

## Prognostic impact of GPR56 in patients with colorectal cancer

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G protein-coupled receptor 56 (GPR56) belongs to the adhesion G protein-coupled receptor subfamily, which plays a role in cell progression and survival. The aim of this study was to investigate the role of the GPR56 gene in a cell line study and the impact of its protein expression on the prognosis of colorectal cancer (CRC) patients. The effect of GPR56 on tumor cell proliferation (WST-1 assay), invasion (Transwell assay), migration (Transwell assay, wound healing assay), and colony-forming ability (semisolid agar colony-forming assay) was explored. The expression levels of GPR56 in tissue samples of 109 CRC patients were evaluated by immunohistochemistry. The prognostic value of GPR56 was analyzed using univariate and multivariate analyses. The downregulation of GPR56 in the CRC cell line reduced cell proliferation as compared with that in a control sample (48 h;  $p=0.042$ , 72 h;  $p=0.001$ ). Downregulation of the GPR56 expression reduced cell invasion and migration abilities and inhibited colony-forming abilities ( $p<0.005$ ). The 5-year overall survival rate was worse in the high-expression group as compared with that in the low-expression group (51.6% vs. 74.4%,  $p=0.008$ ). High GPR56 expression was a significant prognostic factor for overall survival of CRC patients in the univariate ( $p=0.001$ ) and multivariate ( $p<0.001$ ) analyses. The expression level of GPR56 plays an important role in tumor progression in CRC, and it may serve as a prognostic indicator in CRC patients.

*Key words: G protein coupled receptor 56, colorectal cancer, progression, prognosis, biomarker*

G protein-coupled receptors constitute a large family of membrane proteins. G protein-coupled receptor 56 (GPR56), which belongs to the adhesive G protein-coupled receptor subfamily, interacts with collagen III [1, 2], transglutaminase 2 [3], the vascular endothelial growth factor [4], and CD81 [5]. Therefore, GPR56 participates in cell survival, proliferation, adhesion, and migration. GPR56 is highly expressed in the brain, thyroid gland, and heart [6]. GPR56 is also found to be moderately expressed in the kidneys, pancreas, small intestine, stomach, and colon [6]. In recent studies, reverse transcription-polymerase chain reaction (RT-PCR), Northern blot analyses, and functional results demonstrated that GPR56 expression is inversely correlated with the metastatic potential in a melanoma cell line [7, 8]. In addition, studies have reported that the expression level of GPR56 is lower in high metastatic cell lines compared with that in low

metastatic cell lines [7, 8]. However, two other studies have reported that high GPR56 expression is positively associated with tumorigenesis in gliomas and digestive cancers [9, 10]. Therefore, the expression level and functions of GPR56 may differ in various tumors. Few studies have examined the association of GPR56 with gastrointestinal tumors, including those of colorectal cancer (CRC), which is one of the most common cancers worldwide [11]. In the USA, CRC is the second leading cause of cancer-related mortality [12], and it is the third most common cancer in South Korea [13]. CRC is the second most common cancer among men and the third most common cancer among women [13]. As noted, the roles of GPR56 may differ depending on the type of cancer. To the best of our knowledge, no studies have investigated the function of GPR56 in CRC. Therefore, the present study explored the expression pattern of GPR56 in CRC, its corre-

lation with the clinicopathological characteristics of CRC patients, and its effects on cell proliferation, migration, and invasion in CRC.

### Patients and methods

**Cell culture.** The present study examined the expression level of GPR56 in human CRC cell lines (SW620, SW480, HCT116, HT29), which were authenticated by short tandem repeat profiling, and the SW620 cell line was selected as it exhibited the strongest expression of GPR56 (Figures 1A–1D). The human CRC cell line SW620 was obtained from the Korean Cell Line Bank. The CRC cells were maintained in an RPMI-1640 medium (Welgene), which was supplemented with 10% fetal bovine serum (FBS; Youngin Frontier) and 1% penicillin-streptomycin (Hyclone; Cytiva). The cells were cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO<sub>2</sub>).

**Downregulation of GPR56 by transfection with small interfering RNA (siRNA) and its effects on cell proliferation, migration, and invasion.** Knockdown experiments were performed to elucidate the molecular mechanisms of GPR56 in regulating the tumor progression of colorectal carcinomas. The transfection efficiencies of the high expression of GPR56 and siRNA were confirmed by western blotting and RT-PCR analysis. The effects of GPR56 downregulation on the cell proliferation, migration, and invasion of SW620 were then investigated.

**siRNA transfection.** In the present study, siRNAs were mixed and transfected into the cells using the HiPerFect transfection reagent (Qiagen GmbH) in a serum-

free RPMI-1640 medium, according to the manufacturer protocol, with a final siRNA concentration of 100 nM. Total RNAs and proteins were collected from a control group (transfection reagent only), a negative control group (transfected with non-mammalian target siRNA), and a siRNA-GPR56 group (transfected with siRNA-GPR56) 24 h post-transfection. The sequence of siRNA-GPR56 was as follows: 5'-GACACUGUCCUGCUGAGU-3'. The transfection efficiency of GPR56 was confirmed through western blotting and RT-PCR analysis.

The SW620 cells were seeded in six-well plates with  $2 \times 10^5$  cells/well and cultured until the cells reached ~50–60% confluence. The SW620 cells were then divided into the control group and siRNA-GPR56 group. Subsequently, the transfection efficiency was assessed using western blotting and RT-PCR analysis.

**Western blotting.** Cells lysates were prepared in a Pro-Prep protein extraction solution (INtRON). Total protein (30 µg) for electrophoresis was loaded in each well of a 15% gel, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membranes (EMD Millipore). Subsequently, the membranes were incubated in 5% non-fat milk in Tris-buffered saline/0.1% Tween-20 for 1 h at room temperature, followed by incubation with primary antibodies at 4°C overnight. The membranes were then incubated with secondary antibodies for 1 h at room temperature. The signal was detected using an electrogenerated chemiluminescence solution (Advansta) for 2 min and a molecular imaging system (ChemiDocXRS+; Bio-Rad Laboratories, Inc.). The following antibodies were used: anti-GPR56 (1:1,000; Abcam,

