TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Characterization of a New Small Bowel Adenocarcinoma Cell Line and Screening of Anti-Cancer Drug against Small Bowel Adenocarcinoma

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Small bowel adenocarcinoma (SBA) is a rare, aggressive malignancy with a poor prognosis, and the mechanisms of carcinogenesis in SBA remain unclear. Our aims were to investigate the molecular mechanisms underlying SBA and to identify treatments by establishing and characterizing an SBA cell line and performing anti-cancer drug screening. SIAC1 cells, established from jejunal SBA, showed epithelial characteristics and formed organoids in 3D culture. SIAC1 cells had a heterozygous β -catenin deletion mutation, resulting in a stable β -catenin protein with enhanced Wnt/ β -catenin activity. SIAC1 cells lacked MLH1 and MSH6 expression, and target genes such as *TGFR2* and *ACVR2* showed frameshift mutations. Among 10 clinical SBA samples, 2 (20%) had interstitial deletions in β -catenin, expression of mismatch repair protein was aberrant in 4 (40%), and heterozygous frameshift mutations of three target genes were found in all 10 samples. On screening assay using 140 compounds, eribulin significantly inhibited SIAC1 cell growth both *in vitro* and *in vivo* by inhibition of the Wnt/ β -catenin pathway via enhanced degradation of β -catenin. In conclusion, we established an SBA cell line with molecular characteristics similar to those of clinical SBA samples, including β -catenin deletion and mismatch repair protein deficiency, that will be useful for SBA research. Eribulin might be a candidate for SBA treatment due to its inhibitory effect on Wnt/ β -catenin signaling. (*Am J Pathol* 2015, 185: 550–562; <http://dx.doi.org/10.1016/j.ajpath.2014.10.006>)

Small bowel adenocarcinoma (SBA) is a rare and aggressive malignancy with a poor prognosis. The small intestine constitutes approximately 75% of the length of the entire gastrointestinal tract, but primary SBA occurs in only <2% to 3% of gastrointestinal cancers.¹ It is difficult to detect early stage SBA because the small bowel is distal from the mouth and anus, although recently, new technologies such as capsule endoscopy and double-balloon endoscopy have enabled early detection, diagnosis, and endoscopic treatment.^{2–4} Despite the development of these techniques, the diagnosis of SBA is often delayed due to nonspecific symptoms such as abdominal pain, nausea, vomiting, and anemia. Current National Comprehensive Cancer Network guidelines recommend that SBA be treated similarly to colorectal cancer (CRC), with surgical resection as the primary treatment.⁵

SBA has many etiological, morphological, and genetic features in common with CRC. Both cancers are associated

with the same carcinogens, such as red meat, alcohol, and tobacco.⁶ Like CRC, an increased frequency of SBA is also related to genetic disorders such as familial adenomatous polyposis, Lynch syndrome, and Peutz-Jeghers syndrome, as well as Crohn disease and celiac disease.⁷ CRC and SBA undergo a histological progression similar to that of invasive cancer, through adenomas mediated by the adenoma–carcinoma sequence.^{8,9} Despite the many similarities between SBA and CRC, the prevalence of SBA is 60- to 70-fold less than that of CRC.¹⁰ Moreover, the 5-year survival rate of SBA is 37%¹¹ compared with 65% with CRC.¹²

Two types of genomic instability, chromosomal instability and microsatellite instability (MSI), are closely associated

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with carcinogenesis throughout the gastrointestinal tract.¹³ Chromosomal instability is defined as an aberrant genomic status with chromosomal aneuploidy and is found in 50% to 70% of CRCs and 60% of SBAs, reportedly.^{14,15} MSI is caused by the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, *PMS1*, and *PMS2*, which are considered to be causative of Lynch syndrome.¹⁶ MSI is further associated with carcinogenesis through replication errors in target genes such as *TGFBR2*, *ACVR2*, and *MARCKS*.^{17,18} This pathway is more prevalent in SBA, 20% to 45%, compared with 15% in CRC.^{13,19–21} Epigenetic changes, such as DNA hypermethylation, are also associated with gastrointestinal carcinogenesis.²² In particular, the CpG island methylator phenotype (CIMP) has been increasingly recognized in sporadic CRC and SBA.^{14,15,23} The methylation status of the promoters for *p16INK4A*, *MLH1*, and *MGMT* has been related to carcinogenesis, as well as invasion and metastasis.^{15,24}

Although the characteristics of SBA have been identified in resected specimens, the lack of an SBA cell line has made it difficult to determine the molecular mechanisms of SBA carcinogenesis or to establish potential SBA treatment strategies. In this study, we established and characterized an SBA cell line and assessed the anti-cancer effects of candidate drugs.

Materials and Methods

Patient Specimens

We analyzed eight surgical specimens and three biopsy specimens from small bowel cancers, excluding ampullary tumors, diagnosed at Tokyo University Hospital (Tokyo, Japan) from June 2005 to May 2013. The specimens were obtained with written informed consent from the patients ($n = 11$; mean age, 54.5 ± 19.9 years; five male patients). All procedures were approved by the ethics committee of the medical faculty at the University of Tokyo, in accordance with generally accepted guidelines for the use of human material.

Establishment of SIAC1 Cells

For the establishment of a cell line termed *small intestinal adenocarcinoma 1* (SIAC1), SBA biopsy specimens were cut into small pieces in digestion medium containing 360 U collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 3.75-U dispase (Wako) in 3 mL of Dulbecco's modified Eagle's medium (Gibco-Life Technologies, Grand Island, NY). The sample was incubated for 40 minutes at 37°C, washed with phosphate-buffered saline containing 1% fetal bovine serum, resuspended in 25% Ham's F12 medium, 25% Dulbecco's modified Eagle's medium, and 50% RPMI medium mixture containing 20% fetal bovine serum and seeded into a 48-well microplate. After culturing for 10 to 14 days, a small colony was

detected and transferred to a larger microplate. We had tried to establish SBA cell lines in eight patients, and only one cell line was successfully established.

Cell Lines, Antibodies, and Anti-cancer Drugs

Cells from an SBA line (SIAC1), CRC lines (HT29, Lovo, DLD1, Hct116, RKO), and human embryonic kidney line (HEK293T) were used in this study. All cell lines were cultured at 37°C with 5% CO₂ in the indicated media ([Supplemental Table S1](#)). RSpol1-Fc HEK293T cells were kindly provided by Toshiro Sato (Keio University, Tokyo, Japan) with permission from Calvin Kuo (Stanford University, Palo Alto, CA).²⁵ The antibodies and anticancer drugs used in this study are listed in [Table 1](#) and [Supplemental Table S2](#), respectively. The Screening Committee of Anti-cancer Drugs kindly provided inhibitor kits 3 and 4.

