next examined ligand-dependent Hh signal pathway activation in gastric cancer cells after NaBU treatment, according to Gli-1 and Ptc-1, using Western blotting. Over-expression of Gli-1 and Ptc-1 proteins was noted in both gastric cancer cell lines after NaBU treatment (Fig. 6). These data suggest that the Hh signaling pathway is enhanced during gastric cancer cell differentiation.

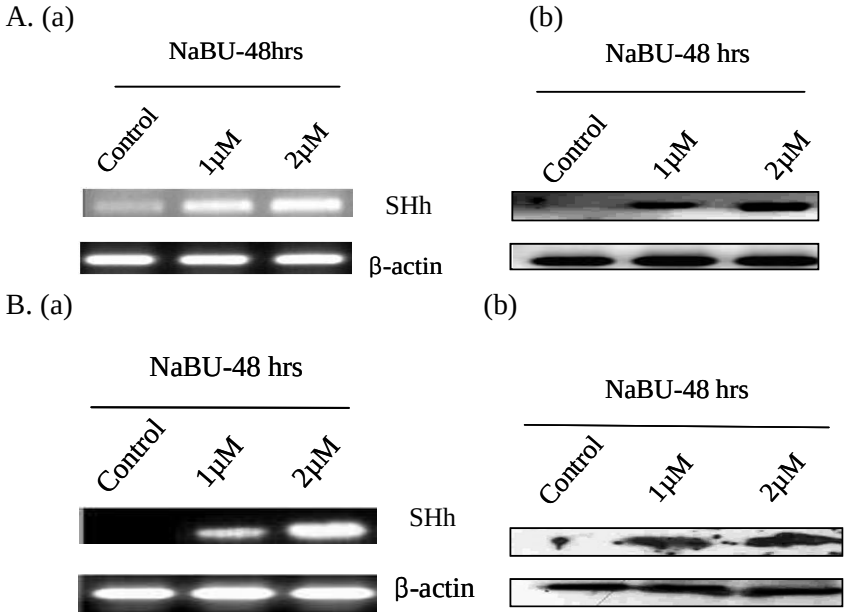


Fig. 5. Overexpression of sonic hedgehog by sodium butyrate treatment for 48 hours in AGS (A) and MKN-45 (B) cells.

RT-PCR (a) and Western blot (b) for SHh were performed with β-actin as internal standard. SHh, sonic hedgehog; NaBU, sodium butyrate.

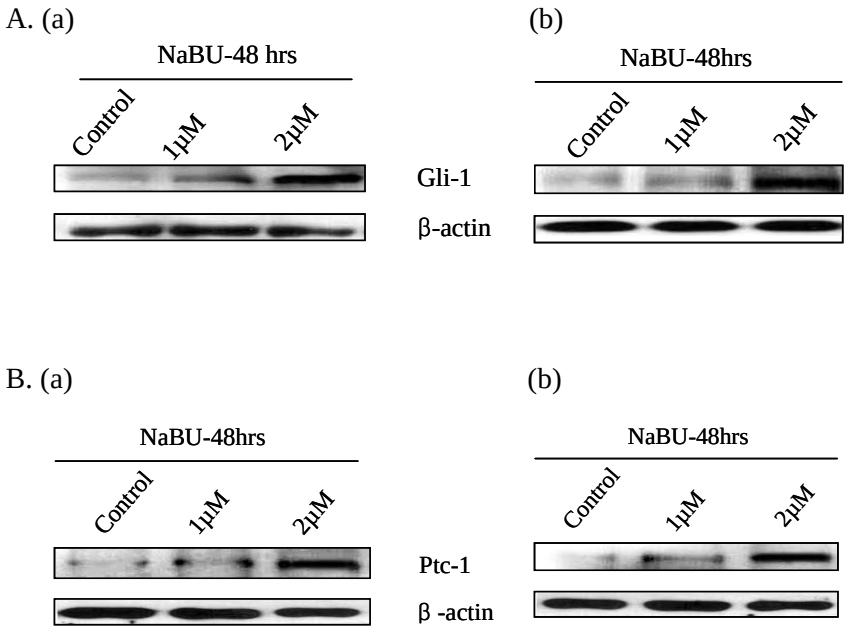


Fig. 6. Overexpression of glioma-associated oncogene-1 (A) and patched-1 (B) proteins after sodium butyrate treatment for 48 hours in AGS (a) and MKN-45 (b) cells. Gli-1, glioma-associated oncogene-1; Ptc-1, patched-1; NaBU, sodium butyrate.

4. Wnt signaling pathway in gastric cancer cell differentiation

To evaluate the Wnt signaling pathway in gastric cancer cell differentiation, the expression of nuclear β-catenin proteins was assessed by Western blotting after sequential extraction of cytosolic and nuclear proteins from cells. NaBU was also treated to 2μM, because gastric cancer cells were maximally differentiated in 2μM of NaBU in our study.

