A study on the role of GPR56 in colorectal cancer progression and its protein expression in tissue on the impact of prognosis in colorectal cancer patient

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A study on the role of GPR56 in colorectal cancer progression and its protein expression in tissue on the impact of prognosis in colorectal cancer patient

Directed by Professor Nam Kyu Kim

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June 2019

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ABSTRACT

A study on the role of GPR56 in colorectal cancer progression and its protein expression in tissue on the impact of prognosis in colorectal cancer patient

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Purpose: G protein-coupled receptor 56 (GPR56) belongs to the adhesion G protein-coupled receptor subfamily, which plays a role in cell progression and survival. Its expression has been known to be correlated with the prognosis in various malignancies. The aim of this study was to investigate the role of GPR56 gene in cell line study and impact of prognosis of colorectal cancer (CRC) patients about its protein expression in CRC.

Material and Methods: The function of GPR56 in a CRC cell line was also explored through studies of downregulation of GPR56 by small interfering RNA (siRNA) transfection. We examined the expression of GPR56 in CRC cell lines by quantitative real time-PCR and western blot analysis. The effect of GPR56 on tumor cell proliferation (WST-1 assay), invasion (Transwell assay), migration (Trans well, 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 GRP56 was analyzed using univariate and multivariate analyses.

Results: The downregulation of GPR56 in the CRC cell line reduced cell proliferation as compared with that in a control sample. (48 h; 53%, $p = 0.042$, 72 h; 55%, $p = 0.001$). Downregulation of GPR56 expression reduced cell invasion and migration abilities and inhibited colony-forming abilities ($p < 0.005$). Among the 109 CRC samples, the expression level of GPR56 was low in 78 samples and high in 31 samples. 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.

Conclusion: The expression level of GPR56 plays an important role in tumoral progression in CRC, and it may serve as a prognostic indicator in CRC patients.

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

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I. INTRODUCTION

G protein-coupled receptors constitute a large family of membrane proteins. G protein-coupled receptor 56 (GPR56), which belongs to the adhesion G protein-coupled receptor subfamily, interacts with collagen III[1-2], transglutaminase 2[3], vascular endothelial growth factor[4], and CD81[5] and thus participates in cell survival, proliferation, adhesion, and migration. GPR56 is highly expressed in the brain, thyroid gland and heart and is moderately expressed in the kidneys, pancreas, small intestine, stomach, and colon[6]. Recently, reverse transcription polymerase chain reaction (RT-PCR), Northern blot analyses, and functional results revealed that GPR56 expression was inversely correlated with metastatic potential in melanoma

cell lines and that the expression level of GPR56 was downregulated in high metastatic cell lines as compared with that in low metastatic cell lines[7-8]. However, several other studies reported that high GPR56 expression appeared to be positively associated with tumorigenesis in gliomas and digestive cancers[9-10]. Therefore, the expression level and function of GPR56 may differ in different tumors. Very few studies have examined the association of GPR56 with gastrointestinal tumors, including colorectal cancer (CRC). CRC is one of the most common cancers worldwide[11]. In the U.S., CRC is the second leading cause of cancer-related deaths[12]. CRC is the third most common cancer in Korea as a whole[13]. It is the second most common cancer among men and the third most common cancer among women[13]. As noted above, the role of GPR56 may differ, depending on the type of cancer. No studies have investigated the function of GPR56 in CRC. The present study explored the expression pattern of GPR56 in CRC, its correlation with the clinicopathological characteristics of CRC patients, and its effect on cell proliferation, migration, and invasion in CRC.

II. MATERIALS AND METHODS

1. Cell culture

The present study has examined the expression level of GPR56 in several cell lines (SW620, SW480, HCT116, HT29), and the SW620 cell line has been selected

because it has the strongest expression of GPR56 (Figure 1.). The human CRC cell line SW620 was obtained from the Korean Cell Line Bank. The CRC cells were maintained in RPMI 1640 medium (Welgene, South Korea), supplemented with 10% Fetal Bovine Serum (Youngin Frontier, South Korea) and 1% penicillin-streptomycin (Hyclone^{TM}, Logan, Utah, USA). The cells were cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2).