3D Culture of SIAC1 Organoids

SIAC1 cells (3.0×10^2 cells/well) were suspended with 25 μ L/well of cold Matrigel and piled up quickly to a 48-well microplate warmed to 37°C. After incubation at 37°C for 5 minutes, Matrigel containing SIAC1 cells was soaked up with 500 μ L/well of ENR (50 ng/mL of murine epidermal growth factor, 100 ng/mL of murine Noggin, human R-spondin-1 condition medium) or EN (without human R-spondin-1 condition medium) culture medium.²⁶ Medium change was performed every 3 days, and the organoids were harvested for histological examination and immunohistochemistry (IHC) analysis 2 weeks after cultivation.

Genomic, Complementary, and Plasmid DNA Preparation

Genomic DNA was extracted from cell lines and 10 μ m of paraffin-embedded SBA samples using the QIAmp DNA Mini Kit and QIAmp DNA Formalin-Fixed, Paraffin-Embedded Tissue Kit, respectively (Qiagen, Hilde, Germany). Total cellular RNA was prepared using the NucleoSpin RNA II kit (Takara Bio Inc., Tokyo, Japan). The Improm-II Reverse Transcription System (Promega Corp., Madison, WI) was used for preparing complementary DNA. Plasmid DNA was prepared using the GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich, St Louis, MO). All procedures were performed according to the manufacturers' recommended protocols.

Mutational Analysis of Proto-Oncogenes

BRAF exon 15, *KRAS* exon 2 (coding exon 1), *APC* exon 15 peripheral mutation cluster region, β -catenin gene (*CTNNB1*) exons 1 to 6, and *EGFR* exons 18, 19, 21, and 23 were analyzed for mutations by direct sequencing as

Table 1 Antibodies Used in This Study

Immunogen	Host	Product	Manufacturer
Pan-cytokeratin (panCK)	Rabbit	H-240	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)
α -Smooth muscle actin (α -SMA)	Mouse	1A4	Dako (Glostrup, Denmark)
Mucin-2 (MUC2)	Mouse	Sc-7314	Santa Cruz Biotechnology
β -Actin	Mouse	AC-15	Sigma-Aldrich (St Louis, MO)
MLH1	Mouse	554073	Becton, Dickinson, and Company (Franklin Lakes, NJ)
MSH2	Mouse	Ab-2	Calbiochem (Darmstadt, Germany)
MSH6	Rabbit	EPR3945	Abcam (Cambridge, UK)
PMS1	Rabbit	C-20	Santa Cruz Biotechnology
PMS2	Mouse	C-20	Santa Cruz Biotechnology
β -Catenin	Rabbit	6B3	Cell Signaling Technology, Inc. (Beverly, MA)
β -Catenin	Mouse	610153	Becton, Dickinson, and Company
SMAD4	Mouse	B-8	Santa Cruz Biotechnology
p53	Rabbit	FL-393	Santa Cruz Biotechnology
TGF β R2	Rabbit	L-21	Santa Cruz Biotechnology
FLAG	Mouse	F1804	Sigma-Aldrich
MLH1 for IHC	Mouse	550838	Becton, Dickinson, and Company
c-MYC	Rabbit	9402	Cell Signaling Technology
Cyclin D1	Mouse	DCS-6	Santa Cruz Biotechnology
Ki-67	Mouse	MIB-1	Dako

IHC, immunohistochemistry.

described previously.²⁷ The primers are shown in Table 2. Conventional PCR was performed on the MyCycler PCR system (Bio-Rad Laboratories, Inc., Hercules, CA), as follows: an initial denaturing step for 10 minutes at 95°C, followed by 40 cycles at 95°C for 45 seconds, 56°C to 64°C for 45 seconds, and 72°C for 30 seconds. Data analysis was performed using the Genetyx version 9 software (Genetyx Corp., Tokyo, Japan).

MSI and Target Gene Analysis

We analyzed the MSI pattern using the following five standard microsatellite markers: *BAT25*, *BAT26*, *D2S123*, *D2S346*, and *D17S250*.²⁸ The primers and thermal cycling protocols were referred to in a previous report.²⁹ Microsatellite sequences of the potential target genes were analyzed by direct sequencing analysis and TA cloning using TOPO TA Cloning Kits (Life Technologies Corp., Carlsbad, CA) according to the manufacturer's protocol. The primers for the target gene analysis are shown in Table 2.

Quantitative PCR

For MMR expression analysis, cDNA was prepared from SIAC1 and RKO cells treated with the indicated amount of 5-aza-2-deoxycytidine (5-aza-dc) for 96 hours and/or 0.5 μ mol/L of trichostatin A for 24 hours. Then, 5 μ L of cDNA was used in 12.5 μ L reactions containing SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA) and 400 nmol/L of gene-specific primer for the target gene or reference gene (glyceraldehyde 3-phosphate dehydrogenase; *GAPDH*). The primer sequences are described in Table 2. Quantitative PCR was performed twice in duplicate

samples using an ABI Prism 7000 real-time PCR system (Life Technologies).

Methylation-Specific PCR of *MLH1* Gene

SIAC1, HT29, and RKO genomic DNA were treated with a EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions, and 20 ng of the bisulfite-modified DNA was used for methylation-specific PCR. The specific primer sets for methylated or unmethylated *MLH1* promoter are shown in Table 2. PCR reactions were started at 95°C for 5 minutes. Amplification was performed for 42 cycles (95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds) and followed by a final extension at 72°C for 10 minutes.

Preparation and Analysis of a β -Catenin Deletion Mutant

A FLAG-epitope tagged β -catenin deletion mutant (MUT) was generated from the wild-type (WT) β -catenin using the KOD -Plus- Mutagenesis Kit (Toyobo Co., Ltd., Osaka, Japan). The primer sequences for generating MUT from WT are shown in Table 2. HEK293T cells were cultured in 12-well plates (2.0×10^5 cells/well) for 24 hours and transfected with 0.3 μ g of WT or MUT vector using Effectene (Qiagen). Cells were harvested 48 hours after transfection and prepared for Western blot analysis.