The expression of nuclear β-catenin decreased without a change in

cytoplasmic β-catenin during differentiation in both gastric cancer cell lines (Fig. 7). These results show that the activity of Wnt signaling decreased in gastric cancer cell differentiation.

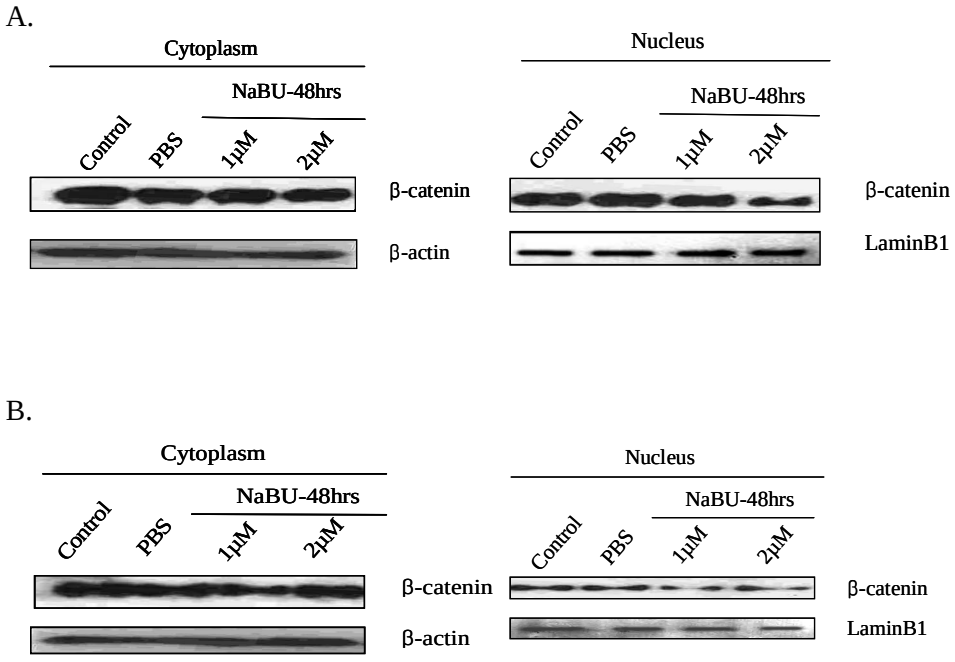


Fig. 7. Decreased nuclear β-catenin expression by butyrate treatment in AGS (A) and MKN-45 (B) cells.

Western blot for β-catenin was performed after sequential extraction of cytosolic and nuclear proteins from cells in non-ionic detergent. Lamin B1 (a nuclear protein) and β-actin were used as internal standard. NaBU, sodium butyrate

5. Regulation of the Wnt signaling pathway by enhanced Hh signaling in gastric cancer cell differentiation

In this study, ligand-dependent Hh signal activation and Wnt signal suppression were found in gastric cancer cell differentiation. To examine the mechanism of the inverse expression patterns between Hh and Wnt signaling during gastric cancer cell differentiation, the expression of sFRP-1 in NaBU-treated gastric cancer cells was analyzed. sFRP-1 is an antagonist of Wnt and a transcriptional target of Hh signaling. In the present study, the expression of sFRP-1 increased in NaBU-treated cancer cells according to RT-PCR and Western blotting (Fig. 8). Furthermore, as shown in Figure 9, Gli-1 regulated the expression of sFRP-1. Ectopically over-expressed Gli-1 increased sFRP-1 transcription and expression. When NaBU was added, over-expressed Gli-1 also increased sFRP-1 transcription and expression. Thus, maximal expression of sFRP-1 was found when Gli-1 was increased by the vector and butyrate. Chip assay was next performed to assess whether Gli-1, increased by the vector or NaBU, was involved in binding the sFRP-1 promoter. As shown in Figure 10, the regulation by Gli-1 involved direct binding to the sFRP-1 promoter. Over-expression of sFRP-1 after NaBU treatment was abolished by a siRNA against Gli-1 (Fig. 11).

To test whether increased Gli-1 expression could decrease Wnt signaling,

western blotting was used to analyze the nuclear pools of β-catenin after Gli-1 over-expression by the vector or NaBU treatment. Ectopically over-expressed Gli-1 (by vector) decreased nuclear β-catenin protein (Fig. 12A). When sodium butyrate was added, the increased Gli-1 due to Hh signal activation also reduced the nuclear β-catenin protein (Fig. 12A). Thus, nuclear β-catenin decreased the most when Gli-1 was over-expressed by the vector and sodium butyrate treatment (Fig. 12A). In addition, when the Topflash or Fopflash reporter assay was performed, TCF activity had been decreased in transfected AGS cells with the Gli-1 expression plasmid (Fig. 12B). When Gli-1 was suppressed by siRNA, the decreased nuclear β-catenin by NaBU recovered (Fig. 13). These findings suggest that increased Gli-1, via activated Hh signaling, in differentiated gastric cancer cells suppressed the Wnt signaling pathway through sFRP-1 over-expression.