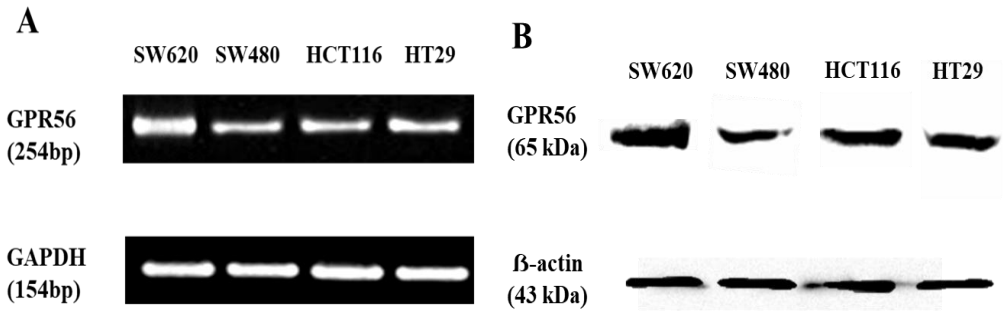


Figure 1. The expression of GPR56 mRNA and protein was analyzed in four CRC cell lines. The expression of GPR56 mRNA and protein was highest in SW620 cells (A: mRNA in RT-PCR, B: Protein in Western blot).

2. Downregulation of GPR56 by small-interfering RNA (siRNA) transfection and its effects on cell proliferation, migration, and invasion

Knockdown experiments were performed to elucidate the molecular mechanisms of GPR56 in regulating tumoral progression of colorectal carcinomas. The transfection

efficiencies of high expression of GPR56 and siRNA were confirmed by a Western blot and RT-PCR analysis. GPR56 expression in the CRC cell line was confirmed by a Western blot. Knockdown of GPR56 expression by siRNA transfection in the cell line was also confirmed by a Western blot. The effect of GPR56 downregulation on cell proliferation, migration, and invasion of the CRC cells was investigated.

3. siRNA transfection

siRNAs were mixed and transfected into cells using HiPerFect transfection reagent (Qiagen, Valencia, California, USA) in serum-free RPMI 1640 medium according to the manufacturer's protocol, with a final siRNA concentration of 100 nM. Total RNA and proteins from the control group and siRNA-GPR56 group were collected after transfection for 24 h. The sequence of GPR56 siRNA was as follows: 5′-GACACUGUUCCUGCUGAGU-3′. The transfection efficiency of GPR56 was confirmed by a Western blot and RT-PCR analysis.

The SW620 cells were seeded in six-well plates with 2×10^5 cells/well and cultured until reaching approximately 50–60% growth. The SW620 cells were then divided into two groups: a control group (transfection reagent only) and a siRNA-GPR56 group (transfected with siRNA-GPR56). Subsequently, the transfection efficiency was assessed using a Western blot and RT-PCR analysis.

4. Western blot

Cells lysates were prepared in Pro-Prep protein extraction solution (INtRON, Korea). Thirty micrograms of total protein for electrophoresis were loaded in each well of a 15% gel, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membranes (Millipore, Burlington, Massachusetts, USA). Subsequently, the membranes were incubated in 5% nonfat 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 incubated with secondary antibodies for 1 h at room temperature. The signal was detected using ECL solution (Advansta, Palo Alto, California USA) for 2 min and a molecular imaging system (ChemiDocXRS+; Bio-Rad Laboratories, USA). The following antibodies were used: anti-GPR56 (1:1000; Abcam), anti-β-actin (1:3000; Sigma, USA), antirabbit IgG (1:5000; Sigma), and antimouse IgG (1:5000; Sigma). Anti-β-actin antibodies were used as an internal control.

5. RT-PCR analysis

Total RNA was extracted from the cells and purified using a Hybrid-RTM RNA extraction kit (Geneall, Korea) according to the manufacturer's instructions. The total RNA quantity was assessed using a NanoDrop instrument (Thermo Scientific,

Waltham, Massachusetts, USA), and 500 ng/μL were applied to the PCR using a ReverTra Ace qPCR kit (Toyobo, Osaka, Japan).

6. Cell proliferation assay using WST-1 assays

Cell proliferation was determined using WST-1 assays and an EZ-Cytox kit (DogenBio, Korea). The control and siRNA-GPR56 transfected SW620 cells were plated in 96-well plates at 1×10^4 cells/well in 100 μL of RPMI 1640 medium containing 10% FBS and then cultured at 37°C in a humidified atmosphere containing 5% CO_2 overnight. After adding 10 μL of the WST-1 reagent to each well, the cells were incubated at 37°C for 4 h in a 5% CO_2 incubator. Subsequently, the absorbance of the supernatant at 450 nm was measured spectrophotometrically. Cell proliferation was measured 24, 48, and 72 h after seeding. Each experiment was repeated in triplicate.