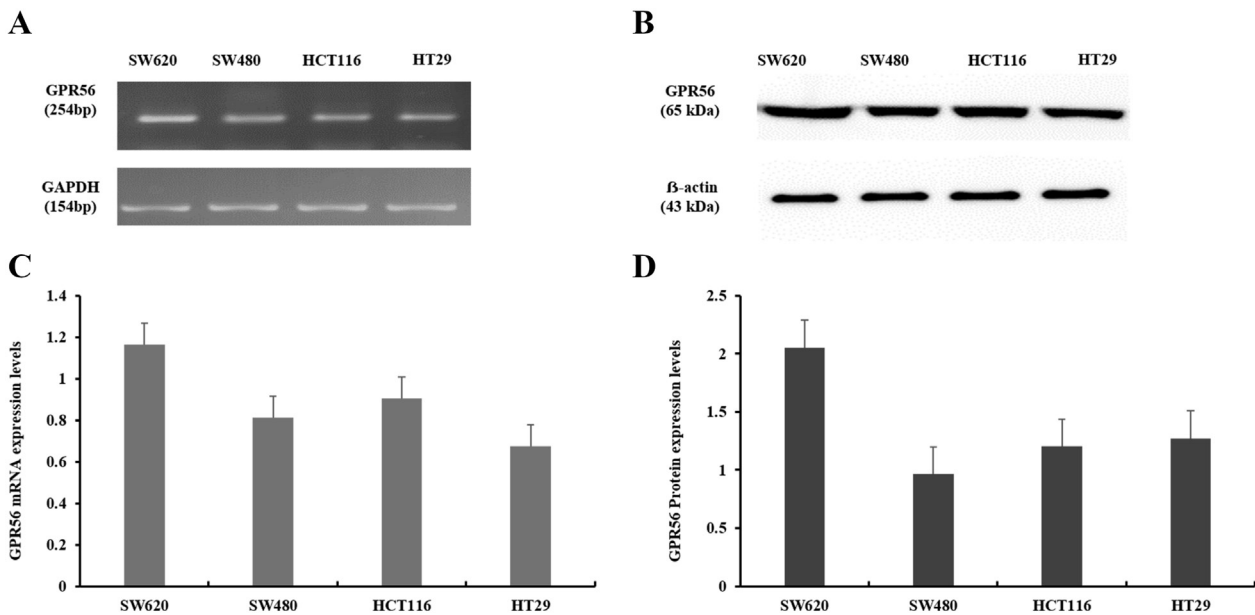


Figure 1. Expression of GPR56 mRNA and protein was analyzed in four CRC cell lines. The expression of GPR56 mRNA and protein was highest in SW620 cells. A, B) mRNA level was assessed by RT-PCR. C, D) Protein level was assessed by western blotting.

#ab75850), anti- $\beta$ -actin (1:3,000; Sigma-Aldrich; Merck KGaA, #A5441), anti-rabbit IgG (1:5,000; Sigma-Aldrich; Merck KGaA, # SAB3700872), and anti-mouse IgG (1:5,000; Sigma-Aldrich; Merck KGaA, #SAB3701212). Anti- $\beta$ -actin antibodies were used as an internal control.

**RT-PCR analysis.** Total RNA was extracted from the cells and purified using a Hybrid-RTM RNA extraction kit (Geneall) according to manufacturer instructions. The total RNA quantity was assessed using a NanoDrop instrument (Thermo Fisher Scientific, Inc.), and 500 ng/ $\mu$ l was applied in the PCR by using a ReverTra Ace qPCR kit (Toyobo Life Science).

**WST-1 assays to assess cell proliferation.** Cell proliferation was determined using WST-1 assays and an EZ-Cytox kit (DogenBio). The control, negative control, and siRNA-GPR56-transfected SW620 cells were plated in 96-well plates at  $1 \times 10^4$  cells/well in 100  $\mu$ l RPMI-1640 medium containing 10% FBS, and then cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> overnight. After 10  $\mu$ l WST-1 reagent was added to each well, the cells were incubated at 37°C for 4 h in a 5% CO<sub>2</sub> incubator. Subsequently, the absorbance of the supernatant was measured spectrophotometrically at 450 nm. Cell proliferation was measured 24, 48, and 72 h after seeding. Each experiment was performed in triplicate.

**Table 1. The association of clinicopathological features and GPR56 expression in colorectal cancer patients.**

Clinicopathological factors	GPR56 expression		Total (n=109)	p-value
	Low (n=78)	High (n=31)		
Age, years, mean (SD)	63.2 $\pm$ 12.5	61.7 $\pm$ 13.5	62.4 $\pm$ 13.0	0.551
Gender, n (%)				0.002
M	23 (54.8%)	19 (45.2%)	42	
F	55 (82.1%)	12 (17.9%)	67	
Stage, n (%)				0.001
I	23 (92.0%)	2 (8.0%)	25	
II	27 (71.1%)	11 (28.9%)	38	
III	26 (68.4%)	12 (31.6%)	38	
IV	2 (25.0%)	6 (75.0%)	8	
pT stage, n (%)				0.001
pT1/T2	41 (91.1%)	5 (8.9%)	46	
pT3/T4	39 (60.9%)	26 (39.1%)	65	
pN stage, n (%)				0.056
-	50 (78.1%)	14 (21.9%)	64	
+	28 (62.2%)	17 (37.8%)	45	
Metastasis, n (%)				0.006
-	76 (75.2%)	25 (24.8%)	101	
+	2 (25.0%)	6 (75.0%)	8	
Vascular invasion, n (%)				0.566
-	66 (71.7%)	26 (28.3%)	92	
+	12 (70.6%)	5 (29.4%)	17	
Lymphatic invasion, n (%)				0.154
-	64 (74.4%)	22 (25.6%)	86	
+	14 (60.9%)	9 (39.1%)	23	

**Cell migration and invasion assays.** Cell migration and invasion assays were carried out using a Transwell chamber kit (Corning, Inc.). The insert chamber was separated by a polycarbonate membrane. The polycarbonate membrane was formed with 8  $\mu$ m pores. The separation was performed in 24-well plates. The SW620 cells were harvested by trypsinization and counted using a Scepter Handheld Automated Cell Counter (EMD Millipore). In total,  $\sim 2.5 \times 10^5$  cells suspended in 100  $\mu$ l serum-free medium were seeded in the upper chambers of the Transwell, and 700  $\mu$ l culture medium containing 10% FBS was added to the lower chambers. The plates with the Transwell insert chambers were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> for 24 h (for migration assays) or 72 h (for invasion assays). After incubation, the polycarbonate membrane was fixed with 3.7% formaldehyde for 10 min and stained with 0.5% methyl green for 1–2 min. Cells that had not migrated to the upper sides of the chambers were carefully removed using cotton swabs. The cell numbers were counted in five random fields under an inverted microscope at 200 $\times$  magnification. For the invasion assays, the upper membranes of the chambers were coated with 50  $\mu$ l Matrigel (2.5 mg/ml; BD Biosciences) before use.