To validate the inverse correlation between Hh and Wnt signal pathways, western blotting was used to evaluate the Hh signal changes after β-catenin over-expression by the vector during gastric cancer cell differentiation. Increased Gli-1 protein by sodium butyrate was decreased after β-catenin over-expression by the vector (Fig. 14).

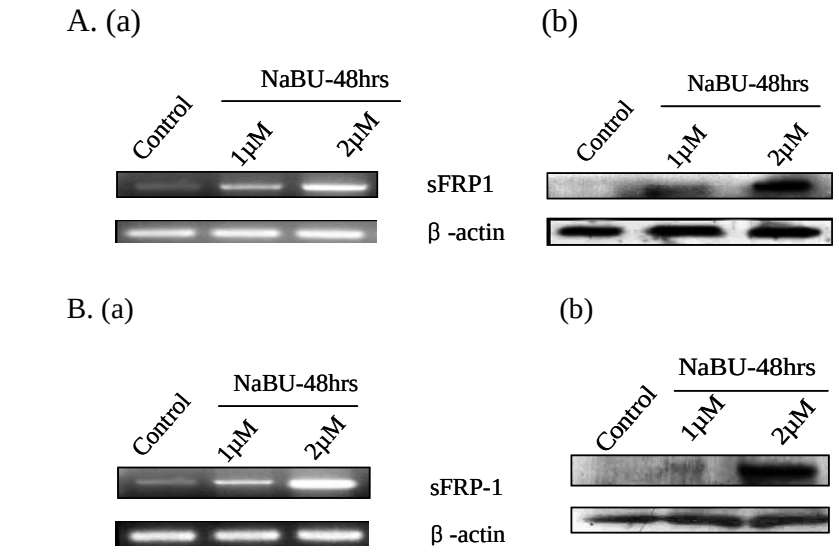


Fig. 8. Overexpression of secreted frizzled-related protein-1 by sodium butyrate treatment for 48 hours in AGS (A) and MKN-45 (B) cells.

RT-PCR (a) and Western blot (b) for sFRP-1 were performed with β-actin as internal standard. sFRP-1, secreted frizzled-related protein-1; NaBU, sodium butyrate.

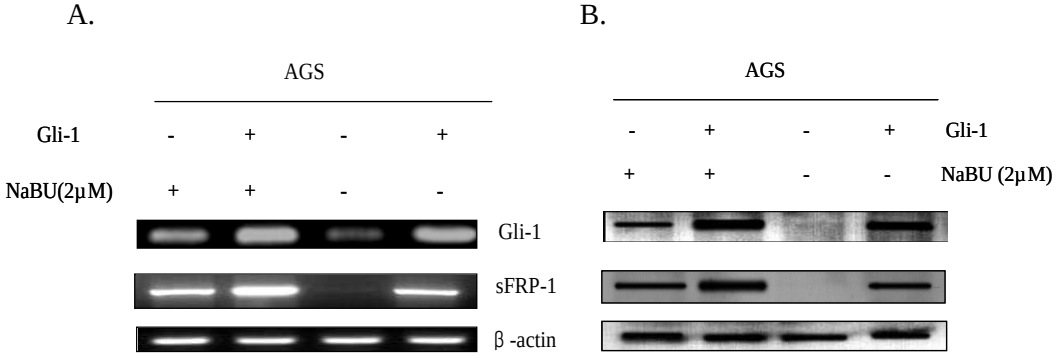


Fig. 9. Glioma-associated oncogene-1 regulates the expression of secreted

frizzled-related protein-1.

Increased Gli-1 expression by Gli-1 vector or 2 μM sodium butyrate increased the expression of sFRP-1 in RT-PCR (A) and western blot (B). The maximal expression of sFRP-1 was found when Gli-1 was overexpressed by vector and butyrate. Gli-1, glioma-associated oncogene-1; sFRP-1, secreted frizzled-related protein-1; NaBU, sodium butyrate.

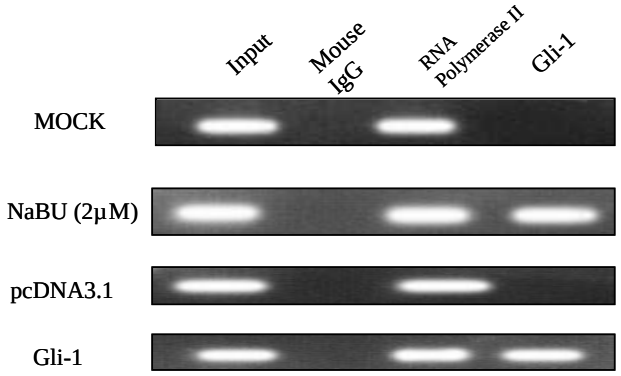


Fig. 10. Chromatin immunoprecipitation analysis of secreted frizzled-related protein-1 promoter region in AGS cells.