7. Cell migration and invasion assays

Cell migration and invasion assays were carried out using a Transwell chamber kit (Coring Inc., Edison, New York, USA). The insert-chamber was separated by a polycarbonate membrane with 8-μm pores in 24-well plates. The SW620 cells were harvested by trypsinization and counted using a Scepter Handle Automated Cell Counter (Millipore, Burlington, Massachusetts, USA). Approximately 2.5×10^5 cells

suspended in 100 μL of serum-free medium were seeded in the upper chambers of the transwells, and 700 μL of 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_2 for 24 h (for migration assays) or 72 h (for invasion assays). After incubation, the transwell 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. Cell numbers were counted in five random fields under an inverted microscope at 200× magnification. For the invasion assays, the upper membranes of the chambers were coated with 50 μL of Matrigel (2.5 mg/mL; BD Bioscience, Billerica, Massachusetts, USA) before use.

8. 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% confluence. Wound healing cell migration assays were performed using a Culture insert system 24 (Ibidi, Munich, Germany). The cells were seeded into the well of the insert. This step was followed by incubation for 12–24 h with medium containing 10% FBS and 1% ABS. The cell-free gap was observed after 12 and 24 h. Images were captured using a phase contrast microscope (AxioCam Camer; Zeiss). The widths of the cell-free gap in five random visual fields were measured, and the average was determined.

9. 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. Subsequently, 2 × 10^3 cells were suspended in serum-free RPMI 1640 medium with 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_2 for 14 d. 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 measured using an inverted microscope at 100× magnification. The experiment was repeated in triplicate.

10. Patient characteristics and tissue specimens

In total, 109 patients diagnosed with primary colorectal adenocarinomas who underwent surgery between January 2009 and December 2013 were selected for inclusion in this retrospective analysis. Data on the following clinical parameters were collected: age, sex, pT stage, pN stage, metastasis, vascular invasion, lymphatic invasion, and 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 7th edition of the TNM classification of the American Joint Committee on Cancer. This study was approved by the ethics committee.

11. Immunohistochemistry (IHC) staining/expression of GPR56 in colorectal adenocarcinomas

IHC staining was performed to explore 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, de-waxed, and rehydrated using a graded series of ethanol, followed by microwave antigen retrieval. After blocking with 0.3% hydrogen peroxidase, the sections were incubated at 4°C overnight with GPR56 primary antibody (Bioss Antibodies, Woburn, Massachusetts, USA, 1:100). IHC staining was conducted using a DAB kit (Dako Denmark). The sections were then stained with hematoxylin, dehydrated, cleared, and mounted. As a negative control, 5% fetal bovine serum (FBS) was used. The samples were divided into two groups based on GPR56 expression: low expression (negative expression: an immunoreactivity score [IRS] < 2) and high expression (positive expression: an IRS ≥ 2) group. The IHC staining results revealed that GPR56 was highly expressed in some clinical colorectal adenocarcinoma samples, mainly in the cytoplasm and cell membrane. To determine the effect of GPR56 expression on clinical characteristics, the relationships between

GPR56 expression and clinicopathological factors were explored.

12. Semiquantitative analysis of IHC results

GPR56 immunoreactivity was observed in the cytoplasm and cell membrane. In the assessment of the IHC staining results, staining intensity was graded as score 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 negative expression (IRS < 2) or positive expression (IRS ≥ 2).

13. Univariate analysis and multivariate analysis

To evaluate the role of GPR56 expression in colorectal adenocarcinomas, the 5-year overall survival rate for the 109 CRC patients was determined using a Kaplan–Meier survival analysis. To further explore their independent predictive value, a multivariate Cox proportional hazards model was performed using all significant parameters detected by the univariate analysis.

14. Statistical analysis

The relationships between GPR56 expression and clinicopathological features were analyzed using the $\chi 2$ or Fisher's exact test, and continuous variables were analyzed using the Student's *t* test or Mann–Whitney *U* rank test. Overall survival curves were generated using the Kaplan–Meier method, and the differences between the high GPR56 expression and control group were examined using log-rank tests. Cox proportional-hazards regression analysis was applied to estimate univariate and multivariate hazard ratios (HRs) for overall survival rate. All *p*-values were two-sided, and *p* values of less than 0.05 were considered statistically significant. All statistical analyses were carried out using SPSS ver. 19.0 software (IBM Incorporation, Armonk, New York, USA).

III. RESULTS

1. GPR56 expression in CRC cells and downregulation by siRNA

GPR56 expression was confirmed in the CRC cell line (SW620) by both a Western blot assay and RT-PCR (Figure. 2A, B). Knockdown of GPR56 expression by siRNA transfection in the SW620 cell line was also confirmed by both the Western blot assay and RT-PCR, with a 28% reduction in GPR56 ($p < 0.001$) (Figure. 2A) and a 59% reduction in GRP56 protein ($p < 0.001$) (Figure. 2B), respectively, as compared with that in the untransfected control SW620 cells. These results suggested that GPR56-

siRNA successfully downregulated GPR56 expression.