**In vitro wound-healing assays.** The SW620 cells were transfected with GPR56-siRNA, seeded in six-well plates 24 h post-transfection, and grown to 60–70% confluency. Wound-healing cell migration assays were performed using a culture insert system 24 (Ibidi). The cells were seeded into the insert chambers. This step was followed by incubation for 12–24 h in a medium containing 10% FBS and 1% adult bovine serum. A cell-free gap was observed after 12 and 24 h. Images were captured using a phase-contrast microscope (AxioCam Camera; Zeiss). The widths of the cell-free gap in five random visual fields were measured. The average width was determined.

**Semisolid agar colony-forming assays.** For measurement of anchorage-independent colony growth, six-well plates coated with two layers of agar were used. First, a 0.5% agar solution was added as a base layer to the six-well plates and left to solidify at room temperature. Next,  $2 \times 10^3$  cells were suspended in a serum-free RPMI-1640 medium with a 0.35% agar solution and seeded over the base layer. The six-well plates containing the cells and double agar layer were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> for 14 days. The cells were then fixed with 3.7% formaldehyde for 10 min and stained with 0.5% methyl green for 20 min. The number of colonies was determined using an inverted microscope at 100 $\times$  magnification. The experiment was repeated in triplicate.

**Patient characteristics and tissue specimens.** In total, 109 patients diagnosed with primary colorectal adenocarcinomas who underwent surgery between January 2009 and December 2013 were included in this retrospective analysis. Data on the following clinical parameters were collected: age, sex, pT stage, pN stage, metastasis, vascular invasion, lymphatic invasion, and the overall stage (Table 1). The 109

patients were divided into two groups: a low-expression GPR56 group (n=78) and a high-expression GPR56 group (n=31). The histological grade and clinical stage of the tumors were identified according to the 7<sup>th</sup> edition of the tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer. This study was approved by the Ethics Committee of our hospital.

**Immunohistochemistry (IHC) staining and expression of GPR56 in colorectal adenocarcinomas.** Immunohistochemistry (IHC) staining was performed to investigate the expression level of GPR56 in clinical colorectal adenocarcinoma tissues, as well as in the adjacent non-tumor tissue. All biopsy specimens were fixed in 10% formalin and embedded in paraffin. They were then cut into sections, dewaxed, and rehydrated using a graded series of ethanol, followed by microwave antigen retrieval. After being blocked with 0.3% hydrogen peroxidase, the sections were incubated at 4°C overnight with the GPR56 primary antibody (Bioss Antibodies; 1:100, #bs-20747R). IHC staining was conducted using a DAB kit (Dako; Agilent Technologies, Inc., #K3468). The sections were then stained with hematoxylin, dehydrated, cleared, and mounted. As a negative control, 5% FBS was used. The samples were divided into two groups based on GPR56 expression: a low expression group [negative expression: immunoreactivity score (IRS) <2] and a high expression group (positive expression: IRS ≥2). The IHC staining results revealed that GPR56 was highly expressed in some of the clinical colorectal adenocarcinoma samples, mainly in the cytoplasm and cell membranes. To determine the effects of GPR56 expression on the clinical characteristics, the relationships between GPR56 expression and clinicopathological factors were explored.

**Semi-quantitative analysis of the IHC results.** The protein expression was scored using a light microscope by two independent investigators blinded to clinical data, and a consensus score was determined for each specimen. GPR56 immunoreactivity was observed in the cytoplasm and cell membrane. In the assessment of the IHC staining results, staining intensity was graded using scores of 0 (negative), 1 (weak, pale yellow), 2 (moderate, dark yellow), or 3 (strong, brown). The staining extent was scored by the percentage of positive cells as 0 (0%), 1 (1–33%), 2 (34–66%) or 3 (67–100%). The final IRS was calculated by multiplying the intensity and percentage scores. GPR56 staining was classified as low expression (IRS ≤2) or high expression (IRS >2).

**Univariate and multivariate analyses.** To evaluate the role of GPR56 expression in colorectal adenocarcinomas, the 5-year overall survival rate for the 109 CRC patients was determined using Kaplan-Meier survival analysis. To further investigate their independent predictive value, a multivariate Cox proportional hazards model was used, with all of the significant parameters detected through univariate analysis.

**Statistical analysis.** The associations between GPR56 expression and clinicopathological features were analyzed using the  $\chi^2$  test or Fisher's exact test, and continuous variables

were analyzed using Student's t-test or the Mann-Whitney U rank test. Overall survival curves were generated using the Kaplan-Meier method, and the differences between the high GPR56 expression and the control group were examined using log-rank tests. Cox proportional-hazards regression model was used to estimate the univariate and multivariate hazard ratios (HRs) for the overall survival rate. All p-values were two-sided, and p<0.05 was considered to indicate a statistically significant difference. All statistical analyses were carried out using SPSS version 19.0 software (IBM Corp.).

## Results

**GPR56 expression in CRC cells and downregulation by siRNA.** GPR56 expression was confirmed in the CRC cell line (SW620) through both western blotting and RT-PCR (Figures 2A–2D). The knockdown of GPR56 mRNA expression by siRNA transfection in the SW620 cell line and inhibition of mRNA and protein expression was also confirmed by RT-PCR and western blotting. The results demonstrated that GPR56 mRNA expression was reduced by 28% in the siRNA-GPR56 group (p<0.005, Figures 2A, 2B) and GPR56 protein was reduced by 59% in the siRNA-GPR56 group (p<0.005, Figures 2C, 2D) compared with that in the untransfected control SW620 cells. However, there was no statically significant difference between the control and negative control groups. These results suggested that GPR56-siRNA successfully downregulated GPR56 expression.

**Downregulation of GPR56 decreased the proliferation ability of SW620 CRC cells.** The proliferation ability of the siRNA-GPR56-transfected SW620 cells was significantly lower than that of the control SW620 cells. The proliferation of the siRNA-GPR56-transfected SW620 cells was 53% (p<0.042) and 55% (p=0.001) after 48 and 72 h, respectively, compared with that of the control SW620 cells, according to the WST-1 assay (Figure 3A). There was no statistically significant difference between the negative control group compared with the control group. These results confirmed that high GPR56 expression increased the proliferative ability of SW620 cells by enhancing cell growth.

