

Clinicopathological and Prognostic Significance of Epithelial Gremlin1 Expression in Gastric Cancer

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Abstract. *Background/Aim:* Gastric cancer (GC) is one of the most common malignancies worldwide. Gremlin1 is an antagonist of bone morphogenetic proteins that plays a critical role in several biological processes including cancer biology. *Materials and Methods:* We immunohistochemically examined the expression and distribution of Gremlin1 in non-neoplastic gastric mucosa in a series of 159 GC cases. *Results:* Among 159 GC primary tumors, 59 (37%) were positive for Gremlin1. Gremlin1-negative GC cases showed significantly more advanced clinicopathologic factors and a trend toward intestinal-type GC. Gremlin1 expression was also frequently observed in MUC5AC-positive and G-type GC cases. Gremlin1-negative GCs had a poorer survival rate than Gremlin1-positive GCs ($p=0.002$). Univariate and multivariate analyses revealed that Gremlin1 expression is an independent predictor of survival in GCs. *Conclusion:* These results indicate that Gremlin1 could be involved in GC progression and may be a good marker of long-term survival in GC.

Gastric cancer (GC) is the fifth leading cause of cancer-related death worldwide and the most frequent cancer in East Asian countries. A deeper understanding of the pathogenesis and biological features of GC is necessary to further inform and enhance early detection and treatment methods. One of the main strategies for the improvement of GC diagnosis and treatment is the identification of novel biomarkers that could be used to develop new tools for diagnosis and targets for

treatment (1). While we have identified several GC-specific genes (2), definitive biomarkers for accurately detecting GC and/or predicting the survival of GC patients are still lacking.

Gremlin1, a product of *GREM1*, is a bone morphogenetic protein (BMP) antagonist against several classes of BMPs (3). BMPs are well known as part of the TGF- β superfamily and play an important regulatory role during organogenesis in the embryonic phase (4). In normal colon crypts, *GREM1* is expressed by the intestinal peri-crypt fibroblasts and smooth muscle cells of the muscular mucosa, creating an increasing gradient toward the crypt. This gradient contributes to maintaining the intestinal stem cell niche in the colonic basal crypt region (5), which indicates that *GREM1* expression may influence the progression of colorectal cancer. Gremlin1 is reportedly expressed in the myofibroblasts and smooth muscle cells at the bottom of crypts (5), as well as the crypt epithelium (6), and has been shown to be overexpressed in the epithelial cells of sporadic colorectal traditional serrated adenomas and in CRC (7, 8). Although one study has investigated the prognostic value of Gremlin1 expression in CRC cells (9), no studies have described the clinical significance of *GREM1*/Gremlin1 expression in GC. The present study evaluated the expression and role of Gremlin1 in GC by analyzing its expression and distribution using immunohistochemistry and examining the relationship between Gremlin1 positivity and clinicopathological features, including the prognostic significance. We also assessed the correlation between Gremlin1 expression and mucin phenotypes, which could provide evidence of a correlation between Gremlin1 expression and genetic background in GC.

Materials and Methods

Patients and tissues. Samples from 159 primary tumors were collected from patients diagnosed with GC who underwent surgery between 2005 and 2008 at Hiroshima University Hospital (Hiroshima, Japan). All patient samples were obtained with consent;

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this study was approved by the Ethics Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan) and conformed to the ethical guidelines of the Declaration of Helsinki.

For immunohistochemical analysis, archival formalin-fixed, paraffin-embedded tissues from 159 patients who had undergone surgical excision for GC at Hiroshima University Hospital were consecutively collected. One or two representative tumor blocks from each patient, including the tumor center and tumor-associated non-neoplastic mucosa, were examined using immunohistochemistry. Tumor staging was determined according to the TNM classification system. Histological classification was carried out according to the Lauren classification (10).

Immunohistochemistry. Immunostaining was performed using the Dako Envision+ Rabbit Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. Peroxidase activity was blocked with 3% H₂O₂-methanol for 10 min and the sections were incubated with normal goat serum (Dako Cytomation, Carpinteria, CA, USA) for 20 min to block non-specific antibody binding sites. The sections were incubated with rabbit anti-Gremlin1 antibody (lot# ab22138, Abcam, Cambridge, UK) for 1 h at room temperature, followed by incubation with Envision+ anti-rabbit peroxidase for 1 h. For color reactions, sections were incubated with the DAB Substrate-Chromogen Solution (Dako Cytomation, Carpinteria, CA, USA) for 10 min. The sections were then counterstained with 0.1% hematoxylin. Negative controls were created by omitting the addition of the primary antibody.