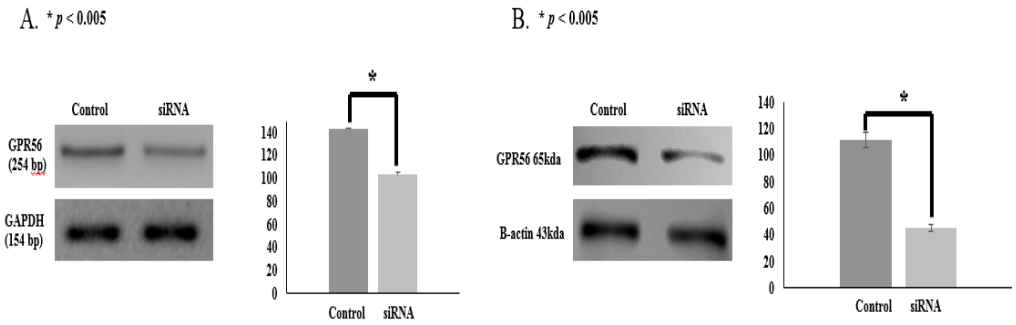


Figure 2. GPR56 mRNA and protein level was confirmed and was down regulated with GPR56 target siRNA. A) In Western blot assay, 28% reduction in siRNA-GPR56 compared control ($p < 0.005$). B) In RT-PCR, 59% reduction in siRNA-GPR56 compared control ($p < 0.005$).

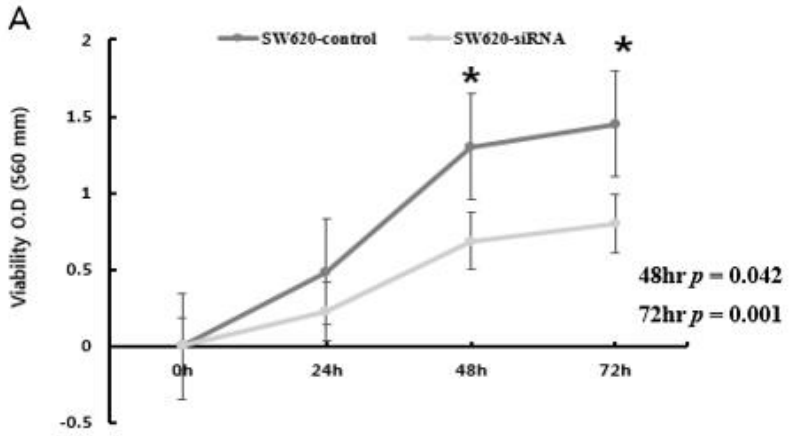
2. Downregulation of GPR56 decreased the proliferation ability of SW620 CRC cells

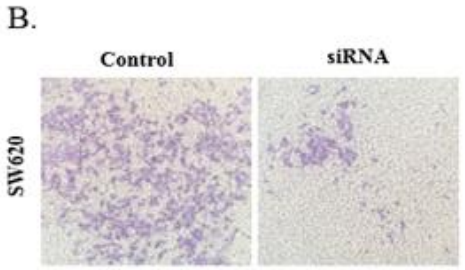
The proliferation ability of the siRNA-GPR56 transfected SW620 cells was significantly decreased as compared with that of the control SW620 cells. The proliferation of the siRNA-GPR56 transfected SW620 cells was 53% ($p < 0.042$) and 55% ($p = 0.001$) after 48 and 72 h, respectively, as compared with that of control SW620 cells in WST-1 assay (Figure. 3A.). These results confirmed that high GPR56 expression increased the proliferative ability of SW620 cells by enhancing cell growth.

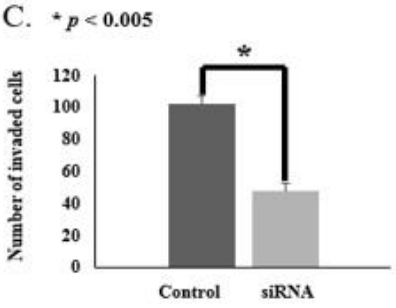
3. Downregulation of GPR56 reduced cell migration and invasion and inhibited colony formation