**Downregulation of GPR56 reduces cell migration and invasion and inhibits colony formation.** The present study analyzed the effects of GPR56 downregulation on the migration, invasion, and anchorage-independent colony formation of SW620 cells. Migration and invasion are functional indicators of cancer aggressiveness, and anchorage-independent colony formation is an indicator of cancer's transformation ability (Figures 3B–3G). In the migration assays, GPR56 downregulation in SW620 cells resulted in reduced cell migration (n=83.3; p<0.005) compared with that of the control SW620 cells (n=141.8; p<0.005) (Figures 3B, 3C). In the *in vitro* wound-healing assay, the downregulation of GPR56 expression inhibited cell migration (p<0.005) compared with that of the control cells (Figures 4A, 4B). Over time, it was observed that the cells in the control groups did not delay

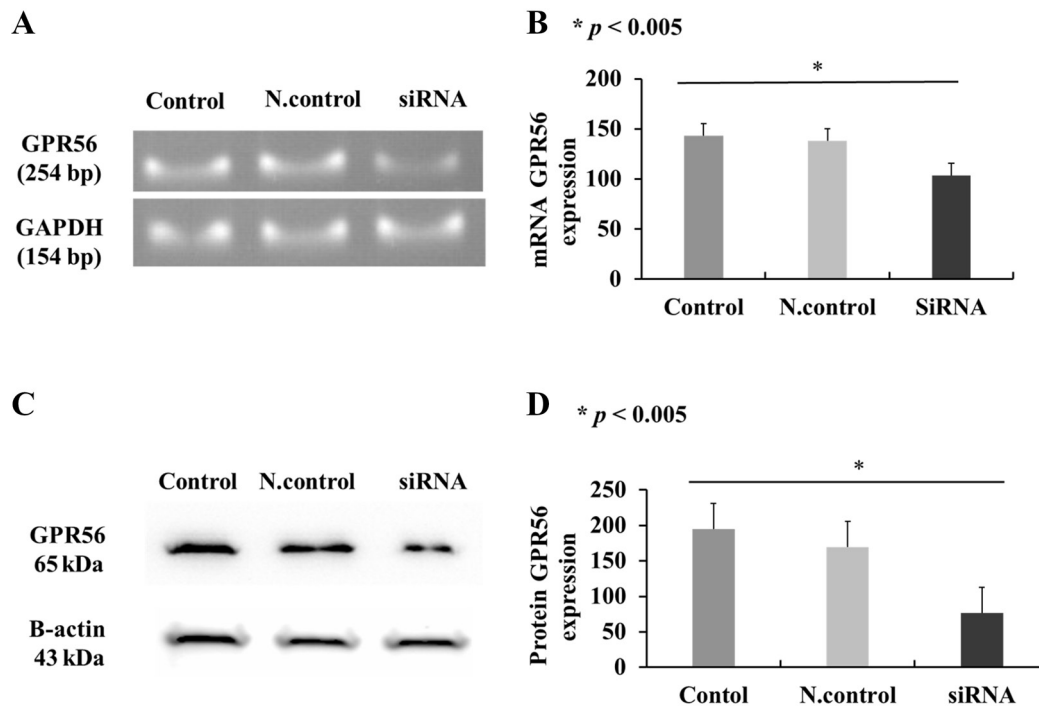


Figure 2. GPR56 mRNA and protein level was confirmed to be downregulated by GPR56 siRNA. A, B) According to western blotting, a 28% reduction was observed in the siRNA-GPR56 compared to control ( $p < 0.005$ ). C, D) According to RT-PCR, a 59% reduction was observed in the siRNA-GPR56 compared with the control group ( $p < 0.005$ ).

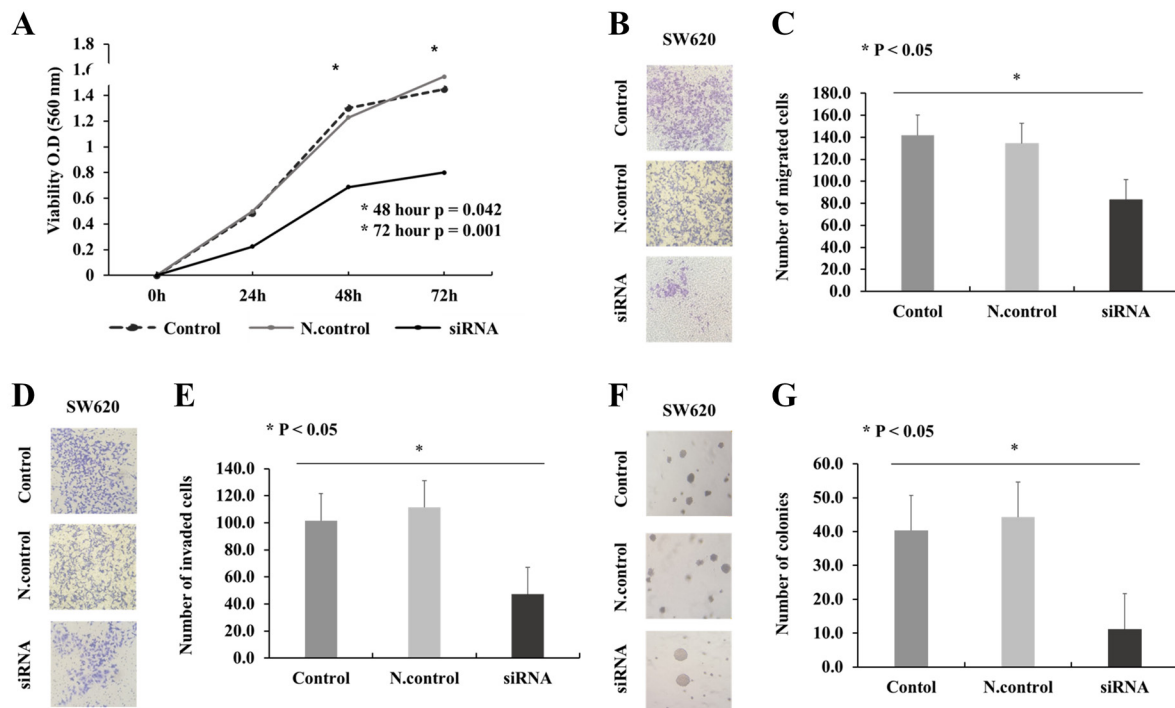


Figure 3. Effects of GPR56 downregulation on CRC cells. A) The downregulation of GPR56 expression in the CRC cell line SW620 reduced cell proliferation. The proliferation rate of SW620 cells was 53% ( $p < 0.042$ ) and 55% ( $p = 0.001$ ) after 48 and 72 h in the WST-1 assay. B, C) Downregulation of GPR56 in SW620 cells resulted in reduced cell migration ( $n = 83.3$ ,  $p < 0.005$ ) compared with the control SW620 cells ( $n = 141.8$ ). D, E) GPR56 downregulation reduced the invasion of SW620 cells ( $n = 47.3$ ,  $p < 0.05$ ) compared with the control cells ( $n = 101.6$ ). F, G) According to the semisolid agar assay, downregulation of GPR56 expression inhibited the colony-forming ability of GPR56 siRNA-transfected SW620 cells ( $n = 11.1$ ) compared with the control SW620 cells ( $n = 40.3$ ) ( $p < 0.005$ ).