Gremlin1 expression was scored as positive or negative in all tumors. When more than 1% of tumor cells were stained for Gremlin1, the immunostaining was considered positive. Using these definitions, two surgical pathologists (NS, AI, and DT) independently reviewed the immunoreactivity in each specimen without knowledge of the clinical and pathological parameters or patient outcome. Inter-observer differences were resolved by consensus review at a double-headed microscope after independent review.

Phenotypic analysis of GC. The GCs were classified into four phenotypes – gastric (G), intestinal (I), gastric intestinal mixed (GI), and Unclassified (N) – as described below. For phenotypic expression analysis of GC, we analyzed the immunohistochemistry as described above, using four antibodies: anti-MUC5AC, anti-MUC6, anti-MUC2, and anti-CD10 (all Novocastra, New Castle, UK). GCs in which more than 10% of the cells in the section expressed at least one gastric epithelial (MUC5AC or MUC6) or intestinal epithelial (MUC2 or CD10) cell markers were classified as G or I-type cancers, respectively; sections that showed both gastric and intestinal phenotypes were classified as GI-type and those that lacked both gastric and intestinal phenotypes were classified as N-type. The details are described in a previous study (11).

Statistical methods. Correlations between clinicopathological parameters and Gremlin1 expression were analyzed using Fisher's exact test. Kaplan–Meier survival curves were constructed for both Gremlin1-positive and Gremlin1-negative patients. The differences between the survival curves were tested for statistical significance using log-rank tests. *p*-Values <0.05 were considered statistically significant.

Results

Gremlin1 expression in non-neoplastic gastric mucosa and GC. We examined Gremlin1 expression based on immunohistochemical staining of normal gastric mucosa, intestinal metaplasia, and a series of 159 GC specimens. In non-neoplastic mucosa, Gremlin1 expression was not detected in most areas (Figure 1A and 1B) and was detected only at the bottom of the crypt in a small number of glands located next to cancerous lesions (data not shown). Cells in the lamina propria and muscular mucosa did not show positivity.

While a previous study in colorectal cancer evaluated Gremlin1 expression by combining the percentages of positive tumor cells and mean intensity, CRC cases with scores above 1 showed significantly better prognosis than CRC cases with a score 0, with no detectable Gremlin1 staining (9). Therefore, we estimated immunostaining positivity as the presence of at least one cancer cell showing any visible signals. In total, 59 (37%) of the 159 GC cases were positive for Gremlin1 expression. The staining was granular and/or cytoplasmic (Figure 1C and D), which was consistent with the staining pattern in colorectal cancer (9). The proportion of Gremlin1 positivity ranged from 0 to 50%; most of the Gremlin1-positive cases had less than 10% positive cancer cells.

Correlation between Gremlin1 expression and clinicopathological features in GC. The correlations between Gremlin1 expression and clinicopathological characteristics were investigated (Table I). Gremlin1-negative cases showed more advanced T grade ($p=0.0466$, Fisher's exact test), N grade ($p=0.0087$, Fisher's exact test), TNM stage ($p=0.0050$, Fisher's exact test), and tended to have intestinal histology ($p=0.0226$, Fisher's exact test).

Correlations between Gremlin1 expression and mucin phenotype in GC. In addition to the Lauren histology-based classification, GC can be subdivided into four phenotypes according to mucin expression(2). Gastric and intestinal markers were detected, including MUC5AC in 78 of 145 (54%) cases, MUC6 in 16 (11%) cases, MUC2 in 37 (25%) cases, and CD10 in 17 (11%) cases. Only MUC5AC expression was significantly correlated with Gremlin1 expression in GC cases (Table II). We further investigated the association between Gremlin1 expression and mucin phenotype, observing that Gremlin1 expression was more frequently detected in GC cases with G types than in cases with other types (Figure 2).