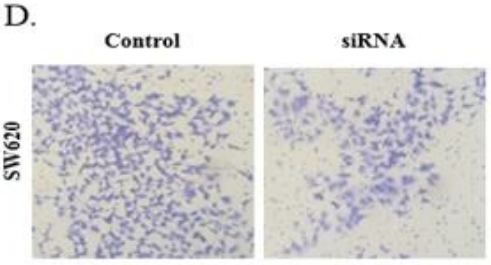
The present study analyzed the effects of GPR56 downregulation on the migration, invasion, and anchorage-independent colony formation of SW620 cells. Migration and invasion are functional indicators of cancer aggressiveness, and anchorage-independent colony formation is an indicator of cancer transformation ability (Figure. 3B–G.). In the migration assays, GPR56 downregulation in SW620 cells resulted in reduced cell migration (n = 83.3, $p < 0.005$) as compared with that of the control SW620 cells (n = 141.8) (Figure. 3D, E). In the in vitro wound healing assay, downregulation of GPR56 expression inhibited cell migration ($p < 0.005$) as compared with that of the control cells (Figure. 4A, B). Over time, the cells in the control groups was no delay migration. However, as compared with the control group, the siRNA-GPR56 transfected group displayed an approximately < 2-fold delay in migration after 12 h (cell-free area in the control group: 82.3 μm; cell-free area in the siRNA-GPR56 group: 189.7 μm) and 24 h (cell-free area in the control group: 70.3 μm; cell-free area in the siRNA-GPR56 group: 169.8 μm) ($p < 0.05$ for both). In the Matrigel-coated transwell assays of the invasion of SW620 cells, GPR56 downregulation reduced the invasion of SW620 cells (n = 47.3, $p < 0.05$) as compared with that of the control cells (n = 101.6) (Figure. 3B, C). In semisolid agar colony-forming assays, normal cells require a solid substratum on which to grow or

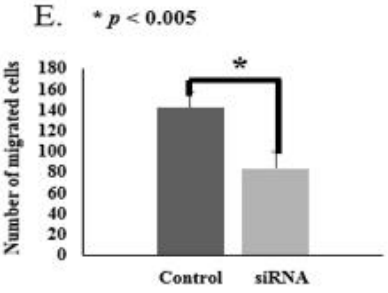
proliferate, whereas cancer cells have the ability to grow, regardless of attachment status. The number of anchorage-independent colonies was significantly lower in the siRNA-GPR56 transfected SW620 cells (n = 11.1) as compared with that in the control SW620 cells (n = 40.3) (p = 0.001) (Figure. 3F, G.). These results showed that GPR56 functioned as an oncogene by enhancing migration, invasion, and anchorage-independent colony formation in SW620 cancer cells.

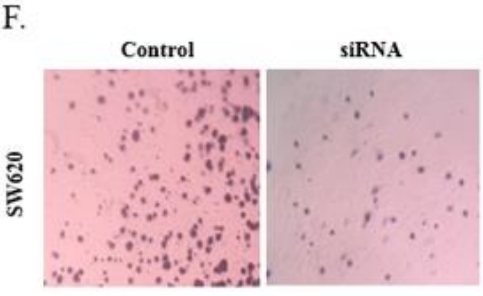












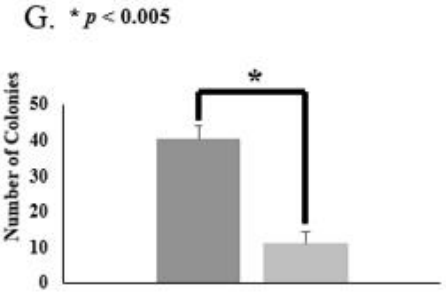


Figure 3. The results of ability of down regulation of GPR 56 expression. A) The down regulation of GPR 56 expression in colorectal cancer cell line SW620 was reduced cell proliferation. SW620 cells was 53% ($p < 0.042$) and 55% ($p = 0.001$) after 48 and 72h in WST-1 assay. B & C) Down regulation of GPR56 expression in invasion assay. GPR56 downregulation reduced the invasion of SW620 cells (n = 47.3, $p < 0.05$) as compared with that of the control cells (n = 101.6). D & E) Down regulation of GPR56 expression in migration assay. GPR56 downregulation in SW620 cells resulted in reduced cell migration (n = 83.3, $p < 0.005$) as compared with that of the control SW620 cells (n = 141.8). F & G) Down regulation of GPR56 expression in semi solid agar assay. Down regulation of GPR56 expression inhibited colorectal cancer cell line SW620 colony forming ability. siRNA-GPR56 transfected SW620 cells (n = 11.1) as compared with that in the control SW620 cells (n = 40.3) ($p < 0.005$).