migration. However, compared with the control group, the siRNA-GPR56-transfected group exhibited an  $\sim$ <2-fold delay in a migration after 12 h (cell-free area in the control group: 82.3  $\mu\text{m}$ ; cell-free area in the siRNA-GPR56 group: 189.7  $\mu\text{m}$ ) and 24 h (cell-free area in the control group: 70.3  $\mu\text{m}$ ; cell-free area in the siRNA-GPR56 group: 169.8  $\mu\text{m}$ ) ( $p < 0.05$  for both). In the Matrigel-coated Transwell assays to assess the invasion of SW620 cells, GPR56 downregulation reduced the invasion of SW620 cells ( $n = 47.3$ ,  $p < 0.05$ ) compared with that

of the control cells ( $n = 101.6$ ) (Figures 3D, 3E). In semisolid agar colony-forming assays, normal cells require a solid substratum to grow or proliferate, whereas cancer cells can grow regardless of their attachment status. In our assays, the number of anchorage-independent colonies was significantly lower in the siRNA-GPR56-transfected SW620 cells ( $n = 11.1$ ) compared with that in the control SW620 cells ( $n = 40.3$ ;  $p = 0.001$ ) (Figures 3F, 3G). In each of these experiments, there was no significant difference between the negative control group and the control group (Figures 3A–3G). These results demonstrated that GPR56 functioned as an oncogene by enhancing the migration, invasion, and anchorage-independent colony formation in SW620 cancer cells.

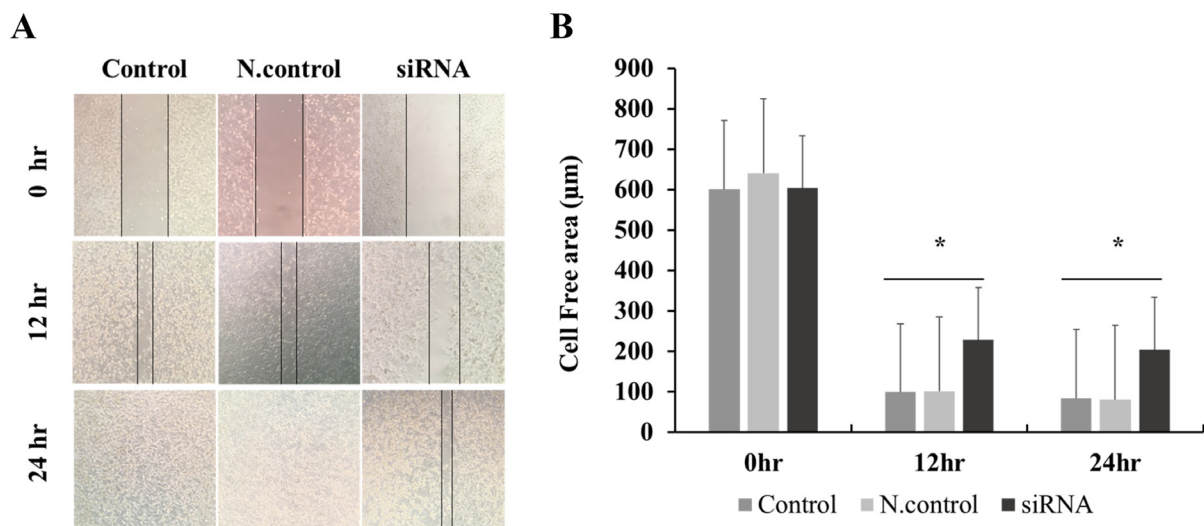
**GPR56 expression in CRC tissues.** IHC was used to assess GPR56 expression in CRC specimens. Overall, 109 specimens from patients with CRC were included. GPR56 was predominantly detected in the cytoplasm and cell membrane of the cancer cells (Figures 5A–5C). According to the GPR56 expression score, there were 78 specimens in the low-expression GPR56 group (78/109; 71.6%) and 31 specimens in the high-expression GPR56 group (31/109; 28.4%).

**Relationship between GPR56 expression and clinicopathological features of CRC.** As shown in Table 1, compared with low GPR56 expression, high GPR56 expression was significantly associated with sex ( $p = 0.002$ ), high pT stage ( $p = 0.001$ ), positive metastasis ( $p = 0.006$ ), and high overall stage ( $p = 0.001$ ) but not with age, pN stage, or lymphatic and vascular invasion. The high GPR56 expression group also demonstrated a tendency toward a higher T stage and overall TNM stage compared with the same parameters in the low GPR56 expression group. In patients with pT stages I/II and III/IV, high GPR56 expression was observed in 16.1% (5/31) and 83.9% (26/31), respectively ( $p = 0.001$ ). In

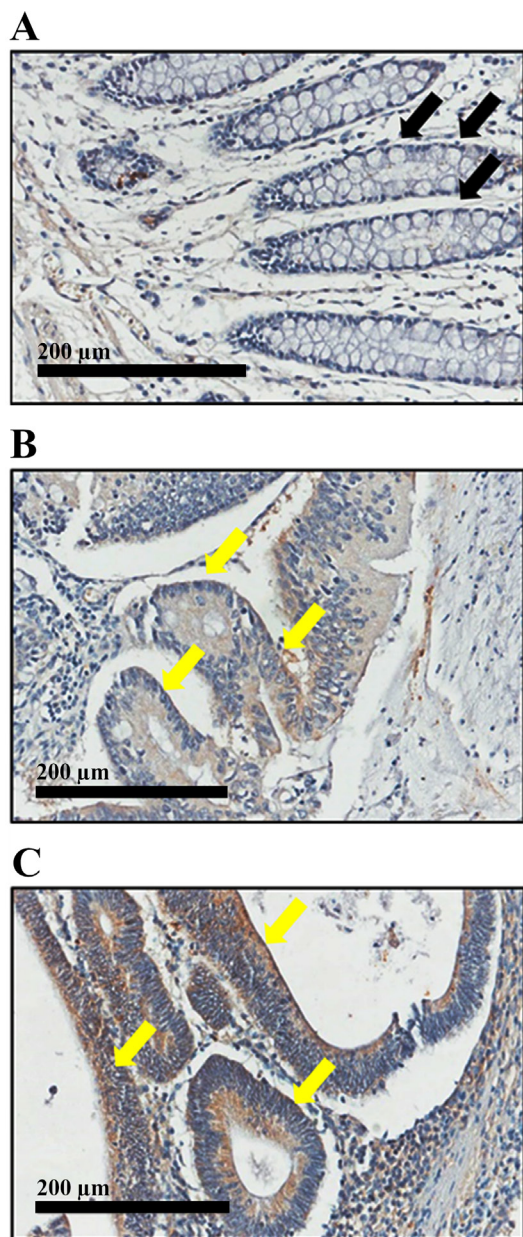
**Table 2. Univariate and multivariate Cox regression analysis of factors potentially influencing overall survival in patients with colorectal cancer.**