Western Blot Analysis

Total protein was extracted from cultured cells using a lysis buffer that contained 50 mmol/L of Tris-HCl (pH 7.4), 1% Triton X-100, 5 mmol/L of EDTA, 1 mmol/L of

Table 2 Sequence of Primers for PCR Mutation Analysis and MSI Target Gene Analysis

Name	Gene	Sequence	Amplicon size
KRAS Ex1 F	<i>KRAS</i>	5'-GCCTGCTGAAAATGACTGAAT-3'	79 Bp
KRAS Ex1 R	<i>KRAS</i>	5'-GCTGTATCGTCAAGGCACCTCT-3'	79 Bp
BRAF Ex11 F	<i>BRAF</i>	5'-TCATAATGCTTGCTCTGATAGGA-3'	224 Bp
BRAF Ex11 R	<i>BRAF</i>	5'-GGCCAAAAATTTAATCAGTGA-3'	224 Bp
APC Ex15(a) F	<i>APC</i>	5'-AGACTTATTGTGTAGAAAGATAC-3'	151 Bp
APC Ex15(a) R	<i>APC</i>	5'-ATGGTTCACTCTGAACGGA-3'	151 Bp
APC Ex15(b) F	<i>APC</i>	5'-TCTGTCAGTTCACCTTGATAG-3'	159 Bp
APC Ex15(b) R	<i>APC</i>	5'-CATTTGATTCTTTAGGCTGC-3'	159 Bp
TGFB2U1	<i>TGFB2</i>	5'-AGATGCTGCTTCTCCAAAGTGC-3'	90 Bp
TGFB2D1	<i>TGFB2</i>	5'-TTGCACTCATCAGAGCTACAGG-3'	90 Bp
IGF2R F	<i>IGF2R</i>	5'-GCAGGTCTCCTGACTCAGAA-3'	110 Bp
IGF2R R	<i>IGF2R</i>	5'-GAAGAAGATGGCTGTGGAGC-3'	110 Bp
BAX F	<i>BAX</i>	5'-ATCCAGGATCGAGCAGGGCG-3'	94 Bp
BAX R	<i>BAX</i>	5'-ACTCGCTCAGCTTCTTGGTG-3'	94 Bp
MSH3 F	<i>MSH3</i>	5'-AGCTGGATGATGCTGTAAAT-3'	150 Bp
MSH3 R	<i>MSH3</i>	5'-TTCCTCACCTGCAAAGTACT-3'	150 Bp
MSH6 F	<i>MSH6</i>	5'-ACTGTGCCTGGCTAACTATA-3'	259 Bp
MSH6 R	<i>MSH6</i>	5'-CTGTCTCATAAGCGTAGACT-3'	259 Bp
FAS F	<i>FAS</i>	5'-ACCCGGACCCAGAATACCAA-3'	82 Bp
FAS R	<i>FAS</i>	5'-GCAAGGGTCACAGTGTTCAC-3'	82 Bp
ACVR2 F	<i>ACVR2</i>	5'-GTTGCCATTTGAGGAGGAAA-3'	167 Bp
ACVR2 R	<i>ACVR2</i>	5'-CCTCTGAAAAGTGTTTTATTTGGAA-3'	167 Bp
CASP5 F	<i>CASP5</i>	5'-TCGCTGGAGACATGGTAAAA-3'	288 Bp
CASP5 R	<i>CASP5</i>	5'-GATCAGGGCCTTGTCTTCAA-3'	288 Bp
MARCKS F	<i>MARCKS</i>	5'-TCTTGAAGGAGAAGCCGC-3'	130 Bp
MARCKS R	<i>MARCKS</i>	5'-CCGCCTCCTCGACTTCTT-3'	130 Bp
NDUFC2 F	<i>NDUFC2</i>	5'-TGAATTTTCAGGTTTGCATCG-3'	103 Bp
NDUFC2 R	<i>NDUFC2</i>	5'-GGTCCCTCACAGCATAACAGG-3'	103 Bp
b-catenin Fr.1F	<i>CTNNB1</i>	5'-GTTTTGAAAATCCAGCGTGG-3'	1.8 kbp
b-catenin Fr.1R	<i>CTNNB1</i>	5'-TTGAAGGCAGTCTGTCTGTA-3'	1.8 kbp
b-cat mut 29	<i>CTNNB1</i>	5'-AACTCCATCAAATCAGCTTGAGTAGC-3'	NA
b-cat mut 411	<i>CTNNB1</i>	5'-AACTTGATTAATCAATCAAGATGATGCAG-3'	NA
MLH1 qPCR-F2	<i>MLH1</i>	5'-TTATCCAGCGGCCAGCTAATG-3'	94 Bp
MLH1 qPCR-R2	<i>MLH1</i>	5'-GCCTCCCTCTTTAACAATCACTT-3'	94 Bp
MSH2 qPCR-F	<i>MSH2</i>	5'-GGAGGAGAGACTGCTGGGAGA-3'	144 Bp
MSH2 qPCR-R	<i>MSH2</i>	5'-TCCCTTTTTCCTTTCAACA-3'	144 Bp
PMS1 qPCR-F	<i>PMS1</i>	5'-GCGGCAACAGTTTCGACTCCTT-3'	174 Bp
PMS1 qPCR-R	<i>PMS1</i>	5'-AGCCTTGATACCCTCCCCGTT-3'	174 Bp
PMS2 qPCR-F	<i>PMS2</i>	5'-GCATGGTTACTGCCTCTGGT-3'	359 Bp
PMS2 qPCR-R	<i>PMS2</i>	5'-GAACCCCTCAGAATCCACGGA-3'	359 Bp
EGFR exon 18F	<i>EGFR</i>	5'-TCCAAATGAGCTGGCAAGTG-3'	397 Bp
EGFR exon 18R	<i>EGFR</i>	5'-TCCCAAACACTCAGTGAAACAAA-3'	397 Bp
EGFR exon 19F	<i>EGFR</i>	5'-ATGTGGCACCATCTCACAATTGCC-3'	178 Bp
EGFR exon 19R	<i>EGFR</i>	5'-ATGTGGCACCATCTCACAATTGCC-3'	178 Bp
EGFR exon 21F	<i>EGFR</i>	5'-GCTCAGAGCCTGGCATGAA-3'	348 Bp
EGFR exon 21R	<i>EGFR</i>	5'-CATCCTCCCTGCATGTGT-3'	348 Bp
EGFR exon 23F	<i>EGFR</i>	5'-TGAAGCAAATTGCCCCAAGAC-3'	383 Bp
EGFR exon 23R	<i>EGFR</i>	5'-TGACATTTCTCCAGGGATGC-3'	383 Bp
MLH1 prom M F	<i>MLH1</i>	5'-ACGTAGACGTTTTATTAGGGTCGC-3'	115 Bp
MLH1 prom M R	<i>MLH1</i>	5'-CCTCATCGTAACTACCCGCG-3'	115 Bp
MLH1 prom U F	<i>MLH1</i>	5'-TTTTGATGTAGATGTTTTATTAGGGTTGT-3'	124 Bp
MLH1 prom U R	<i>MLH1</i>	5'-ACCACCTCATCATAACTACCCACA-3'	124 Bp