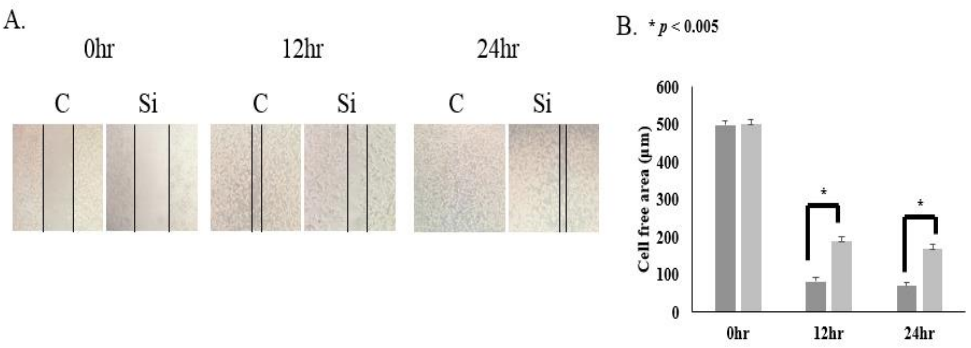


Figure 4. A & B) Down regulation of GPR56 expression in vitro wound healing assay. Down regulation of GPR56 expression inhibited cell migration on in vitro wound healing assay. Cell free area: 12h (siRNA-GPR56: 189.7 μm, control: 82.3 μm), 24h (siRNA-GPR56: 169.8 μm, control: 70.3 μm), $p<0.005$.

4. GPR56 expression in CRC tissues

IHC was used to assess GPR56 expression in the 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 (Figure. 5.). 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 GRP56 group (31/109; 28.4%).

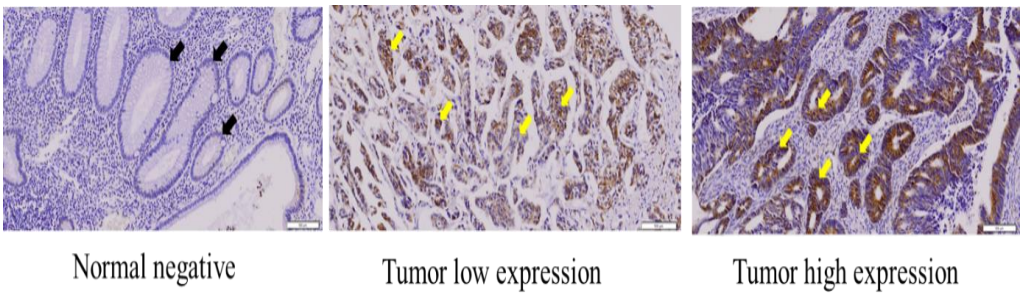


Figure 5. GPR56 protein level was confirmed in colorectal cancer tissue by immunohistochemistry stain. GPR56 protein was mainly detected in the cytoplasm and membrane of colorectal tissue (brown color). Arrow indicate normal and tumor cells (black: normal colon epithelial cells, yellow: tumor cells)

5. The relationship between GPR56 expression and clinicopathological features of CRC

As shown in Table 1, as 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 showed a tendency toward a higher T stage and overall TNM stage as compared with the same parameters in the low GPR56 expression group. In patients with pT stage I/II and III/IV, high GPR56 expression was observed in 16.1% (5/31) and 83.9% (26/31) of patients, respectively ($p = 0.001$). In patients with stage 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) of patients, respectively ($p = 0.001$).

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

Clinicopathological factors	GPR56 Expression		Total	*p* value
	Low (N=78)	High (N=31)	(N=109)	
Age, years, mean (SD)	63.2±12.5	61.7±13.5	62.4±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	

6. 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 after surgery according to the univariate analysis. The overall TNM stage and high GPR56 expression were also poor prognostic indicators of the overall survival rate after surgery in the multivariate analysis. High GPR5 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-y overall survival rate was significantly worse in the high GPR56 expression group as compared with that in the low GPR56 expression group (51.6% vs. 74.4%, p = 0.008) (Figure. 6).

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. ≥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)	**0.003**	2.863 (1.400 - 5.857)	**0.004**
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)	**0.007**	1.278 (0.362 - 4.511)	0.703
Metastasis (absent vs. present)	5.173 (2.124 - 12.60)	**<0.001**	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)	**0.010**	2.305 (1.176 - 4.517)	**0.015**