Correlation between Gremlin1 expression and survival in GC patients. The relationship between Gremlin1 expression and survival probability was examined in 159 GC cases. The 5-year overall survival rates were 86% and 58% for Gremlin1-positive and -negative cases, respectively, and the Gremlin1-negative GC cases had significantly lower survival probability

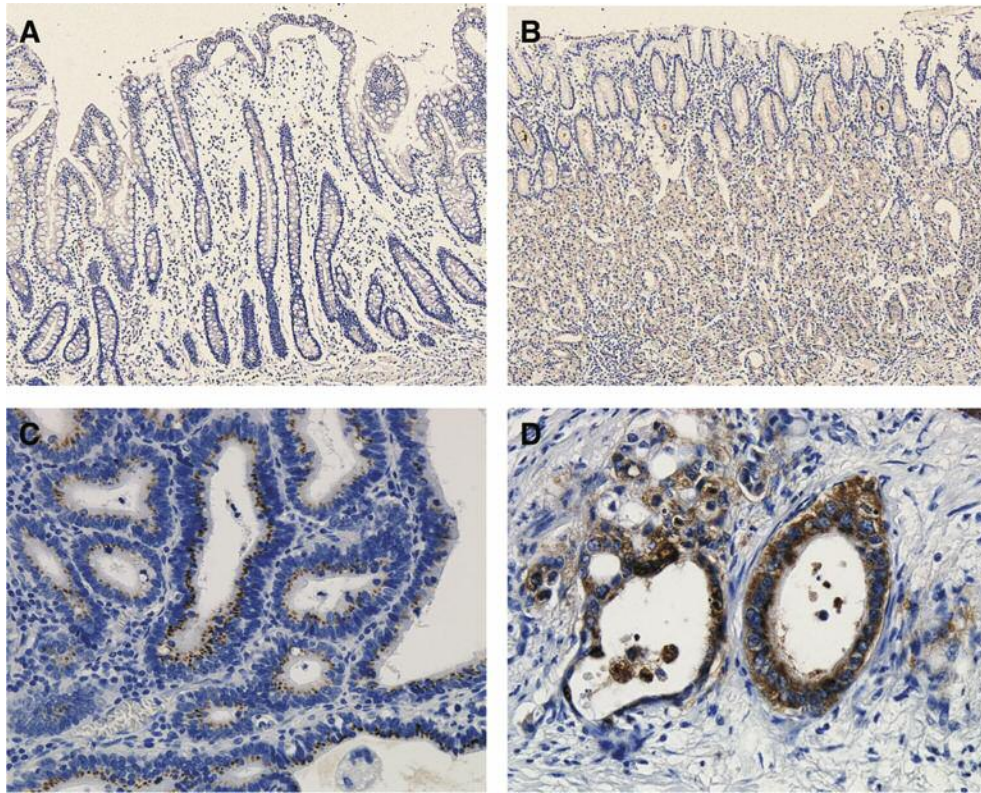


Figure 1. Immunohistochemical analysis of Gremlin1 in non-neoplastic gastric mucosa and gastric cancer (GC). No visible staining was observed in the fundic gland (A) and intestinal metaplasia (B) (Original magnification: $\times 40$). Granular (C) and cytoplasmic (D) staining was visible in GC (original magnification: $\times 400$).

