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Expression study of the metastasis suppressor gene CD82 by DNA methylation in gastric cancer

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글로벌바이오융합학과

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위암에서 DNA 메틸화에 의한 전이 억제 유전자 CD82의 발현 연구

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위암은 전 세계적으로 높은 발병률과 사망률을 보이는 암종이다. 대부분이 아시아에서 발생하며 그중에서도 동아시아에 특이적으로 발생한다. 위암은 전이가 발생하면 생존율이 급격하게 감소한다. 전이는 암의 특성 중의 하나로 원발 암 부위에서부터 멀리 떨어진 기관으로 이동하는 것을 말한다. 전이 억제 유전자 중의 하나이며 tetraspanin super family에 속하는 CD82는 KAI1 이라고 하는 단백질로도 알려져 있다. 여러 암종에서 CD82가 전이 억제 유전자로써 작용하지만, CD82는 암의 진행 시 대부분 하향조절 되어있으며 정확한 원인은 밝혀져 있지 않다. 따라서 이번 연구에서는 위암에서의 CD82의 역할에 대해 검증하고 CD82를 하향조절 하는 후생유전학적 조절에대한 기전에 대하여 알아보고자 한다.

CD82 발현이 낮은 환자군은 높은 환자군에 비하여 좋지 않은 예후를 보였으며 위암 환자에서 전이의 진행 시 CD82의 발현이 감소한 것을 확인하였다. 실제 위암에서 하향조절 되어있는지 확인하기 위해 열 가지의 위암 세포주와 한 가지의 정상위 상피 세포주에서 CD82의 발현을 확인하였을 때 대부분의 위암 세포주에서 낮은 발현을 보였다. CD82가 실제로 전이 억제 유전자로서 역할을 하는지 알아보기 위해 CD82 특이적인 작은 간섭 RNA와 과발현 플라스미드 벡터를 이용하여 위암 세포주에서 CD82의 발현을 조절하였다. CD82 발현의 감소는 위암

세포의 침습과 이동을 증가시켰으며, CD82의 과발현은 반대로 침습과 이동을 감소시켰다. 추가로 CD82의 발현조절은 암세포의 전이에 중요한 역할을 하는 상피-중간엽 전이 관련 유전자들의 변화에 영향을 미쳤다. 이를 통해 위암에서 CD82가 전이에 영향을 미치는 것을 확인하였다. CD82의 발현이 하향되어있는 요인으로써 DNA methylation을 예상하였고 탈메틸화제 5-AZA-2′-deoxycytidine 처리 시 CD82의 발현이 증가하는 것을 확인하였으며 메틸 특이적 PCR을 통해 실제로 CD82가 과 메틸화되어있음을 확인하였다. 또한 탈메틸화제 처리로 인한 CD82 발현의 증가는 세포의 이동성을 감소시켰다.

이 연구는 CD82가 위암에서 세포의 이동성을 감소시킴으로써 전이 억제 유전자로써의 기능을 하며, 위암의 진행 중 DNA methylation 에 의해 CD82의 발현이 조절될 수 있다는 것을 보여준다. 이러한 결과는 위암의 진단에 표적 유전자로써 CD82의 가능성을 제시하며 CD82의 후성유전학적 조절에 대한 기전을 밝히는 데에 도움을 줄 것이라고 예상한다.

ABSTRACT

Expression study of the metastasis suppressor gene CD82 by DNA methylation in gastric cancer

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Gastric cancer is a type of cancer with a high incidence and mortality worldwide. Most of them occur in Asia, especially in East Asia. When gastric cancer metastasizes, the survival rate decreases drastically. Metastasis is one of the characteristics of cancer and refers to the movement from the primary cancer site to distant organs. CD82, one of the metastasis suppressor genes and belonging to the tetraspanin super family, is also known as a protein called KAI1. Although CD82 acts as a metastasis suppressor gene in many cancer types, CD82 is mostly down-regulated during cancer progression, and the exact cause is not known. Therefore, in this study, Iaimed to verify the role of CD82 in gastric cancer and investigate the mechanism of epigenetic modificationthat downregulates CD82.