CI: confident index

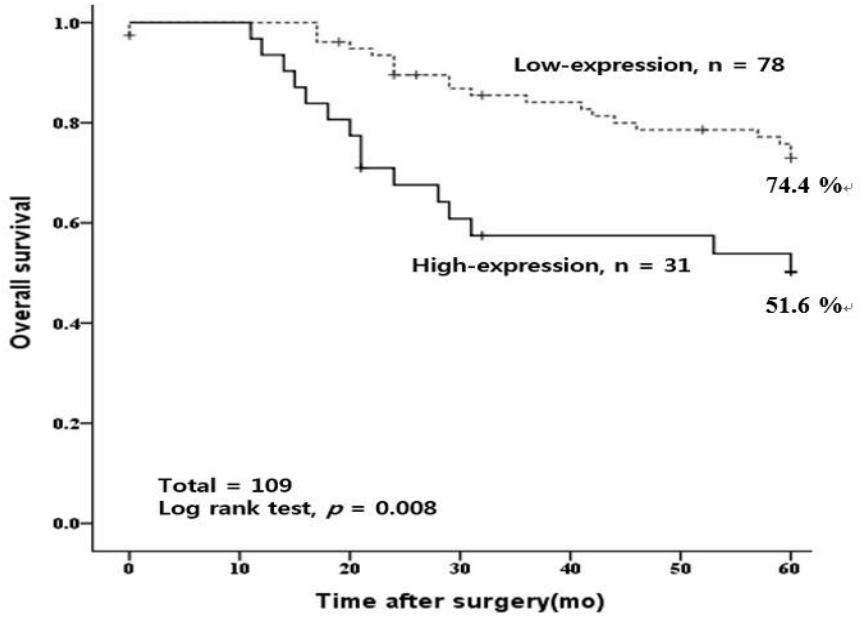


Figure 6. The Kaplan-Meier curve estimates the association of GPR56 and the survival in serum of colorectal cancer patients.

IV. DISCUSSION

The process of carcinogenesis of GPR 56 and mechanism of action of GPR56 in regulating tumorigenesis is not clear because of GPR56 mediated signal transduction pathways and adhesion GPR 56 are generally poorly understood. However, many studies demonstrated that in terms of its mechanism of action, GPR 56 has been involved in proliferation, migration, angiogenesis, cell adhesion, apoptosis, and cell cycle regulation[14-15]. Recent study showed that overexpression of human progastrin in mice stimulated GPR56 expression in colonic mucosa cells and progastrin was able

to bind to cell surface GPR56 and the proliferation of GPR56 expressing colorectal cancer cells was increased in the presence of progastrin[16]. Progastrin has been known to enhance the proliferation of colonic epithelial cells and the progression of colorectal cancer in mice[17]. In vivo, progastrin dependent colonic proliferation was inhibited by the loss of GPR56 and deficiency of GPR56 also inhibited progastrin dependent colonic crypt fission and colorectal carcinogenesis in a mouse model[17]. Base on this study, the progastrin-GPR56 axis is thought as an important promoter of colorectal tumorigenesis. GPR56 expression has been detected in various malignancies, with research reporting that it is associated with the prognosis in several types of cancer. One study reported that GPR56 expression was significantly associated with advanced FIGO stage ($p = 0.01$) and positive lymph node invasion ($p = 0.016$) in epithelial ovarian cancer and that it served as an independent unfavorable prognostic marker in epithelial ovarian cancer[18]. Another study reported that GPR56 expression served as an independent prognostic indicator of osteosarcomas and that GPR56-siRNA downregulated the expression of GTP-Rho A and Ki-6, both of which participate in cell migration and cell proliferation, as shown by a Western blot[19]. In addition, the expression level of GPR56 in tumoral tissue was significantly correlated with the TNM stage in non-small cell lung cancer ($p = 0.005$), and GPR56 acted as an independent prognostic marker of overall survival in univariate and multivariate analyses[20]. Furthermore, GPR56 promoted the proliferation and invasion of non-small cell lung cancer in overexpression and knockdown experiments[20]. In other research, transcript levels of GPR56 were increased in 48% of esophageal squamous

cell carcinomas, whereas GPR56 was not expressed in adjacent non-malignant esophageal tissue[21].

Recently, CRC has been comprehensively studied using ever more refined molecular biology methods[22-24]. A large number of expression and genomic profiling studies have contributed to current understanding of the different cellular mechanisms underlying CRC tumoral formation, maintenance, and the development of metastasis[25]. GPR56 expression was significantly correlated with the location of proximal CRC and with the expression of mismatch repair genes[26]. In addition, GPR56 was expressed in CRC tissue but not in surrounding normal tissues[26]. A recent study, which included a proteomic analysis of microdissected material from formalin-fixed and paraffin embedded CRC, proteins that are significantly upregulated in CRC samples can be considered potential biomarkers. In IHC, antibodies against PALM3, MFI, and GPR56 strongly stained CRC cells, whereas only weak staining was observed in normal tissue, thus validating upregulation of the proteins[27].