Overexpressed Gli-1 by NaBU or Gli-1 vector was direct binding to the sFRP-1 promoter region. Mouse IgG was used as the negative control and anti-RNA polymerase beads were used as the positive control. Gli-1, glioma-associated oncogene-1; sFRP-1, secreted frizzled-related protein-1; NaBU, sodium butyrate.

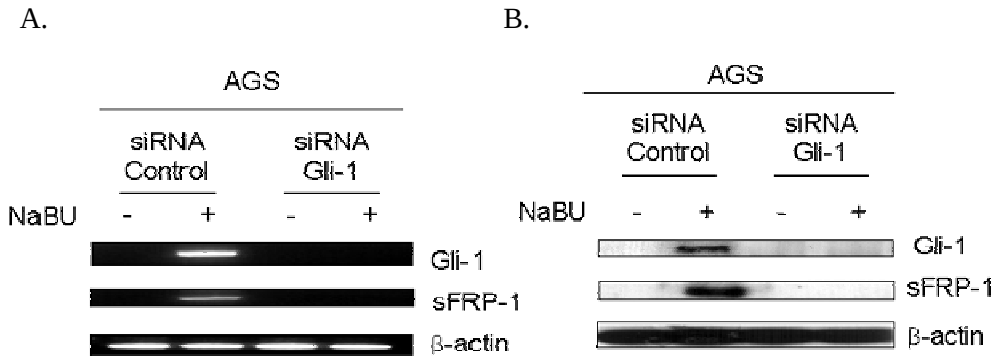
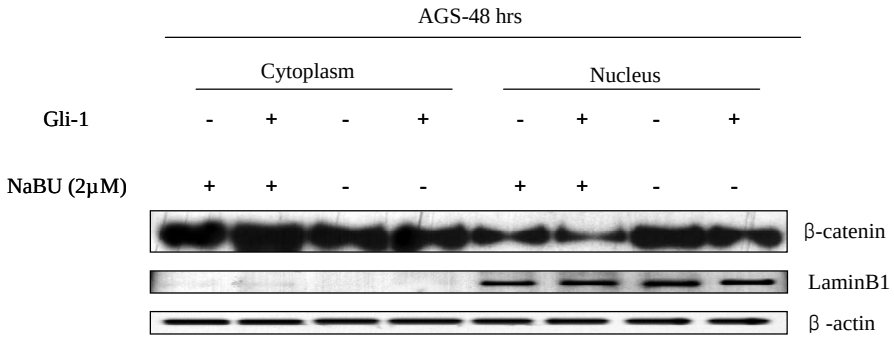


Fig. 11. Decreased expression of secreted frizzled-related protein-1 by siRNA against glioma-associated oncogene-1.

The increased expression of sFRP-1 by Hh signal activation after NaBU treatment was much decreased by siRNA against Gli-1 in RT-PCR (A) and western blot (B). Gli-1, glioma-associated oncogene-1; sFRP-1, secreted frizzled-related protein-1; NaBU, sodium butyrate.

A.



B.

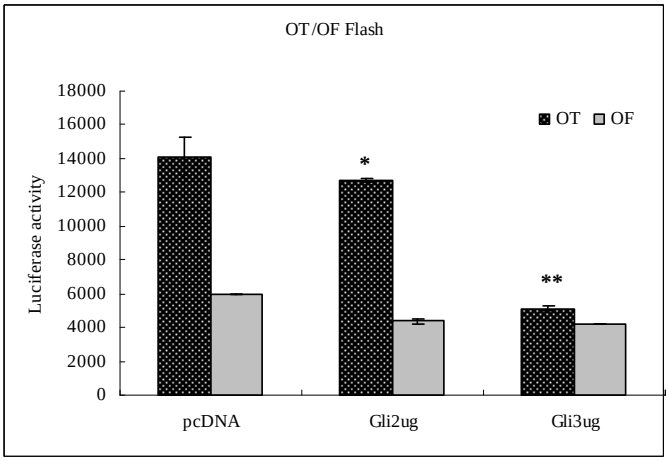


Fig. 12. Overexpressed glioma-associated oncogene-1 suppressed nuclear β-catenin in AGS cells.