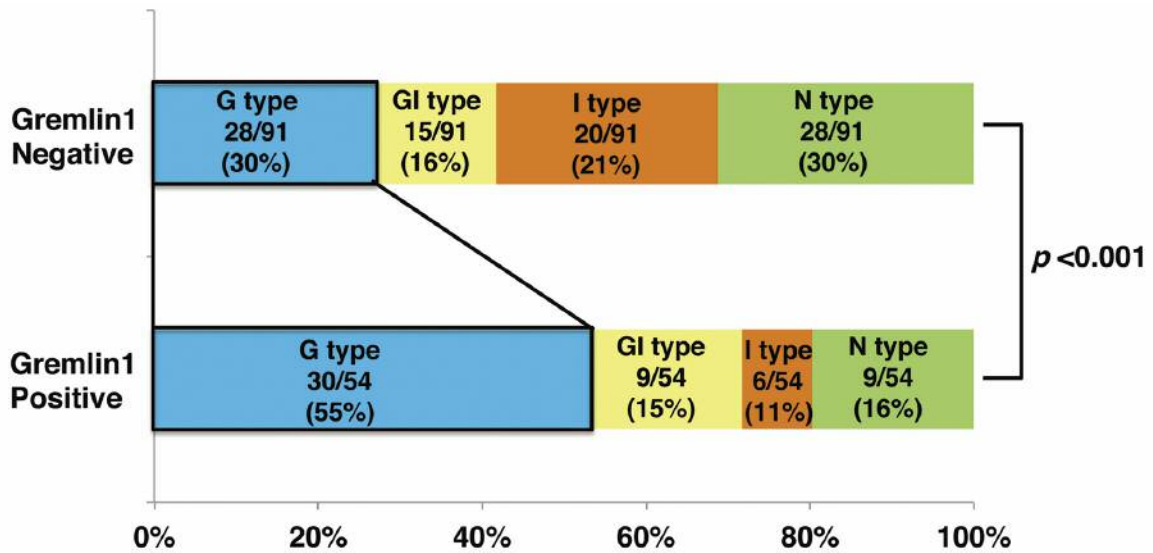


Figure 2. Distribution of gastric (G), intestinal (I), gastric and intestinal mixed (GI), and unclassified (N) phenotypes in Gremlin1-positive gastric cancer (GC) cases. Gremlin1-positive cases were more frequently found in G-type GC than in the other types. *p*-Values were analyzed using Fisher's exact tests.

Table I. Clinicopathologic characteristics of 159 GC cases.

		Gremlin1 expression			
		Negative	Positive		<i>p</i> -Value*
Median age, years (range)	65 (34-87)	<65	53	30	0.8698
		≥65	47	29	
Gender (%)					
Male	99 (61)	Male	64	35	0.6128
Female	60 (39)	Female	36	24	
T grade (%)					
T1	69 (30)	T1	37	32	0.0466
T2	43 (26)	T2/3/4	63	27	
T3	39 (31)				
T4	8 (13)				
N grade (%)					
N0	86 (58)	N0	46	40	0.0087
N1	36 (58)	N1/2/3	54	19	
N2	26 (27)				
N3	11 (46)				
Stage (%)					
I	81 (14)	I	42	39	0.0050
II	22 (21)	II/III/IV	58	20	
III	47 (47)				
IV	9 (18)				
Histology(%)					
Intestinal		Intestinal	42	36	0.0226
Diffuse		Diffuse	58	23	

*Fisher's exact test.

than that of the Gremlin 1-positive GC cases ($p=0.002$; Figure 3A). As Gremlin1 expression was frequently found in G-type GC, we further investigated the association between its expression and patient survival in the 58 G-type GC cases. Kaplan–Meier analysis showed significantly poorer survival in Gremlin1-negative GC cases than that in Gremlin1-positive GC cases ($p=0.001$; Figure 3B).

In order to evaluate Gremlin1 as a prognostic classifier, the association between its expression and cancer-specific mortality was evaluated in univariate and multivariate Cox proportional hazards analyses. In univariate analysis, T grade (hazard ratio [HR]=14.801; 95% confidence interval [CI]=5.408-60.886; $p=0.001$), N grade (HR=9.128; 95%CI=1.267-16.392; $p<0.001$), TNM stage (HR=8.460; 95%CI=2.083-11.111; $p<0.001$), histological type (HR=3.369; 95%CI=1.928–6.477; $p=0.001$), and Gremlin1 expression (HR=2.734; 95%CI=1.132-5.694; $p=0.003$) were associated with survival. In multivariate modeling, Gremlin1 expression (HR=3.468; 95%CI=1.214-8.776; $p<0.001$) was an independent prognostic indicator (Table III). Thus, immunohistochemical examination of Gremlin1 in GC samples reveals the potential of Gremlin1 as a prognostic biomarker for GC.

Table II. Comparison between Gremlin 1 and markers for mucin phenotype in GC.