However, these achievements of GPR56 did not provide direct information on the composition of the cell and effect for CRC. In a recent study on the association of GPR56 with CRC, of 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)[24]. In the same study, the overall survival rate of patients in a low GPR56 expression group was significantly better than that of patients in a high GRP56 expression group (p = 0.013). In addition, high GPR56 expression was associated with proliferation, migration, and invasion of CRC cells in

vitro[28]. However, there are still few studies have investigated the relationship between GPR56 and CRC. Therefore, the present study explored the expression pattern of GPR56 in CRC and demonstrated its correlation with clinicopathological characteristics, as well as its effect on tumoral cell proliferation, migration, invasion, and colony formation. The present study was conducted in two stages. The first stage involved downregulation of GPR56 by siRNA transfection. The results of a Western blot, RT-PCR analysis, transwell assays, wound healing assays, and semisolid agar colony-forming assays confirmed the effect of GPR56 expression on CRC cell proliferation, migration, invasion, and colony formation. Briefly, the function of GPR56 in a CRC cell line was explored through a knockdown experiment. In present study, the results of the knockdown experiment showed that high GPR56 expression increased the proliferative, migration, invasion ability, and colony forming ability in CRC. In the second stage, we analyzed the level of GPR56 by IHC in colorectal adenocarcinoma tissue and the association between GPR56 expression and the clinicopathological characteristics of CRC patients. We conducted univariate and multivariate analyses to examine the prognostic value of GPR56. The results revealed a significant association between the GPR56 expression level and 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 in CRC. In present study, high GPR56 expression exhibited a poorer outcome compared to those with low GPR56 expression. The present study results demonstrated that GPR56 has the potential to

be a clinical marker for prognosis in CRC. In addition, present study results suggest that the possible role for GPR56 as a tumor suppressor gene as it is downregulated in the setting of metastasis in CRC. In fact, previous study reported that knockdown of GPR56 activated the apoptosis of CRC cells[28] and other study reported that inactivation of GPR56 in vivo inhibited progastrin-induced colonic proliferation, and increased colonic epithelial apoptosis[17].

The present study has several limitations, including its retrospective nature, significant selection biases, and small sample size.

V. CONCLUSION

In conclusion, based on the results of the present study, high GPR56 expression was associated with tumoral proliferation, migration, invasion, and colony formation in CRC patients. The high GPR56 expression was a poor prognostic factor in CRC patients. The expression level of GPR56 plays an important role in tumoral progression in CRC, and it may serve as a prognostic indicator in CRC patients. Further various studies of the association between GPR56 and CRC progression are required.

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ABSTRACT(IN KOREAN)

GPR56발현과 대장암의 암화과정 연관성 및 대장암 환자의 임상적 예후의 상관성에 관한 연구

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임대로

목적: G 단백질-결합 수용체 56 (GPR56)은 세포의 성장 및 생존에 중요한 역할을 하는 부착성 G 단백질-결합 수용체의 아과에 속한다. 그 발현은 다양한 악성 종양의 예후와 상관 관계가 있는 것으로 알려져 있다. 이 연구의 목적은 세포주 연구에서 GPR56 유전자의 역할과 대장암에서의 발현이 대장암 환자의 예후에 미치는 영향을 조사하고자 한다.

재료 및 방법: 대장암 세포주에서 GPR56의 기능은 small interfering RNA (siRNA) 형질 감염에 의한 GRP56 발현의 하향 조절을 통해 연구가 진행되었다. 우리는 양적 실시간 PCR과 western blot 분석에 의해 대장암 세포주에서 GPR56의 발현을 조사하였다. 종양 세포 증식 (WST-1 분석), 침윤 (Transwell 분석), 이동 (Transwell, 상처 치유분석) 및 콜로니 형성 능력 (반고체 한천 콜로니 형성 분석)에 대한 GPR56의 영향을 조사하였다. 109명의 대장암 환자의 조직 샘플에서 GPR56의 발현 수준을 면역 조직 화학법으로 평가 하였다. GPR56의 예후적 가치는 단변량 및 다변량 분석을 사용하여 분석하였다.

결과: 대장암 세포주에서 GPR56의 하향 조절은 대조군 샘플과 비교하였을 때 세포의 증식을 감소시켰다 (48시간; 53%, p = 0.042, 72시간; 55%, p = 0.001). GPR56 발현의 하향 조절 군에서 세포 침윤 및 이동 능력이 감소

되었고 콜로니 형성 능력이 억제되었다. ($p < 0.005$). 109명의 대장암 샘플 중 GPR56의 발현 수준은 78명에서 낮은 발현을 보였고 31명에서 높은 발현을 보였다. 5년 전체 생존율은 낮은 발현 군에 비해 높은 발현 군에서 더 나빴다 (51.6% 대 74.4%, $p = 0.008$). 높은 GPR56 발현은 단변량 ($p = 0.001$) 및 다변량 ($p < 0.001$) 분석에서 대장암 환자의 전반적인 생존율에 영향을 미치는 중요한 예후 인자로 분석 되었다.

결론: 본 연구결과를 토대로 GPR56 의 발현 수준은 대장암의 종양 진행에 중요한 역할을 하며, 대장암 환자의 예후 지표로 사용 될 가능성이 있다.

핵심되는 말: G 단백질-결합 수용체 56, 대장암, 암화과정