EGFR, epidermal growth factor receptor; F, forward; MSI, microsatellite instability; NA, not applicable; qPCR, real-time quantitative PCR; R, reverse.

Na₃VO₄ and protease inhibitor cocktail tablets [cOmplete Mini (F. Hoffmann-La Roche, Ltd., Basel, Switzerland)] (1 tablet/10 mL). For the evaluation of protein stability, cells were treated with 10 µg/mL cycloheximide (Wako)

for the indicated time. The lysate was centrifuged for 10 minutes at 10,000 rpm, and the supernatant was collected as total protein. Western blot analysis was performed as described previously.²⁷

Reporter Gene Assay

SIAC1, RKO, HT29, and Hct116 cells were grown in 48-well plates until 60% to 70% confluence. The luciferase-reporter plasmids pTOP-Flash, pFOP-Flash, or pD1luc (0.15 µg/well) were mixed with 0.4 µg/well of thymidine kinase promoter-Renilla luciferase reporter plasmid, which was used to correct for transfection efficiency. Transfection was performed using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Reporter gene activity was assessed after 48 hours using PicaGene Dual (Toyo B-Net Co., Ltd., Tokyo, Japan) in triplicate. Representative data from more than three experiments are shown.

Immunofluorescence

SIAC1 or HT29 cells were seeded into two- or four-well plastic chamber slides (Thermo Fisher Scientific, Rochester, NY). The cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 5 minutes, and then blocked with 5% goat normal serum for 1 hour at room temperature. Primary antibodies were added, followed by incubation overnight at 4°C. Detection was performed as described previously.²⁷

IHC Analysis

Formalin-fixed, paraffin-embedded small bowel specimens and SIAC1 organoids were cut to a thickness of 3 µm and deparaffinized. After boiling with antigen retrieval buffer (10 mmol/L of citric acid monohydrate, pH 6.0) for 20 minutes, endogenous peroxidase activity was blocked using 3% H₂O₂. The primary antibody was added, and incubated overnight. Subsequent immunostaining was conducted using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), following the manufacturer's instructions. The staining was developed by reaction with Histofine Simple Stain Diaminobenzide solution (Nichirei Biosciences, Tokyo, Japan), followed by counterstaining with hematoxylin. For SIAC1 organoids, rhodamine-conjugated Ulex europaeus agglutinin I lectin (1:1000; Vector Laboratories) was mixed with primary antibody: anti-MUC2 antibody, and Alexa Fluor 488 goat anti-mouse IgG (1:400, A11001; Invitrogen, Carlsbad, CA) was used as secondary antibody. Nucleic acid staining was performed by Hoechst33342 (1:1000; Dojindo Chemical Co., Kumamoto, Japan).

Cell Proliferation Assay

For anti-cancer drug screening, SIAC1 cells were seeded at 3.0×10^3 cells per well in flat-bottomed 96-well microplates. After 48 hours, cells were treated with 10 µmol/L of the anti-cancer drugs listed in [Supplemental Table S2](#) for the indicated times. The cells were enumerated using the Cell Counting Kit-8 (Dojindo Chemical Co.).

Tumor Xenograft Model

Nude mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were inoculated s.c. at four sites in the back with 3.0×10^6 SIAC1 cells per site as described previously.³⁰ After 2 weeks, the mice were randomly assigned to receive bortezomib 0.1 mg/kg i.p. b.i.w., eribulin 0.25 mg/kg i.p. t.i.w., or saline (control) i.p. t.i.w., as described previously.^{31,32} We measured the largest diameter of the s.c. tumor every 3 days, and the volume of each tumor was calculated according to the following formula: $\text{volume} = 4\pi r^3/3$, where π = circular constant and r = half of largest diameter. The experimental protocols were approved by the Ethics Committee for Animal Experimentation at the University of Tokyo and were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the Graduate School of Medicine, the University of Tokyo.

Statistical Analysis

Statistical analysis was performed using the Student's *t*-test, two-sided, or one-way analysis of variance with the Dunnett multiple-comparison test, or the Tukey honestly significant difference test. Differences were considered statistically significant if the *P* value was <0.05. Data analysis was performed using JMP Pro version 10.0.2 software (SAS institute Japan Inc., Tokyo, Japan).

Results

Establishment and Characterization of SIAC1 Cells

A cell line was established from SBA tumor of 70-year-old woman who had no history of Lynch syndrome, celiac disease, and Crohn's disease or family history of inherited disease. The original tumor was jejunal adenocarcinoma located about 5 cm from the ligament of Treitz. The SIAC1 cells displayed a short, polygonal, and epithelial-like morphology ([Figure 1A](#)). Western blot analysis demonstrated that PanCK, but not α -small muscle actin (SMA), was present in SIAC1 cells, as well as in the CRC cell line, HT29 ([Figure 1B](#)). These results verified that SIAC1 cells were derived from intestinal epithelial cells. SIAC1 cells also grew in 3D culture condition ([Figure 1C](#)), which has been reported in CRC cells.³³ When cultured in Matrigel, SIAC1 cells formed cystic organoids with budding structures. IHC analysis showed that a few SIAC1 cells differentiated into MUC2 or Ulex europaeus agglutinin I lectin-positive secretory cells, suggesting their intestinal cell origin ([Figure 1C](#)).