		Gremlin1 expression		
		Negative	Positive	<i>p</i> -Value*
Gastric markers				
MUC5AC				
Positive		30	48	<0.001
Negative		56	11	
MUC6				
Positive		7	9	0.1902
Negative		79	50	
Intestinal markers				
MUC2				
Positive		21	16	0.8465
Negative		65	43	
CD10				
Positive		10	7	0.9882
Negative		76	52	

*Fisher's exact test.

Discussion

The results of the present study demonstrate the clinical significance of Gremlin1 expression in GC, including evidence that Gremlin1-negative GC cases are more likely to be advanced cancer and have a poorer prognosis, and that Gremlin1 expression is an independent prognostic factor in GC. BMPs have been established as functional regulators in the maintenance of homeostasis and sequential metaplastic and dysplastic changes in normal stomach tissue (12). In the light of the functional role of Gremlin1, an antagonist of several BMPs, Gremlin1 is presumably involved in biological processes in normal stomach and/or GC tissue, although no studies have described the functional roles of Gremlin1 in GC tissue.

A previous study reported Gremlin1 to be preferentially expressed in CRC cases with serrated morphology (9). Colorectal serrated tumors often display gastric features (13) and CRCs with serrated morphology are often categorized as “hypermethylated” cases that are frequently accompanied by microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and several mutations in the genes related to the TGF-β signal pathway, including *ACVR2* and *TGFBR2* (14). In the present study, Gremlin1 expression was significantly associated with MUC5AC expression and was often detected in G-type GC. G-type GC may represent MSI-H, a frequent methylation in the promoter region of tumor suppressor genes and less frequent p53 mutations (2). Recent evidence suggest that GCs in the “MSI-group”,

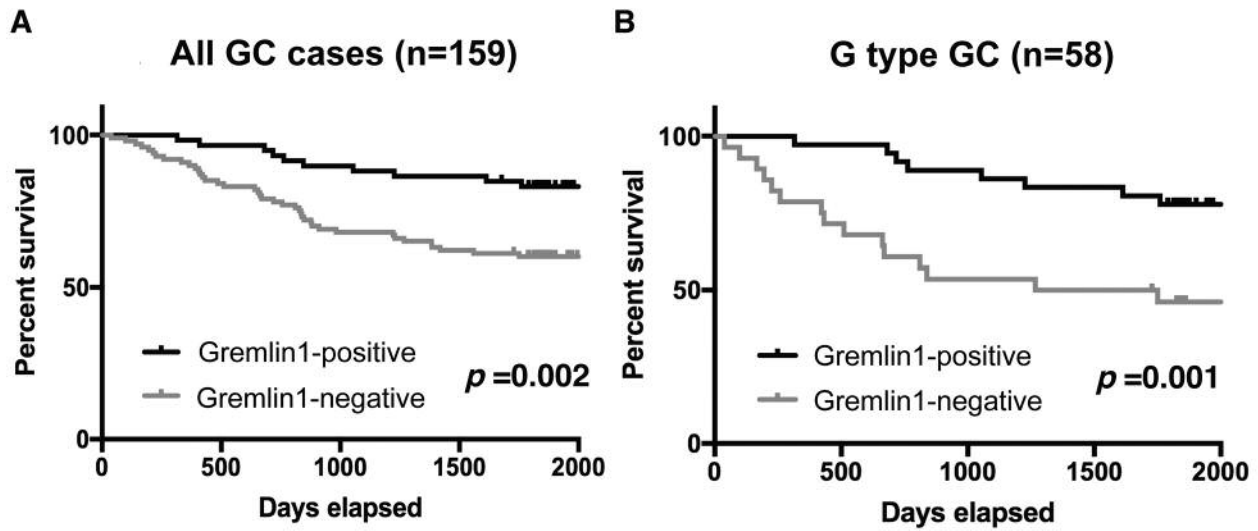


Figure 3. Survival of patients with Gremlin1-positive and -negative gastric cancer (GC). (A) Kaplan-Meier curves of patients with Gremlin1-positive and -negative GC in a series of 159 GC cases. (B) Kaplan-Meier curves of patients with Gremlin1-positive and -negative GC in 59 G-type GC cases.

Table III. Univariate and multivariate analysis of factors influencing survival in 159 GC cases.