Clinicopathologic factors	Univariate analysis		Multivariate analysis	
	Hazard ratio (95%/CI)	p-value	Hazard ratio (95%/CI)	p-value
Age (<60 vs. $\geq$ 60)	1.216 (0.662–2.232)	0.529		
Gender (male vs. female)	1.065 (0.599–1.892)	0.830		
Stage (I/II vs. III/IV)	2.958 (1.448–6.044)	<b>0.003</b>	2.863 (1.400–5.857)	<b>0.004</b>
pT stage (T1/2 vs. T3/4)	0.766 (0.394–1.490)	0.431		
pN stage (0 vs. 1–2)	2.546 (1.294–5.011)	<b>0.007</b>	1.278 (0.362–4.511)	0.703
Metastasis (absent vs. present)	5.173 (2.124–12.60)	<b>&lt;0.001</b>	2.395 (0.820–6.995)	0.110
Vascular invasion (absent vs. present)	1.848 (0.838–4.064)	0.128		
Lymphatic invasion (absent vs. present)	1.859 (0.892–3.873)	0.089		
GPR56 expression (low vs. high)	2.413 (1.233–4.720)	<b>0.010</b>	2.305 (1.176–4.517)	<b>0.015</b>

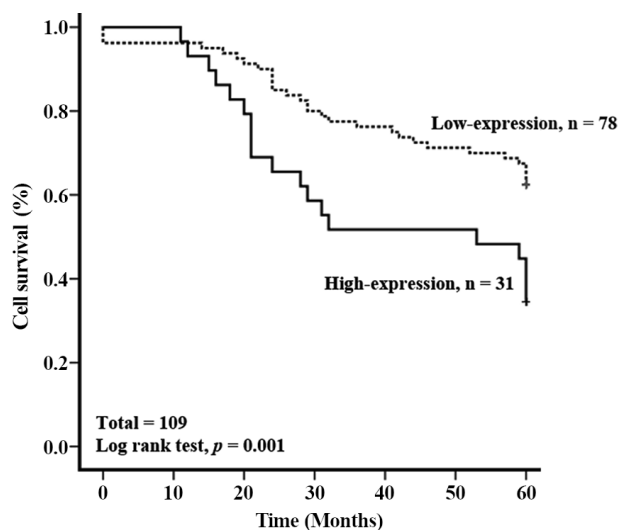
Abbreviation: CI—confidence index



**Figure 4. A, B) Downregulation of GPR56 expression in the *in vitro* wound-healing assay. Downregulation of GPR56 expression inhibited cell migration in the *in vitro* wound-healing assay. Cell-free areas at 12 h (siRNA-GPR56: 189.7  $\mu\text{m}$ , control: 82.3  $\mu\text{m}$ ) and 24 h (siRNA-GPR56: 169.8  $\mu\text{m}$ , control: 70.3  $\mu\text{m}$ ) were significantly different ( $p < 0.005$ ).**



**Figure 5.** GPR56 protein level was confirmed in colorectal cancer tissue by immunohistochemistry staining. GPR56 protein was mainly detected in the cytoplasm and membrane of colorectal tissue (brown color). A total of 109 CRC samples were stained with GPR56 antibody and graded based on both staining intensity and staining frequency. Arrows indicate normal and tumor cells (black, normal colon epithelial cells; yellow, tumor cells). **A)** Representative GPR56 expression in adjacent normal colorectal tissues. In the field, the score of staining intensity is 0 and the percentage score of GPR56 positive cells is 0. The score of 0 is thus defined as the GPR56 negative group. **B, C)** Defined as a positive group of GPR expression. **(B)** The total score is 2 and was thus defined as GPR56 low expression. Representative lower GPR56 staining in colorectal cancer tissues. In this field, the score of staining intensity is 1 and the percentage score of GPR56 positive cells is 2. **(C)** Representative higher GPR56 staining in colorectal cancer tissues. The score of staining intensity was 3 and the score of GPR56-positive cell percentage was 3. Therefore, the total score of 9 was defined as GPR56 high expression. Magnification:  $\times 100$ ; scale bar: 200  $\mu\text{m}$



**Figure 6.** Kaplan-Meier curve estimates of the association of GPR56 expression in the serum and the survival of patients with colorectal cancer.

patients with stages I, II, III, and IV, high GPR56 expression was observed in 6.5% (2/31), 35.5% (11/31), 38.7% (12/31), and 19.3% (6/31), respectively ( $p=0.001$ ).

**High GPR56 expression as a prognostic factor.** The overall TNM stage, N stage, distant metastasis, and GPR56 expression were poor prognostic indicators of the overall survival rate post-surgery according to the univariate analysis. Furthermore, the overall TNM stage and high GPR56 expression were poor prognostic indicators of the overall survival rate after surgery in the multivariate analysis. High GPR56 expression was an independent prognostic factor in the univariate Cox regression analysis [HR, 2.413, 95% confidence interval (CI), 1.233–4.720;  $p=0.010$ ] and multivariate Cox regression analysis (HR, 2.305; 95% CI, 1.176–4.517;  $p=0.015$ ) (Table 2). The 5-year overall survival rate was significantly lower in the high GPR56 expression group compared with that in the low GPR56 expression group (51.6 vs. 74.4%;  $p=0.008$ ) (Figure 6).

## Discussion

Because the GPR56-mediated signaling pathways and the adhesion of GPR56 are generally not well understood, the mechanisms of action of GPR56 in regulating carcinogenesis and tumorigenesis remain unclear. However, a number of studies have demonstrated that in relation to the mechanism of action of GPR56, GPR56 is involved in proliferation, migration, angiogenesis, cell adhesion, apoptosis, and cell cycle regulation [4, 14]. The present study demonstrated the effect of GPR56 on CRC progression, invasion, migration, and colony formation ability by various assays. The present study also found that the overexpression of GPR56 is related to tumoral progression by demonstrating that the downregu-

lation of GPR56 expression inhibited progression, invasion, migration, and colony-forming ability.