Aberrant MMR gene expression is frequently found in SBA.^{13,21} Therefore, we evaluated MMR expression in SIAC1 cells. MLH1 and MSH6 expression was deficient and MSH2 expression was reduced in SIAC1 cells ([Figure 1D](#)). PMS1 and PMS2 expression levels were also decreased compared with those in the HT29 cells, which are known to have normal MMR gene expression.³⁴ Findings on immunofluorescence for MLH1

Figure 1 Characterization of SIAC1 cells established from adenocarcinoma of the jejunum. **A:** Photomicroscopy of SIAC1 cells. **B:** Western blot analysis of α -smooth muscle actin (α -SMA) as a fibroblast marker and pan-cytokeratin (panCK) as an epithelial marker in SIAC1, HT29, and human fibroblast cells. **C:** SIAC1 cells form organoids. Photomicroscopy (left) and hematoxylin and eosin staining (middle) of SIAC1 organoids cultured for 14 days. Double-staining of anti-MUC2 (green) and rhodamine-conjugated Ulex europaeus agglutinin I lectin (red) for goblet cells (right). Nuclear staining was performed by Hoechst 33342 (blue). **D:** SIAC1 and human colorectal cancer cell lines were analyzed for mismatch repair (MMR) expression by Western blot analysis. HT29 cells are microsatellite stable. Lovo, DLD1, and Hct116 cell lines lack MSH2, MSH6, and MLH1 expression, respectively. **E:** Immunofluorescence microscopy of SIAC1 cells for MMR proteins. **F:** MMR expression was analyzed by Western blot analysis in SIAC1 and RKO cells treated with the indicated concentrations of 5-aza-2-deoxycytidine (5-aza-dc) alone or with trichostatin A (TSA). **G:** Relative MMR mRNA levels were analyzed in SIAC1 cells treated with 5-aza-dc and/or TSA by real-time PCR. The ratio was calculated relative to the basal mRNA level in control SIAC1 cells. **H:** Methylation-specific PCR for the *MLH1* promoter. Bisulfite-modified genomic DNA was amplified with unmethylated (U) and methylated (M) specific primer sets. H₂O represents blank control. HT29 and RKO are positive controls for unmethylated and methylated cell lines, respectively. Original magnification: $\times 200$ (**A** and **C**, right); $\times 100$ (**C**, middle); and $\times 40$ (**C**, left); $\times 400$ (**E**).

and MSH6 correlated with the findings from the Western blot analysis (Figure 1E), suggesting that altered MMR gene expression was involved in the development of SIAC1 cells.

Since MMR deficiency causes MSI, we examined MSI markers in SIAC1 cells. MSI analysis showed that all five markers—*BAT25*, *BAT26*, *D2S123*, *D2S346*, and *D17S250*²⁸—were positive in SIAC1 cells, indicating this cell line to be MSI-high (H). Regarding MSI target genes in SIAC1, we found monoallelic frameshift mutations in *BAX*, *MSH3*, *CASP5*, *TGFBR2*, and *MARCKS*, whereas *ACVR2* and *MSH6* had bi-allelic frameshift mutations. No mutations were detected in the *FAS*, *IGF2R*, and *NDUFC2* genes in SIAC1 cells (Table 3).

Some tumor suppressor genes, including *MLH1*, undergo CIMP epigenetic changes in a variety of cancers.^{13,22,35} It has also been reported that methylation of *MLH1* was a causative factor of MSI-H tumors in SBA and CRC.^{15,36} Thus, we examined epigenetic modification of MMR gene expression in SIAC1 cells treated with 5-aza-dc and trichostatin A. Western blot analysis indicated that neither agent increased MMR

protein levels (Figure 1F). However, by qualitative PCR, *MLH1* mRNA level was enhanced by 5-aza-dc alone and further in combination with trichostatin A (Figure 1G). Moreover, methylation-specific PCR assay showed that the *MLH1* promoter region was methylated in SIAC1 cells (Figure 1H). These results suggest that MSI-H is caused by *MLH1* promoter methylation and a lack of *MLH1* expression in SIAC1 cells.

Table 3 Frameshift Mutations in SIAC1 Cells

Target gene	Target site	SIAC1 genomic DNA
<i>FAS</i>	(T) ₇	(T) ₇
<i>ACVR2</i>	(A) ₈	(A) ₇
<i>BAX</i>	(G) ₈	(G) ₇ /(G) ₈
<i>IGF2R</i>	(C) ₈	(C) ₈
<i>MSH3</i>	(A) ₈	(A) ₇ /(A) ₈
<i>MSH6</i>	(C) ₈	(C) ₇
<i>NDUFC2</i>	(T) ₉	(T) ₉
<i>CASP5</i>	(A) ₁₀	(A) ₉ /(A) ₁₀
<i>TGFBR2</i>	(A) ₁₀	(A) ₉ /(A) ₁₀
<i>MARCKS</i>	(A) ₁₁	(A) ₉ /(A) ₁₀ /(A) ₁₁

Role of β -Catenin Mutation in SIAC1 Cells

Next, we examined mutation or aberrant expression of other proto-oncogenes and tumor suppressor genes reported to be involved in SBA, such as *KRAS*, *BRAF*, *CTNNB1*, *APC*, *SMAD4*, and *TP53*.^{15,21,37–39} DNA sequence analysis of genomic DNA of SIAC1 cells showed no mutations at codons 12 and 13 in *KRAS*, codon 600 in *BRAF*, mutation cluster region of *APC*, and exons 18, 19, 21, and 23 in *EGFR*. Western blot analysis revealed a truncated form of β -catenin and decreased TGF β R2 levels in SIAC1 cells, whereas SMAD4 and p53 levels were similar to those in Lovo and Hct116 cells, which have intact SMAD4 and p53 (Figure 2A). Aberrant p53 accumulation was not seen in SIAC1 cells.

Interstitial deletion of *CTNNB1* exon 3 is frequently found in SBA.⁴⁰ Agarose gel electrophoresis of PCR products corresponding to *CTNNB1* exons 1 to 6 using SIAC1 cell genomic DNA showed both a normal-sized band and a smaller band (Figure 2B). DNA sequencing revealed a 381-Bp in-frame deletion spanning exons 3 to 4, resulting in a mutant

β -catenin protein that lacked amino acids 11 to 137 (Figure 2C). Since the deleted region of β -catenin included the degradation box, we examined the stability of this β -catenin mutant. We generated a β -catenin deletion (MUT) vector that lacked amino acids 11 to 137 of the WT β -catenin, and examined its stability after transfection into HEK293T cells. Treatment with a protein synthesis inhibitor, cycloheximide, decreased exogenous WT more rapidly than MUT β -catenin, indicating that the deletion mutant was more resistant to degradation (Figure 2D). We also evaluated the endogenous β -catenin protein in SIAC1 cells. The treatment of SIAC1 cells with cycloheximide decreased the amount of WT β -catenin in a time-dependent manner similar to that in HT29 cells (Figure 2E). In contrast, mutant β -catenin in SIAC1 cells was retained 12 hours after cycloheximide treatment, indicating that SIAC1 cells possess stable β -catenin protein.