Parameter	Univariate analysis			Multivariate analysis		
	HR ^a	95% CI ^b	p-Value	HR	95% CI	p-Value
Gender						
Female	Ref					
Male	1.131	0.685-2.152	0.6894			
Age						
<65 y.o	Ref					
>65 y.o	1.122	0.442-1.483	0.8593			
T grade						
pT1	Ref			Ref		
pT2/T3/T4	14.801	5.408-60.886	0.001	1.322	0.248-9.085	0.774
N grade						
pN0	Ref			Ref		
pN1/ N2/ N3	9.128	1.267-16.392	<0.001	4.979	0.928-89.438	0.0325
TNM stage						
pStage I	Ref			Ref		
pStage II/III/IV	8.460	2.083-11.111	<0.001	34.579	6.293-222.27	<0.001
Histological type						
Intestinal	Ref			Ref		
Diffuse	3.369	1.928-6.477	0.001	0.652	0.333-1.381	0.267
Gremlin1 expression						
Positive	Ref			Ref		
Negative	2.734	1.132-5.694	0.003	3.468	1.214-8.776	0.017

^aHR: Hazard ratio; ^bCI: confidence interval.

defined as TCGA based on data from the comprehensive characterization of GC by whole-genome sequencing, frequently have mutations in activin type II receptor (*ACVR2*) and transforming growth factor beta receptor 2

(*TGFBR2*) (15). Taken together, the previous data and our own indicate that Gremlin1 expression is probably disturbed in both CRC and GC cells with similar genetic and phenotypic backgrounds. Only one paper has described the

details of a proposed mechanism concerning Gremlin1 overexpression, which is confirmed by a rare hereditary polyposis syndrome; human hereditary mixed polyposis syndrome (16). Further in-depth studies are still needed to characterize the mechanism underlying the dysregulation of Gremlin1 expression, which may also reveal the fundamental mechanisms commonly involved in GC and CRC progression.

Another important issue is that the antibody used in the present study does not detect Gremlin1 expression in cells from the stromal components. Evidence indicates that Gremlin1 expression in intestinal pericryptal fibroblasts and smooth muscle cells is present in an increasing gradient alongside the crypts, which may contribute to the maintenance of the intestinal stem cell niche in the colonic basal crypt region (5). *In situ* hybridization for *GREM1* has identified the expression of GREM1 in both epithelial and stromal cells in the colon and GREM1-negative CRCs in the stromal cells showed significantly more advanced TNM stage and worse prognosis compared to those of GREM1-positive CRCs, whereas there were no significant correlations among epithelial GREM1 positivity and these factors (4). In terms of the difference in the stromal tissue of the gastrointestinal tract, there is a definitive signature in both the stomach and colon. One of the biggest differences is BARX1, which could interact with CDX2, an essential transcription factor of the colon epithelium (17). Thus, stromal Gremlin1 expression in normal stomach and GC tissue could be regulated in a different manner than that in the normal colon and CRC. Additional studies are needed to measure the stromal Gremlin1 expression in normal stomach and GC, which could further verify the importance of Gremlin1 expression in these tissues.

In summary, we verified the staining property of Gremlin1 in normal, metaplastic, and cancerous tissues of the stomach. The expression status of epithelial Gremlin1 was significantly associated with tumor progression and was also an independent marker for poor survival in patients with GC. Although the detailed function of Gremlin1 in GC remains unclear, these data highlight its potential as a prognostic classifier in patients with GC.