A. Western blot for β-catenin was performed after sequential extraction of cytosolic and nuclear proteins from cells in non-ionic detergent. Lamin B1 (a nuclear protein) and β-actin were used as internal standard. Increased Gli-1 expression by Gli-1 vector or NaBU decreased nuclear β-catenin proteins. The most decreased nuclear β-catenin proteins were found when Gli-1 was overexpressed by vector and NaBU. B. AGS cells were transfected with Topflash or Fopflash, a control, and the indicated amount of a Gli-1 vector. Transfection with Gli-1 vector decreased TCF activity compared with control (Bars, s.d.; * vs. control, p=0.044; ** vs. control, p<0.001).

Gli-1, glioma-associated oncogene-1; NaBU, sodium butyrate.

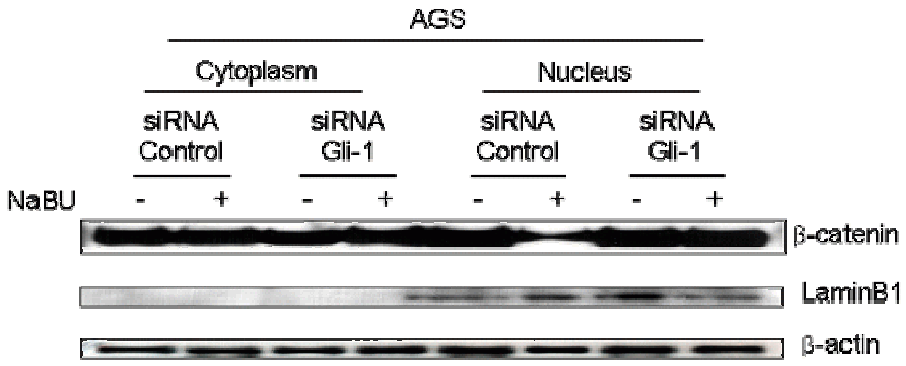


Fig. 13. Decreased nuclear β-catenin after sodium butyrate treatment was recovered by siRNA against glioma-associated oncogene-1 in AGS cells.

Gli-1, glioma-associated oncogene-1; NaBU, sodium butyrate.

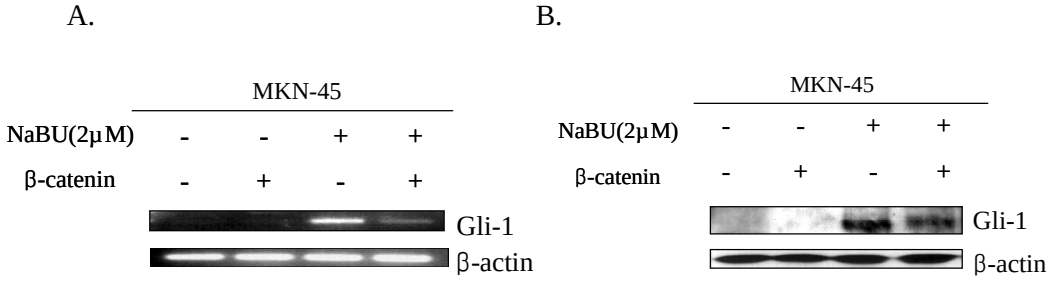


Fig. 14. Increased expression of glioma-associated oncogene-1 by sodium butyrate treatment was decreased after β-catenin over-expression by the vector in MKN-45 cells in RT-PCR (A) and western blot (B).

β-actin was used for internal control. Gli-1, glioma-associated oncogene-1; NaBU, sodium butyrate.

IV. DISCUSSION

Several histopathological classifications of gastric cancers exist, such as the WHO, Lauren, Ming, and Japanese systems. These classifications divide gastric cancers based on histopathological morphology. However, these classifications can largely be simplified to two growth patterns. One involves more gland formation-like growth, and the other involves less gland formation-like growth. Generally, the more gland formation growth pattern is believed to have a less aggressive biological behavior. Thus, the clinical therapeutic approach is different according to the histological type. For example, local endoscopic treatment is not generally accepted in undifferentiated-type gastric cancers because undifferentiated-type gastric cancer has more lymph node metastasis compared to differentiated-type gastric cancer. The different growth patterns of gastric cancer may involve different predominant molecular signaling pathways, especially based on cancer-initiating cell theory.[5]