Recent mouse studies have shown that the overexpression of human progastrin stimulates GPR56 expression in colonic mucosa cells [15]. The progastrin may bind to GPR56 cell surfaces and increase the proliferation of CRC cells expressing GPR56 in the presence of progastrin [15]. Progastrin has been reported to be associated with the proliferation of colonic epithelial cells and the progression of colorectal cancer in mice [16]. Based on these studies, the relationship between progastrin and GPR56 is considered to be associated with colorectal tumorigenesis. GPR56 expression has been found in a variety of malignant tumors, and there is research demonstrating that it is associated with the prognosis of several types of cancer. One study reported that GPR56 expression is significantly associated with advanced FIGO stages ( $p=0.01$ ) and positive lymph node invasion ( $p=0.016$ ) in epithelial ovarian cancer and that it serves as an independent unfavorable prognostic marker of epithelial ovarian cancer [17]. Another study reported that GPR56 expression serves as an independent prognostic indicator of osteosarcomas and that GPR56-siRNA downregulates the expression of GTP-Rho A and Ki-6, both of which participate in cell migration and cell proliferation, as shown by western blot analysis [18]. In addition, the expression level of GPR56 in tumoral tissue is significantly correlated with the TNM stage of non-small cell lung cancer ( $p=0.005$ ), and GPR56 acts as an independent prognostic marker of overall survival in univariate and multivariate analyses [19]. Furthermore, GPR56 promoted the proliferation and invasion of non-small cell lung cancer in overexpression and knockdown experiments [20]. In other research, the transcript levels of GPR56 were increased in 48% of esophageal squamous cell carcinomas, whereas GPR56 was not expressed in adjacent non-malignant esophageal tissue [20]. However, only a few studies have investigated the relationship between GPR56 and CRC.

Recently, CRC has been extensively studied using molecular biology methods that are more sophisticated [21–23]. Numerous expression and genomic profiling studies have contributed to the current understanding of the various cellular mechanisms leading to the development of CRC tumoral formation, maintenance, and metastasis [24]. A recent study on cell surface markers for colon adenocarcinoma reported that GPR56 is expressed in CRC tissues but not in surrounding normal tissues [25]. In addition, GPR56 expression was found to be significantly correlated with the location of proximal CRC and with the expression of mismatch repair genes [25]. In another recent study, antibodies against PALM3, MFI, and GPR56 strongly stained CRC cells, whereas weak staining was observed in normal tissues by IHC, thereby confirming the upregulation of the protein [26].

However, these achievements of GPR56 have not provided direct information on the composition of the cell or its effect on CRC. In a recent study on the association of GPR56

with CRC, GPR56 expression was significantly associated with the TNM stage ( $p=0.017$ ), lymph node metastasis ( $p=0.009$ ), depth of invasion ( $p=0.023$ ), and distant metastasis ( $p=0.019$ ) [21]. In the same study, the overall survival rate of patients in a low GPR56 expression group was significantly improved compared with that of patients in a high GPR56 expression group ( $p=0.013$ ). In addition, high GPR56 expression was associated with the proliferation, migration, and invasion of CRC cells *in vitro* [27]. However, only a few studies have investigated the relationship between GPR56 and CRC. Therefore, the present study explored the expression patterns of GPR56 in CRC and demonstrated their correlation with clinicopathological characteristics, as well as their effects on tumoral cell proliferation, migration, invasion, and colony formation. The present study was conducted in two stages. The first stage involved the downregulation of GPR56 by siRNA transfection. The results of western blotting, RT-PCR, Transwell assays, wound-healing assays, and semisolid agar colony-forming assays revealed the effects of GPR56 expression on CRC cell proliferation, migration, invasion, and colony formation. In brief, the function of GPR56 in a CRC cell line was investigated through a knockdown experiment. In the present study, the results of the knockdown experiment demonstrated that high GPR56 expression increased the proliferation, migration, invasion, and colony-forming ability of CRC cells. In the second stage, the level of GPR56 was analyzed by IHC with colorectal adenocarcinoma tissue, and the association between GPR56 expression and the clinicopathological characteristics of CRC patients was evaluated. Univariate and multivariate analyses were conducted to examine the prognostic value of GPR56. The results demonstrated that the GPR56 expression level was significantly associated with the overall TNM stage ( $p=0.001$ ), T stage ( $p=0.001$ ), and distant metastasis ( $p=0.006$ ). The results of the multivariate analysis confirmed that high GPR56 expression alone was a poor prognostic indicator of the overall survival rate of patients with CRC. Patients with high GPR56 expression exhibited a poorer outcome compared with patients with low GPR56 expression. The present results demonstrate that GPR56 has the potential to be a clinical marker for the prognosis of patients with CRC. In addition, the present results suggest a possible role for GPR56 as a tumor suppressor gene when it is downregulated in the setting of metastasis in CRC. In fact, a previous study reported that the knockdown of GPR56 activates the apoptosis of CRC cells [27], and another study reported that the inactivation of GPR56 *in vivo* inhibits progastrin-induced colonic proliferation and increases colonic epithelial apoptosis [16].

The present study has several limitations, including its retrospective nature, significant selection biases, and small sample size. In addition, this study was conducted using cell lines only; therefore, *in vivo* studies are required to improve understanding of the role of GPR56 in the tumor microenvironment.



In conclusion, based on the results of the present study, high GPR56 expression is associated with tumor cell proliferation, migration, invasion, and colony formation in patients with CRC. The high GPR56 expression is a poor prognostic factor for patients with CRC. The expression level of GPR56 may play an important role in tumor progression of CRC and seems to have potential as a prognostic indicator of CRC in patients although further study is required to confirm this assertion. Therefore, further various studies on the association between GPR56 and CRC progression are required.