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References

- 1 Yasui W, Oue N, Sentani K, Sakamoto N and Motoshita J: Transcriptome dissection of gastric cancer: identification of novel diagnostic and therapeutic targets from pathology specimens. *Pathol Int* 59: 121-136, 2009.
- 2 Oue N, Sentani K, Sakamoto N and Yasui W: Clinico-pathologic and molecular characteristics of gastric cancer showing gastric and intestinal mucin phenotype. *Cancer Sci* 106: 951-958, 2015.
- 3 Wordinger RJ, Zode G and Clark AF: Focus on molecules: gremlin. *Exp Eye Res* 87: 78-79, 2008.
- 4 Bragdon B, Moseychuk O, Saldanha S, King D, Julian J and Nohe A: Bone morphogenetic proteins: a critical review. *Cell Signal* 23: 609-620, 2011.
- 5 Kosinski C, Li VS, Chan AS, Zhang J, Ho C, Tsui WY, Chan TL, Mifflin RC, Powell DW, Yuen ST, Leung SY and Chen X: Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *PNAS* 104: 15418-15423, 2007.
- 6 Namkoong H, Shin SM, Kim HK, Ha SA, Cho GW, Hur SY, Kim TE and Kim JW: The bone morphogenetic protein antagonist gremlin 1 is overexpressed in human cancers and interacts with YWHAH protein. *BMC Cancer* 6: 74, 2006.
- 7 Laurila R, Parkkila S, Isola J, Kallioniemi A and Alarimo EL: The expression patterns of gremlin 1 and noggin in normal adult and tumor tissues. *Int J Exp Pathol* 6: 1400-1408, 2013.
- 8 Davis H, Irshad S, Bansal M, Rafferty H, Boitsova T, Bardella C, Jaeger E, Lewis A, Freeman-Mills L, Giner FC, Rodenas-Cuadrado P, Mallappa S, Clark S, Thomas H, Jeffery R, Poulosom R, Rodriguez-Justo M, Novelli M, Chetty R, Silver A, Sansom OJ, Greten FR, Wang LM, East JE, Tomlinson I and Leedham SJ: Aberrant epithelial GREM1 expression initiates colonic tumorigenesis from cells outside the stem cell niche. *Nat Med* 21: 62-70, 2015.
- 9 Pelli A, Vayrynen JP, Klintrup K, Makela J, Makinen MJ, Tuomisto A and Karttunen TJ: Gremlin1 expression associates with serrated pathway and favourable prognosis in colorectal cancer. *Histopathology* 69: 831-838, 2016.
- 10 Lauren P: The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. an attempt at a histo-clinical classification. *Acta Microbiol Scand* 64: 31-49, 1965.
- 11 Sakamoto N, Oue N, Sentani K, Anami K, Uraoka N, Naito Y, Oo HZ, Hinoi T, Ohdan H, Yanagihara K, Aoyagi K, Sasaki H and Yasui W: Liver-intestine cadherin induction by epidermal growth factor receptor is associated with intestinal differentiation of gastric cancer. *Cancer Sci* 103: 1744-1750, 2012.
- 12 Todisco A: Regulation of gastric metaplasia, dysplasia and neoplasia by bone morphogenetic protein signaling. *Cell Mol Gastroenterol Hepatol* 3: 339-347, 2017.
- 13 Sakamoto N, Feng Y, Stolfi C, Kurosu Y, Green M, Lin J, Green ME, Sentani K, Yasui W, McMahon M, Hardiman KM, Spence JR, Horita N, Greenson JK, Kuick R, Cho KR and Fearon ER: BRAF(V600E) cooperates with CDX2 inactivation to promote serrated colorectal tumorigenesis. *Elife* 6: e20331, 2017.
- 14 Cancer Genome Atlas N: Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 487: 330-337, 2012.

- 15 Wang K, Yuen ST, Xu J, Lee SP, Yan HH, Shi ST, Siu HC, Deng S, Chu KM, Law S, Chan KH, Chan AS, Tsui WY, Ho SL, Chan AK, Man JL, Foglizzo V, Ng MK, Chan AS, Ching YP, Cheng GH, Xie T, Fernandez J, Li VS, Clevers H, Rejto PA, Mao M and Leung SY: Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. *Nat Genet* 46: 573-582, 2014.
- 16 Jaeger E, Leedham S, Lewis A, Segditsas S, Becker M, Cuadrado PR, Davis H, Kaur K, Heinemann K, Howarth K, Collaboration H, East J, Taylor J, Thomas H and Tomlinson I: Hereditary mixed polyposis syndrome is caused by a 40-kb upstream duplication that leads to increased and ectopic expression of the BMP antagonist GREM1. *Nat Genet* 44: 699-703, 2012.
- 17 Stringer EJ, Duluc I, Saandi T, Davidson I, Bialecka M, Sato T, Barker N, Clevers H, Pritchard CA, Winton DJ, Wright NA, Freund JN, Deschamps J and Beck F: Cdx2 determines the fate of postnatal intestinal endoderm. *Development* 139: 465-474, 2012.

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