The patient group with low CD82 expression showed a poorer prognosis than the patient group with high CD82 expression, and it was confirmed that CD82 expression decreased during metastasis in gastric cancer patients. When the expression of CD82 was checked in ten gastric cancer cell lines and one normal gastric epithelial cell line to confirm whether it was actually down-regulated in gastric cancer, CD82 expression was found to be down-regulated in most gastric cancer cell lines. To determine whether

CD82 actually acts as a metastasis suppressor gene, CD82 expression was controlled in gastric cancer cell lines using CD82-specific small interfering RNA and an over-expression plasmid vector. Decreased CD82 expression increased invasion and migration of gastric cancer cells, while over-expression of CD82 decreased invasion and migration. Additionally, regulating the expression of CD82 affected the changes in epithelial-mesenchymal transition related genes, which play an important role in the metastasis of cancer cells. Through this, it was confirmed that CD82 affects metastasis in gastric cancer. DNA methylation was expected to be a factor in the down-regulation of CD82 expression, and it was confirmed that CD82 expression increased upon treatment with a demethylating agent 5-AZA-2′-deoxycytidine. Through methyl-specific PCR, it was confirmed that CD82 was actually hypermethylated. DNA methylation was expected to be a factor in the down-regulation of CD82 expression, and it was confirmed that CD82 expression increased upon treatment with the demethylating agent 5-AZA. Through methyl-specific PCR, it was confirmed that CD82 was actually hypermethylated. Additionally, CD82, whose expression increased due to demethylating agent treatment, similarly reduced migration.

This study shows that CD82 functions as a metastasis suppressor gene by reducing cell mobility in gastric cancer, and that the expression of CD82 can be regulated by DNA methylation during the progression of gastric cancer. These results suggest the possibility of CD82 as a target gene in the diagnosis of gastric cancer and are expected to help reveal the epigenetic mechanism of CD82.

I. INTRODUCTION

Gastric cancer is a cancer that ranks 5th in incidence and 4th in mortality worldwide. The incidence of gastric cancer is high in Asia, and it is a cancer type with a particularly high incidence in East Asia [1]. According to Korean statistics in 2020, cancer is the number one cause of death in Korea, and among them, gastric cancer has the fourth highest incidence and ranks fourth in mortality. Gastric cancer has a 5-year survival rate of over 95% when diagnosed at a local stage in both men and women, but when metastasis occurs, the survival rate decreases dramatically [2]. This shows that most deaths from cancer are caused by metastatic tumors, not primary tumors, and that metastasis is a very dangerous event in cancer.

Metastasis is known as one of the hallmarks of cancer, including inflammation, cell death resistance, and angiogenesis [3]. Metastasis refers to the growth of cancer cells in an organ distant from the organ where the cancer cells originated. Metastasis occurs through the process of cancer cells invading tissues, entering blood vessels, moving, spreading to other tissues, and colonizing. In the early stages of metastasis, a program called Epithelial-Mesenchymal Transition (EMT) is experienced [4]. EMT refers to an epithelial cell losing its polarity and adhesion and changing into a mesenchymal cell with the ability to migrate and invade [5]. Therefore, I wanted to study this by targeting tetraspanin, which regulates cell adhesion and mobility.

Tetraspanin is a protein with four transmembrane domains and extra cellular loops of different lengths [6, 7]. It is a membrane protein that regulates cell adhesion and mobility, cell signaling, protein transport, cell proliferation, neuro transmission, and immune activation [8]. Tetraspanins form tetraspanin-enriched micro domains (TEMs) through interactions with different tetraspanins or proteins, and depending on the composition of the TEM, they regulate the activity of cell surface signaling molecules and regulate several functions [9]. It is known that there are 33 tetraspanin in mammals, and many tetraspanins, including TSPAN1, TSPAN8, CD9, CD63, and CD82, are associated with cancer [10]. Among these, CD82 is known to be a tumor metastasis

suppressor gene [7].