Indeed, several studies have sought to analyze signaling changes according to histological classification in human gastric cancer tissues. These studies, however, have shown inconsistent results.[16, 18-20] These discrepancies may be the result of the different extents of cancer progression or migration in the studies, and/or failure to take into account the interaction(s) between

signaling pathways. Thus, two signaling pathways, Hh and Wnt signals, in gastric cancer cells *in vitro* were investigated to examine changes purely according to histological differentiation, minimizing the effect of different cancer stages. An *in vitro* differentiation model of human gastric cancer cell lines is similar to that used in colon cancer studies.[22, 29, 32, 33] This *in vitro* differentiation model of gastric cancer cell lines may reflect the molecular conditions of *in vivo* histological differentiation, based on previous biomorphological and molecular studies.[22, 30, 31, 34] When evaluating morphological changes after applying differentiation-inducing agents under electron microscopy, numerous microvilli, more desmosomes with tightly cohesive clusters, and intercellular lumens along cell junctions were observed, mimicking primitive gland formation.[22] In addition, when cancer cells were heterotransplanted into SCID mice with or without differentiation-inducing agents, the tumor originating from gastric cancer cells grown in the presence of the differentiation-inducing agent showed numerous well developed gland formations, the lumina of which were lined by many microvilli and filled with secretions.[22] These results suggest that gastric cancer cells treated with differentiation-inducing agents may have the characteristics of the gland formation growth pattern, as seen in histopathologically differentiated-type gastric cancers *in vivo*. Furthermore, several studies have revealed that

molecular markers for cancer cell differentiation, by inducing agents *in vitro*, such as ALP or Brm, were correlated with histological differentiated-type gastric cancer tissues.[30, 31, 34] The expression of one of the ALP isoenzymes was stronger in well differentiated than in poorly differentiated gastric carcinomas.[31] Brm is also an important factor for determining the differentiation status of gastric cancers.[30] A tendency toward a Brm decrease was more prominent in poorly differentiated than in well differentiated gastric cancers.[30] This study also showed increased ALP and Brm expression during gastric cancer cell differentiation by NaBU.

These findings suggest that ligand-dependent Hh signal activation and inverse Wnt signal suppression by Hh signaling occurred in gastric cancer cell differentiation. These results also suggest that the Hh signal pathway may be activated predominantly in gastric cancer tissues that have a more gland formation-like growth pattern. In contrast, the Wnt signaling pathway may be activated predominantly in gastric cancer tissues that have a less gland formation-like growth pattern. Immunohistochemical analysis of gastric cancer specimens in our study showed stronger expression of Hh signaling in well differentiated than in poorly differentiated cancer tissues.

Immunohistochemistry on specimens was performed limited to stages I and II gastric cancer to exclude any effect of cancer stage on the signals. These

consistent results between gastric cancer cells and human tissues may suggest that variable cancer stages could have caused the inconsistent results in previous studies.[16, 18-20]

According to this study, the inverse correlation between Hh and Wnt signaling was the result of sFRP-1, acting through Gli-1. Gli-1 bound directly to the sFRP-1 promoter region, consistent with the results of He *et al*.[23] However, Yanai *et al*. reported a different result because the promoter region of the sFRP-1 gene was methylated in AGS cells.[18] Thus, siRNA against Gli-1 was used to investigate whether over-expression of sFRP-1 in gastric cancer cell differentiation was derived from demethylation by a differentiation-inducing agent, not by Gli-1 binding to sFRP-1. When Gli-1 was suppressed by siRNA, sFRP-1 was also suppressed, regardless of NaBU treatment. It was also showed that Gli-1 was directly bound to the sFRP-1 promoter region based on the Chip assay. These results suggest that the transcription of sFRP-1 was increased by Gli-1 binding to the sFRP-1 promoter region during gastric cancer cell differentiation. Increased expression of sFRP-1 then suppressed the Wnt signaling pathway. That is, cross talk between the Hh and Wnt signal pathways occurred in gastric cancer cell differentiation.

The present study suggests that the predominant signaling pathway may be dissimilar in diverse differentiation-type gastric cancers. However, variable

signaling pathways may be involved in clinically presenting gastric cancers, such as signals related to carcinogenesis, progression, and migration. Despite that, the existence of baseline signals related to carcinogenesis in differentiated-type or undifferentiated-type gastric cancers may be important in the investigation of signaling pathways related to progression or migration.

V. CONCLUSION

The aim of this study was to investigate the differentiation-specific signal changes between Hh and Wnt pathways in gastric cancer cells. The present study revealed these findings.

1. During gastric cancer cells differentiation, activation of the Hh pathway and suppression of the Wnt pathway occurred.

2. The inverse correlation between Hh and Wnt pathway during differentiation was originated from crosstalk between Gli-1 and β-catenin though the sFRP-1.

3. Hh signal positive immunoexpression was higher in well differentiated than poorly differentiated tissues. However, nuclear β-catenin-positive immunoexpression was lower in well differentiated compared to poorly differentiated tissues.

In conclusion, the activation of the Hh pathway and suppression of the Wnt

pathway by Hh signaling occurred during gastric cancer cell differentiation. If these differentiation-specific signal changes are analyzed combined with signals related to the process of tumor progression or migration, it may give a clue in discovering the molecular heterogeneity of gastric cancers.