Next, we investigated the function of mutant β -catenin in SIAC1 cells. A luciferase assay indicated that Wnt/ β -catenin signaling activity was significantly greater in SIAC1 cells than in RKO cells, which have normal Wnt/ β -catenin signaling

Figure 2 β -Catenin deletion mutant in SIAC1 cells. **A:** SIAC1 cells and colorectal cancer cell lines were analyzed by Western blot analysis for β -catenin and tumor suppressor gene proteins. **B:** The 5' region of β -catenin gene (*CTNNB1*) of SIAC1 was analyzed by PCR amplification and sequencing of the indicated PCR fragments. Normal- and smaller-sized fragments were noted as the wild type (WT) and deletion mutant (MUT), respectively. The sequences encoding the phosphorylation sites that function as a degradation targeting box are underlined. **C:** A schematic representation of β -catenin protein in SIAC1 cells. **D:** FLAG-tagged WT and deletion MUT β -catenin expressed in HEK293T cells were analyzed by Western blot analysis after treatment with 10 μ M cycloheximide (CHX). An anti-FLAG antibody was used to detect exogenous β -catenin. **E:** Endogenous β -catenin in SIAC1 and HT29 cells was analyzed by Western blot analysis after treatment with CHX at the indicated times. **F:** TCF transcriptional activities of SIAC1 and colorectal cancer cell lines. The activities in RKO, HT29, and Hct116 cells are shown as ratios of TOP/FOP luciferase activity. **G:** TOP/FOP and cyclin D1 promoter activities were compared between WT and MUT β -catenin plasmid-transfected HEK293T cells. **H:** Photomicroscopy of SIAC1 organoids under culture condition with [50 ng/mL of murine epidermal growth factor, 100 ng/mL of murine Noggin, human R-spondin-1 condition (ENR medium; left) or without (EN medium; right) R-spondin-1 for 14 days. Data are expressed as means $\pm$ SEM (F and G). * P < 0.05. Scale bar = 100 μ m. Original magnification, $\times 40$ (H).

activity (Figure 2F). The function of the mutant β -catenin protein was also evaluated in an overexpression experiment. When transfected into HEK293T cells, MUT β -catenin exhibited increased Wnt/ β -catenin signaling activity compared with the WT vector (Figure 2G). Moreover, the MUT vector stimulated transcriptional activity of the cyclin D1 promoter, which is a known oncogene and one of the target genes of Wnt/ β -catenin signaling (Figure 2G). Interestingly, we also found that, like CRC cells, SIAC1 cells formed organoids in 3D culture without R-spondin-1, which is an agonist of the canonical Wnt/ β -catenin signaling and is essential for the organoid formation from normal crypt (Figure 2H).^{26,33} These results indicate that the deletion of exon 3 of *CTNNB1* is a carcinogenic factor in SIAC1 cells through aberrant activation of intracellular Wnt/ β -catenin signaling.

Comparison with Clinical SBA Specimens

We compared the molecular characteristics of SIAC1 cells to those of clinical SBA samples. The SBA patient tumor characteristics are summarized in Table 4. We used 10 SBA samples for comparison, excluding the tumor from sample 1, which was the origin of the SIAC1 cell line. One case (sample 9) was considered to have familial adenomatous polyposis as a predisposing disease, based on the patient's family history.

MMR expression was evaluated by IHC analysis. MLH1, MSH2, and MSH6 proteins were localized in the nucleus of the glands and lymphoid cells of normal small bowel tissue. IHC analysis demonstrated that MLH1, MSH2, or MSH6 expression was lost or weak in three cases (30%), one case (10%), and two cases (20%), respectively, and that four

cases (40%) had lost expression of at least one MMR protein (Figure 3A and Table 4).

Subsequently, genomic DNA extracted from SBA specimens was analyzed for mutations of MSI target genes. Heterozygous frameshift mutations of *CASP5*, *TGFBR2*, and *MARCKS* were found in all samples (Table 4). In contrast, no frameshift mutations in *IGF2R* were present in any samples. Frameshift mutations in the other MSI target genes, *BAX*, *MSH3*, and *MSH6*, were found in one tissue (10%), two tissues (20%), and three tissues (30%), respectively. We also investigated the mutation of proto-oncogenes reported in SBA, that is, *KRAS* and *BRAF*.^{14,15,41} Mutation at codon 12 or 13 in the *KRAS* gene was present in three cases (30%), whereas mutation at codon 600 in *BRAF* was not detected in any clinical SBA samples (Table 4).

Since we found significant abnormalities in *CTNNB1* in SIAC1 cells, we examined the β -catenin status in clinical SBA tissues. It has been reported that WT β -catenin protein localizes along the intercellular borders of normal small bowel mucosa; however, the interstitial deletion mutant of exon 3 exhibits altered localizations to the nucleus and cytoplasm.^{38,40} IHC analysis for β -catenin localization revealed a cytoplasmic pattern in two cases (20%), and that one case (10%) was nuclear and cytoplasmic, rather than membranous (Figure 3B and Table 2). PCR of genomic DNA showed deletion mutants in two cases (20%). Samples 3 and 10 had interstitial deletion of *CTNNB1* at Bps 44 to 430 and 29 to 251 (+1 Bp insertion), respectively. These results indicate that SIAC1 cells have many molecular characteristics found in clinical SBA specimens, suggesting this cell line to be a promising *in vitro* model of SBA.