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## References

- [1] LUO R, JIN Z, DENG Y, STROKES N, PIAO X. Disease-associated mutations prevent GPR56-collagen III interaction. *PLoS One* 2012; 7: e29818. <https://doi.org/10.1371/journal.pone.0029818>
- [2] CASEY BJ, SOMERVILLE LH, GOTLIB IH, AYDUK O, FRANKLIN NT et al. Behavioral and neural correlates of delay of gratification 40 years later. *Proc Natl Acad Sci USA* 2011; 108: 14998–15003. <https://doi.org/10.1073/pnas.1108561108>
- [3] XU L, HYNES RO. GPR56 and TG2: possible roles in suppression of tumor growth by the microenvironment. *Cell Cycle* 2007; 6: 160–165. <https://doi.org/10.4161/cc.6.2.3760>
- [4] YANG L, CHEN G, MOHANTY S, SCOTT G, FAZAL F et al. GPR56 Regulates VEGF production and angiogenesis during melanoma progression. *Cancer Res* 2011; 71: 5558–5568. <https://doi.org/10.1158/0008-5472.CAN-10-4543>
- [5] LITTLE KD, HEMLER ME, STIPP CS. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-Galpa q/11 association. *Mol Biol Cell* 2004; 15: 2375–2387. <https://doi.org/10.1091/mbc.e03-12-0886>
- [6] Liu M, Parker RM, Darby K, Eyre HJ, Copeland NG et al. GPR56, a novel secretin-like human G-protein-coupled receptor gene. *Genomics* 1999; 55: 296–305. <https://doi.org/10.1006/geno.1998.5644>
- [7] ZENDMAN AJ, CORNELISSEN IM, WEIDLE UH, RUITER DJ, VAN MUIJEN GN et al. TM7XN1, a novel human EGF-TM7-like cDNA, detected with mRNA differential display using human melanoma cell lines with different metastatic potential. *FEBS Lett* 1999; 446: 292–298. [https://doi.org/10.1016/s0014-5793\(99\)00230-6](https://doi.org/10.1016/s0014-5793(99)00230-6)
- [8] XU L, BEGUM S, HEARN JD, HYNES RO. GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. *Proc Natl Acad Sci U S A* 2006; 103: 9023–9028. <https://doi.org/10.1073/pnas.0602681103>
- [9] SHASHIDHAR S, LORENTE G, NAGAVARAPU U, KUO J, CUMMINS J et al. GPR56 is a GPCR that is overexpressed in gliomas and functions in tumor cell adhesion. *Oncogene* 2005; 24: 1673–1682. <https://doi.org/10.1038/sj.onc.1208395>
- [10] KE N, SUNDARAM R, LIU G, CHIONIS J, FAN W et al. Orphan G protein-coupled receptor GPR56 plays a role in cell transformation and tumorigenesis involving the cell adhesion pathway. *Mol Cancer Ther* 2007; 6: 1840–1850. <https://doi.org/10.1158/1535-7163.MCT-07-0066>
- [11] BRENNER H, KLOOR M, POX CP. Colorectal cancer. *Lancet* 2014; 383: 1490–1502. [https://doi.org/10.1016/S0140-6736\(13\)61649-9](https://doi.org/10.1016/S0140-6736(13)61649-9)
- [12] SIEGEL RL, MILLER KD, JEMAL A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; 68: 7–30. <https://doi.org/10.3322/caac.21442>
- [13] NATIONAL CANCER CENTER (Eds.) Annual report of cancer statistics in Korea in 2013. National Cancer Center (South Korea) 2015. <http://ghdx.healthdata.org/record/south-korea-annual-report-cancer-statistics-korea-2013> [As accessed online on December, 2020]
- [14] CHIANG NY, PENG YM, JUANG HH, CHEN TC, PAN HL et al. GPR56/ADGRG1 Activation Promotes Melanoma Cell Migration via NTF Dissociation and CTF-Mediated Galpha12/13/RhoA Signaling. *J Invest Dermatol* 2017; 137: 727–736. <https://doi.org/10.1016/j.jid.2016.10.031>
- [15] WANG TC, KOH TJ, VARRO A, CAHILL RJ, DANGLER CA et al. Processing and proliferative effects of human progastrin in transgenic mice. *J Clin Invest* 1996; 98: 1918–1929. <https://doi.org/10.1172/JCI118993>
- [16] JIN G, SAKITANI K, WANG H, JIN Y, DUBEYKOVSKIY A et al. The G-protein coupled receptor 56, expressed in colonic stem and cancer cells, binds progastrin to promote proliferation and carcinogenesis. *Oncotarget* 2017; 8: 40606–40619. <https://doi.org/10.18632/oncotarget.16506>
- [17] LIU Z, HUANG Z, YANG W, LI Z, XING S et al. Expression of orphan GPR56 correlates with tumor progression in human epithelial ovarian cancer. *Neoplasma* 2017; 64: 32–39. [https://doi.org/10.4149/neo\\_2017\\_104](https://doi.org/10.4149/neo_2017_104)
- [18] CHEN Z, GAO P, LI Z. Expression of G Protein-coupled Receptor 56 Is an Unfavorable Prognostic Factor in Osteosarcoma Patients. *Tohoku J Exp Med* 2016; 239: 203–211. <https://doi.org/10.1620/tjem.239.203>
- [19] SONG Y, LI A, ZHANG L, DUAN L. Expression of G protein-coupled receptor 56 is associated with tumor progression in non-small-cell lung carcinoma patients. *Onco Targets Ther* 2016; 9: 4105–4112. <https://doi.org/10.2147/OTT.S106907>
- [20] SUD N, SHARMA R, RAY R, CHATTOPADHYAY TK, RALHAN R et al. Differential expression of G-protein coupled receptor 56 in human esophageal squamous cell carcinoma. *Cancer Lett* 2006; 233: 265–270. <https://doi.org/10.1016/j.canlet.2005.03.018>
- [21] MEHTA P, PIAO X. Adhesion G-protein coupled receptors and extracellular matrix proteins: Roles in myelination and glial cell development. *Dev Dyn* 2017; 246: 275–284. <https://doi.org/10.1002/dvdy.24473>

- [22] KISHORE A, HALL RA. Disease-associated extracellular loop mutations in the adhesion G protein-coupled receptor G1 (ADGRG1; GPR56) differentially regulate downstream signaling. *J Biol Chem* 2017; 292: 9711–9720. <https://doi.org/10.1074/jbc.M117.780551>
- [23] FEARON ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol* 2011; 6: 479–507. <https://doi.org/10.1146/annurev-pathol-011110-130235>
- [24] CARDOSO J, BOER J, MORREAU H, FODDE R. Expression and genomic profiling of colorectal cancer. *Biochim Biophys Acta* 2007; 1775: 103–137. <https://doi.org/10.1016/j.bbcan.2006.08.004>
- [25] SEWDA K, COPPOLA D, ENKEMANN S, YUE B, KIM J et al. Cell-surface markers for colon adenoma and adenocarcinoma. *Oncotarget* 2016; 7: 17773–17789. <https://doi.org/10.18632/oncotarget.7402>
- [26] WISNIEWSKI JR, OSTASIEWICZ P, DUS K, ZIELIŃSKA DF, GNAD F et al. Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma. *Mol Syst Biol* 2012; 8: 611. <https://doi.org/10.1038/msb.2012.44>
- [27] JI B, FENG Y, SUN Y, JI D, QIAN W et al. GPR56 promotes proliferation of colorectal cancer cells and enhances metastasis via epithelial-mesenchymal transition through PI3K/AKT signaling activation. *Oncol Rep* 2018; 40: 1885–1896. <https://doi.org/10.3892/or.2018.6582>