V. REFERENCES

1. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. Oncogene 2004;23:7274-82.
2. Sell S. Stem cell origin of cancer and differentiation therapy. Crit Rev Oncol Hematol 2004;51:1-28.
3. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. Nature 2004;432:324-31.
4. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer 2005;5:744-9.
5. Mimeault M, Batra SK. Interplay of distinct growth factors during epithelial mesenchymal transition of cancer progenitor cells and molecular targeting as novel cancer therapies. Ann Oncol 2007;18:1605-19.
6. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature 2001;411:349-54.
7. Ma X, Chen K, Huang S, Zhang X, Adegboyega PA, Evers BM, et al. Frequent activation of the hedgehog pathway in advanced gastric adenocarcinomas. Carcinogenesis 2005;26:1698-705.
8. Fukaya M, Isohata N, Ohta H, Aoyagi K, Ochiya T, Saeki N, et al. Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. Gastroenterology 2006;131:14-29.
9. Yanai K, Nagai S, Wada J, Yamanaka N, Nakamura M, Torata N, et al. Hedgehog signaling pathway is a possible therapeutic target for gastric cancer. J Surg Oncol 2007;95:55-62.
10. Horii A, Nakatsuru S, Miyoshi Y, Ichii S, Nagase H, Kato Y, et al. The APC gene, responsible for familial adenomatous polyposis, is mutated in human gastric cancer. Cancer Res 1992;52:3231-3.
11. Sasaki Y, Morimoto I, Kusano M, Hosokawa M, Itoh F, Yanagihara K,

et al. Mutational analysis of the beta-catenin gene in gastric carcinomas. Tumour Biol 2001;22:123-30.

12. Tong JH, To KF, Ng EK, Lau JY, Lee TL, Lo KW, et al. Somatic beta-catenin mutation in gastric carcinoma--an infrequent event that is not specific for microsatellite instability. Cancer Lett 2001;163:125-30.

13. Clements WM, Wang J, Sarnaik A, Kim OJ, MacDonald J, Fenoglio-Preiser C, et al. beta-Catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. Cancer Res 2002;62:3503-6.

14. Yuasa Y. Control of gut differentiation and intestinal-type gastric carcinogenesis. Nat Rev Cancer 2003;3:592-600.

15. Tamura G. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. World J Gastroenterol 2006;12:192-8.

16. Miyazawa K, Iwaya K, Kuroda M, Harada M, Serizawa H, Koyanagi Y, et al. Nuclear accumulation of beta-catenin in intestinal-type gastric carcinoma: correlation with early tumor invasion. Virchows Arch 2000;437:508-13.

17. Grabsch H, Takeno S, Noguchi T, Hommel G, Gabbert HE, Mueller W. Different patterns of beta-catenin expression in gastric carcinomas: relationship with clinicopathological parameters and prognostic outcome. Histopathology 2001;39:141-9.

18. Yanai K, Nakamura M, Akiyoshi T, Nagai S, Wada J, Koga K, et al. Crosstalk of hedgehog and Wnt pathways in gastric cancer. Cancer Lett 2008;263:145-56.

19. Wang LH, Choi YL, Hua XY, Shin YK, Song YJ, Youn SJ, et al. Increased expression of sonic hedgehog and altered methylation of its promoter region in gastric cancer and its related lesions. Mod Pathol 2006;19:675-83.

20. Kim B, Koo H, Yang S, Bang S, Jung Y, Kim Y, et al. TC1(C8orf4) correlates with Wnt/beta-catenin target genes and aggressive biological behavior in gastric cancer. Clin Cancer Res 2006;12:3541-8.

21. Nakamura T, Yao T, Kabashima A, Nishiyama K, Maehara Y, Tsuneyoshi M. Loss of phenotypic expression is related to tumour progression in early gastric differentiated adenocarcinoma. Histopathology 2005;47:357-67.
22. Choe G, Kim WH, Park JG, Kim YI. Effect of suramin on differentiation of human stomach cancer cell lines. J Korean Med Sci 1997;12:433-42.
23. He J, Sheng T, Stelter AA, Li C, Zhang X, Sinha M, et al. Suppressing Wnt signaling by the hedgehog pathway through sFRP-1. J Biol Chem 2006;281:35598-602.
24. Li X, Deng W, Lobo-Ruppert SM, Ruppert JM. Gli1 acts through Snail and E-cadherin to promote nuclear signaling by beta-catenin. Oncogene 2007;26:4489-98.
25. Maeda O, Kondo M, Fujita T, Usami N, Fukui T, Shimokata K, et al. Enhancement of GLI1-transcriptional activity by beta-catenin in human cancer cells. Oncol Rep 2006;16:91-6.
26. Nakashima H, Nakamura M, Yamaguchi H, Yamanaka N, Akiyoshi T, Koga K, et al. Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. Cancer Res 2006;66:7041-9.
27. Ohki K, Kumamoto H, Ichinohasama R, Sato T, Takahashi N, Ooya K. PTC gene mutations and expression of SHH, PTC, SMO, and GLI-1 in odontogenic keratocysts. Int J Oral Maxillofac Surg 2004;33:584-92.
28. Buras RR, Shabahang M, Davoodi F, Schumaker LM, Cullen KJ, Byers S, et al. The effect of extracellular calcium on colonocytes: evidence for differential responsiveness based upon degree of cell differentiation. Cell Prolif 1995;28:245-62.
29. Ribiczey P, Tordai A, Andrikovics H, Filoteo AG, Penniston JT, Enouf J, et al. Isoform-specific up-regulation of plasma membrane Ca2+ATPase expression during colon and gastric cancer cell differentiation. Cell Calcium 2007;42:590-605.