Table 4 Results of Sequence Analysis and IHC Analysis of the SBA Samples

Sample no.	1*	2	3	4	5	6	7	8	9	10	11
Location	Jejunum	Jejunum	Jejunum	Ileum	Jejunum	Duodenum	Duodenum	Duodenum	Jejunum	Ileum	Ileum
Pathology	tub1 > por	tub1	tub1	tub2	tub1 > pap	tub1 > 2	pap > tub1	por	tub1 > 2	tub1	tub1,2
Stage	III	IV	III	IV	I	II	II	IV	IV	IV	II
MLH1 IHC	Lost	Lost	Lost					Lost			
MSH2 IHC	Weak							Lost			
MSH6 IHC	Lost	Lost							Lost		
β -Catenin IHC	C	M	C	M	M	M	M	M	M	C	C + N
<i>KRAS</i> mutation	WT	G12D	G13D	G13D	WT	WT	WT	WT	WT	WT	WT
<i>BRAF</i> mutation	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
<i>CTNNB1</i> (Bp)	Del. (30–410)	WT	Del. (44–430)	WT	WT	WT	WT	WT	WT	Del. + 1 (29–251)	ND
<i>ACVR2</i> (A)8	(A) _{7/8}	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
<i>BAX</i> (C)8	(C) _{7/8}	(C) _{7/8/9}	WT	WT	WT	WT	WT	WT	WT	WT	WT
<i>IGF2R</i> (G)8	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
<i>MSH3</i> (A)8	WT	WT	WT	WT	WT	WT	WT	(A) _{7/8}	(A) _{7/8}	WT	WT
<i>MSH6</i> (C)8	(C) _{7/8}	WT	(C) _{7/8}	WT	WT	WT	WT	(C) _{7/8}	(C) _{7/8}	WT	WT
<i>CASP5</i> (A)10	(A) _{8/9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}	(A) _{9/10}
<i>TGFBR2</i> (T)10	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}	(T) _{9/10}
<i>MARCKS</i> (A)11	(A) _{9/10/11}	(A) _{10/11}	(A) _{9/10/11}	(A) _{10/11}	(A) _{10/11}	(A) _{10/11}	(A) _{10/11}	(A) _{9/10/11}	(A) _{10/11}	(A) _{10/11}	(A) _{10/11}

Blank spaces indicate normal expression of MMR genes.

*Sample 1 was original tissue from the SIAC1 cell line and was excluded from the comparative analysis of clinical SBA specimens.

C, cytoplasmic localization; IHC, immunohistochemical analysis; M, cell membranous localization; N, nuclear localization; ND, not determined; SBA, small bowel adenocarcinoma; WT, wild type.

Figure 3 Immunohistochemistry analysis of clinical small bowel adenocarcinoma (SBA) tissues. **A:** Immunohistochemical staining for MLH1, MSH2, and MSH6 proteins was performed with SBA samples. The staining is lost for MLH1, MSH2, and MSH6 in the cancerous lesions of sample 1. Positive staining is observed for the indicated mismatch repair (MMR) protein. **Insets** show high-magnification images of the cancerous lesions. **B:** β-Catenin staining is found along the cellular membrane of normal mucosa and cancers with intact β-catenin. Aberrant expression of β-catenin is indicated by decreased membranous staining and accumulation in the cytoplasm and nucleus. **Insets** show high-magnification image (upper right) and with hematoxylin and eosin staining (lower right) of the cancerous lesions. Original magnification: $\times 100$ (main images, A and B); $\times 200$ (insets, A); $\times 400$ (insets, B).

Screening and Mechanistic Analysis of Anti-Cancer Drugs for SBA Treatment Using SIAC1 Cells

Since a specific or molecular-targeted therapy against SBA has not been established, we assessed the effect of 140 anti-cancer drugs on the death of SIAC1 cells. These drugs included molecular-targeted drugs commonly used for the treatment of gastric cancer and CRC, such as vascular endothelial growth factor, epidermal growth factor receptor, and human epidermal growth factor receptor 2 inhibitors. The results of the first drug screening are shown in [Figure 4A](#) and [Supplemental Table S2](#). Twenty-five drugs, including AKT inhibitors, platelet-derived growth factor receptor inhibitors, and epidermal growth factor receptor inhibitors such

as gefitinib and erlotinib, inhibited SIAC1 cell growth by 50% at 10 $\mu\text{mol/L}$ for 96 hours.

These 25 drugs were used in a second screening experiment at concentrations of 1, 0.1, and 0.01 $\mu\text{mol/L}$. The protease inhibitor bortezomib and the tubulin and microtubule inhibitor eribulin showed efficacy at an optimal concentration of 0.01 $\mu\text{mol/L}$ ([Figure 4B](#)). In this second screening, molecular-targeted drugs against digestive malignancies, such as bevacizumab, cetuximab, trastuzumab, and erlotinib, were also examined but were not effective against SIAC1 cells.

To assess the effects of bortezomib and eribulin against SIAC1 cells, we examined the activity of Wnt/β-catenin signaling. Eribulin, but not bortezomib, decreased Wnt/β-catenin signaling ([Figure 4C](#)). The expression levels of its

Figure 4 Drug screening for small bowel adenocarcinoma (SBA) treatment using SIAC1 cells. **A:** Waterfall plot showing the results of screening of 140 anticancer drugs. Dimethyl sulfoxide was administered as a control, and evaluation was performed 96 hours after treatment. **B:** Proliferation of SIAC1 cells was evaluated using a dilution series of bortezomib, eribulin, and erlotinib. Optical density at 450 nm was measured at 0, 48, and 96 hours after the administration of the anticancer drugs at the indicated concentrations. **C:** Effect of eribulin and bortezomib on Wnt/β-catenin signaling was analyzed by luciferase assay. Evaluation was performed by calculating the relative ratio of TOP/FOP to saline. **D:** SIAC1 cells were analyzed by Western blot analysis for β-catenin, c-Myc, and cyclin D1 proteins after treatment with the indicated concentrations of bortezomib and eribulin for 24 hours (right) or with 100 μmol/L eribulin for the indicated times (left). **E:** Nude mice with s.c. SIAC1 tumors were dosed with bortezomib (white square), eribulin (triangle), or saline (circle), and the volumes of the tumors were measured. **F:** Images on days 1 and 27 of mouse xenografts treated with saline, bortezomib, and eribulin. **G:** Immunohistochemical staining for β-catenin and Ki67 was performed with SIAC1 xenograft tumors treated with saline or eribulin. Inset shows a high magnification image of the cancerous lesion. Data are expressed as means ± SD (**B**), means ± SEM (**C** and **E**). * $P < 0.05$. Original magnification: ×40 (**G**, main image); ×400 (**G**, inset).

downstream targets, c-MYC and cyclin D1, were decreased by eribulin treatment in a dose- and time-dependent manner (Figure 4D). Interestingly, eribulin treatment significantly decreased WT and mutant β-catenin protein levels in SIAC1 cells (Figure 4D), suggesting that eribulin affects β-catenin stability.

Finally, we used a SIAC1 xenograft mouse model to validate the efficacy of bortezomib and eribulin in SBA *in vivo*. Treatment with eribulin significantly inhibited tumor growth compared with saline ($177 \pm 39 \text{ mm}^3$ versus $575 \pm 120 \text{ mm}^3$; $P = 0.01$); however, bortezomib did not decrease the tumor volume (Figure 4, E and F). Immunostaining of xenograft tumors for β-catenin supported the results of the *in vitro* eribulin treatment experiment (Figure 4G). Ki-67—positive cells were also decreased in tumors treated with eribulin (Figure 4G). These results suggest eribulin to be an effective drug for the treatment of SBA.

Discussion

In this study we established an SBA cell line termed SIAC1 and used it to unravel the molecular abnormalities associated with, and to identify a treatment of, SBA. To our knowledge, this is the first report of a cell line derived from

SBA, which will increase our understanding of this rare malignancy. The established SIAC1 cell line was successfully cultured in a plastic dish, as well as in a 3D culture condition, resulting in organoid formation, and on the s.c. tissue of nude mice. The merit of establishing a cell line is the supply of stable experimental material, which enables detailed characteristic analysis, functional assays, and drug screening. A comparison of SIAC1 cells to clinical SBA samples showed molecular similarities, indicating that this cell line is representative of SBA in several important respects.

Recent studies of cancer genetics have indicated that three major events, that is, chromosomal instability, MSI, and CIMP, were closely associated with gastrointestinal carcinogenesis.^{13–15} In CRC development, these molecular disorders represent distinct carcinogenic pathways.¹⁴ However, in SBA, the contribution of these events is relatively unknown.¹⁵ In our analysis, the expression of several MMR genes was lost in some SBA specimens, including in SIAC1 cells. The analysis of microsatellite markers revealed that SIAC1 cells are MSI-H. Previous reports of MSI-H SBA showed a high frequency of frameshift mutations in target genes such as *TGFB2*, *CASP5*, and *MARCKS*,¹⁸ which our data confirmed in SIAC1 cells and SBA specimens. Interestingly, we found that all SBA specimens had heterogeneous

mutations of target genes, which resulted in >10 repeats. In colorectal and gastric cancers, MLH1 expression is frequently suppressed by CIMP.^{23,36,42} We found that 5-aza-dc treatment significantly increased MLH1 mRNA levels (Figure 1G), and methylated modification of *MLH1* promoter region was found in SIAC1 cells (Figure 1H). However, the MLH1 protein levels were not restored by treatment with 5-aza-dc alone or in combination with trichostatin A (Figure 1F). This result suggests the contribution of other mechanisms of regulation of MLH1 protein synthesis, such as post-transcriptional modifications or microRNAs.^{43,44}

We also examined the status of oncogenes and tumor suppressor genes in SBA. We found no mutations in *BRAF* in our clinical SBA specimens, which are frequently observed in CIMP, although three tumors showed a loss of MLH1 protein. The rarity of *BRAF* mutations in SBA was similar to that in previous reports.^{15,45,46} Thus, the contribution of CIMP and MLH1 suppression may be different in SBA compared with the transition from serrated adenoma to CRC.¹⁴

One of the important findings of this study is that SIAC1 cells had a mono-allelic in-frame deletion of *CTNNB1* spanning exons 3 to 4, as reported previously.⁴⁰ Exon 3 of *CTNNB1* contains a degradation box. Deletion of this site stabilizes β -catenin, resulting in abnormal activation of Wnt/ β -catenin signaling and downstream targets, such as c-MYC and cyclin D1,⁴⁷ which leads to large and small intestinal carcinogenesis.⁴⁰ Thus, in SIAC1 cells, the amount of mutant β -catenin protein was greater than that of WT β -catenin (Figure 2A). Moreover, mutant β -catenin was more resistant to degradation (Figure 2E), which elevated Wnt/ β -catenin signaling activity compared with that in RKO cells (Figure 2F). The formation of SIAC1 organoids without R-spondin-1 suggests that mutant β -catenin functionally complements the activity of Wnt/ β -catenin signaling. The crucial role of Wnt/ β -catenin and transforming growth factor β signaling in invasive tumor formation in the small intestine was reported in a genetically engineered mouse model.⁴⁸ Thus, we considered that aberrant β -catenin accumulation due to mutation, coupled with dysregulated transforming growth factor β signaling by MSI, contribute to the development of human SBA.

Another novel finding of this study comes from the drug screening. We tested >100 drugs with known target molecules and found that 2 drugs inhibited the growth of SIAC1 cells under optimal conditions. One of the candidate drugs was a proteasome inhibitor, bortezomib, which is an established agent for the treatment of multiple myeloma.⁴⁹ *In vitro* experiments showed that bortezomib has cytotoxic effects against multiple tumors, including gastric cancer.³⁰ Unfortunately, a xenograft experiment using SIAC1 cells failed to verify its anti-cancer efficacy. Thus, it is necessary to test additional cell lines to verify the therapeutic effect of bortezomib against SBA.

Eribulin is an anti-cancer agent used for breast cancer treatment. It is a synthetic analogue of halichondrin B, which is extracted from a variety of sponges, including

Halichondria okadai.⁵⁰ The activity of eribulin is based on a tubulin-based antimitotic mechanism similar to that of a Vinca alkaloid.⁵¹ We found that eribulin inhibited SIAC1 cell growth and xenograft tumor growth via suppressed Wnt/ β -catenin signaling. Western blot analysis showed accelerated degradation of β -catenin protein after treatment with eribulin and subsequent decreases in c-MYC and cyclin D1 protein levels. Importantly, the mutant β -catenin protein level in SIAC1 cells also decreased, suggesting that eribulin affects β -catenin stabilization independently of its degradation box, which is encoded by exon 3. The stability and degradation of β -catenin are regulated by several kinases at multiple phosphorylation sites.⁵² Eribulin may affect the degradation mechanism independently of GSK3 β , AXIN, APC, and CK1 complex. Among the drugs screened, three Wnt inhibitors did not inhibit the growth of SIAC1 cells. Thus, targeting β -catenin by eribulin may be useful in the treatment of SBA.

In summary, we established an SBA cell line and found MSI and β -catenin mutations to be molecular characteristics of SBA. Furthermore, eribulin exerted an anti-cancer effect both *in vitro* and *in vivo*, suggesting its potential as a therapy for SBA.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.10.006>.

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