30. Yamamichi N, Inada K, Ichinose M, Yamamichi-Nishina M, Mizutani T, Watanabe H, et al. Frequent loss of Brm expression in gastric cancer correlates with histologic features and differentiation state. Cancer Res 2007;67:10727-35.

31. Watanabe H, Tokuyama H, Ohta H, Satomura Y, Okai T, Ooi A, et al. Expression of placental alkaline phosphatase in gastric and colorectal cancers. An immunohistochemical study using the prepared monoclonal antibody. Cancer 1990;66:2575-82.

32. Hung MW, Tsai LC, Lin YL, Chen YH, Chang GG, Chang TC. Differential regulation of placental and germ cell alkaline phosphatases by glucocorticoid and sodium butyrate in human gastric carcinoma cell line TMK-1. Arch Biochem Biophys 2001;388:45-54.

33. Gelebart P, Kovacs T, Brouland JP, van Gorp R, Grossmann J, Rivard N, et al. Expression of endomembrane calcium pumps in colon and gastric cancer cells. Induction of SERCA3 expression during differentiation. J Biol Chem 2002;277:26310-20.

34. Itoh T, Miyake K, Iijima S. Differentiation-specific expression of chromatin remodeling factor BRM. Biochem Biophys Res Commun 2008;366:827-33.

< ABSTRACT(IN KOREAN)>

위암세포 분화와 관련된 Hedgehog과 Wnt 신호전달의 상호작용

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김 지 현

위암은 조직학적 형태에 따라 다른 생물학적 행태를 가지고 있다고 알려져 있으며, 다른 생물학적 행태에 대한 차별적 신호전달체계가 존재할 수 있다. 본 연구의 목적은 위암세포에서 조직학적 분화도에 따른 Hedgehog (Hh)과 Wnt 신호전달의 차이를 분석하고자 하였다. 위암세포주로 AGS, MKN-45를 사용하였으며, 실험실적 분화에 대한 양적 대조군으로 대장암 세포주인 HT-29를 사용하였다. 암세포의 분화는 sodium butyrate와 all-*trans* retinoid acid로 유도하였다. Hh 신호분석을 위하여 sonic hedgehog (SHh), Patched (Ptc)-1, glioma-associated oncogene (Gli)-1을 측정하였으며, Wnt 신호분석을 위하여 핵내 β-catenin을 측정하였다. 위암환자들의 파라핀 조직을 통하여 생체조직의 Hh과 Wnt 신호전달을 분석하였고, Hh과 Wnt 신호전달의 상호작용은 secreted frizzled-related protein (sFRP-1)으로 분석하였다. 위암세포도 대장암세포와 비슷한 양상으로 실험실적 분화가 유도되었는데, 분화되는 동안 Hh신호전달은 활성화되었으나, Wnt 신호전달은 억제되는 양상을

보였다. Gli-1을 과발현 시켰을 때 sFRP-1의 전사가 증가하였으며, Gli-1를 억제하였을 때에는 sFRP-1의 전사가 감소하였고, 이는 chromatin immunoprecipitation (Chip) assay를 통하여 Gli-1이 sFRP-1의 전사를 조절함을 알 수 있었다. Wnt 신호전달에 있어서는 Gli-1의 과발현시 핵내 β-catenin 발현이 감소하였으나, Gli-1을 억제하였을 때 핵내 β-catenin 발현이 회복되었다. 위암환자 조직에서 시행한 면역화학염색에서는 SHh, Gli-1 단백이 고분화 선암에서 저분화 선암보다 강하게 발현되었으며, 핵내 β-catenin 단백은 저분화 선암에서 고분화 선암보다 강하게 발현되었다. 결론적으로 위암세포가 분화하는 동안 Hh신호전달은 활성화되고 Wnt신호전달은 억제되었다. 이와 같이 분화 특이 신호전달변화가 암의 진행이나 전이와 관련된 신호전달과 같이 분석이 된다면 위암의 분자생물학적 이질성을 이해하는 데에 도움이 될 것이다.

핵심되는 말: 분화, Hedgehog, Wnt, 발암, 상호작용