Tumor metastasis suppressor genes are genes that negatively regulate metastasis without affecting the growth of the primary tumor. Tumor suppressor genes have several pathways depending on their mode of action, including the MAPK pathway, adhesion proteins, cytoskeletal signaling, and apoptotic pathway. Among them, CD82 is a pathway driven by adhesion protein [11]. Adhesion protein is a protein that regulates metastasis by regulating cell-cell and cell-extracellular matrix interactions. CD82's role as a metastasis suppressor gene was revealed in studies using rat prostate cancer cells [12]. Studies have shown that CD82 expression suppresses metastasis in several cancers. However, CD82 is down-regulated in many different cancers [13-17].

Gene repression occurs due to genetic modification or epigenetic changes, but it is known that down-regulation of CD82 rarely occurs due to genetic mutation [18]. Therefore, it was predicted that CD82 suppression would occur due to epigenetic changes rather than genetic modification. Among epigenetic modification processes including histone modification, chromatin remodeling, non-coding RNAs, and DNA methylation, DNA methylation is known to play an important role in cancer progression and metastasis [19]. DNA methylation causes excessive activation of some genes by hypomethylation or inactivation of genes by hypermethylation [20].

Currently, not much research has been conducted on CD82 in gastric cancer, and only miRNA is known to play a role in regulating CD82 [21]. Therefore, in this study, I first sought to determine the effect of CD82 on metastasis in gastric cancer and to determine whether the expression of CD82 is regulated by DNA methylation.

II. MATERIALS AND METHODS

1. Cell line and cell culture

Human gastric cancer cell lines (AGS, MKN-28, MKN-74, NCI-N87, KATO-III, SNU216, SNU601, SNU638, and SNU668) were purchased from the Korea Cellular Bank, and human gastric epithelial cell line GES-1 and gastric cancer cell line YCC-2 were obtained from Yonsei Cancer Center. All cell lines were cultured in a 37°C incubator supplied with 5% CO2 with RPMI-1640 (Welgene, Gyengsan, Korea) medium containing 10% fetal bovine serum (FBS) (Corning costar, Cambridge, MA, USA) and 1% Antibiotic-antimycotic (Gibco, Carlsbad, CA, USA).

2. Transfection of siRNA and construction

CD82 cDNA was amplified through CD82 primers with HindIII and XhoI restriction enzyme sites. Human cDNA was isolated from SNU-216 cells. The human CD82 construct was cloned into pCMV_3Tag_1A Plasmid at the HindIII/XhoI restriction enzyme site to produce pCMV_3Tag_1A_CD82. The sequence of human CD82 siRNA was purchased from Genolution, and the base sequence is as follows: CD82_siRNA#1, 5'-CAUCGUGACUGAGCUCAUUUU-3' (forward) and 5'-AACGAGCUCAGUCACGAU GUU-3' (reverse); CD82_siRNA#2, 5'-GCACGUCCAUUCCGAAGACUU-3' (forward) and 5'-GUCUUCGGAAUGGACGUGCUU-3' (reverse). For transfection of siRNA, Lipofectamin RNA imax was used, and for transfection of plasmid DNA, Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) was used. First, cells were cultured overnight in RPMI1640 media containing only 5% FBS but without antibiotics. Lipofectamin, human siRNA, and plasmid were each diluted in Opti-MEM medium (Gibco, Waltham, MA, USA) and placed at room temperature for 5 minutes. Afterwards, each dilution was mixed and reacted again at room temperature for 15 minutes. Cells cultured overnight were washed with DPBS (Welgene, Gyengsan, Korea) after removing the media, and

cultured with mixed dilutions and Opti-MEM in a 37°C incubator for 5 hours. Afterwards, the culture medium was replaced with RPMI1640.

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

Total RNA from human gastric cancer cell lines was isolated from RNAiso reagent (Takara, Shiga, Japan). The cells were washed with DPBS after removing the culture medium, harvested with 1 ml of RNAiso reagent, placed in a 1.5 ml tube, and chloroform equivalent to 1/5 of the RNAiso reagent was added. Afterwards, it was lightly inverted 10-15 times, reacted at room temperature for 5 minutes, and centrifuged at 13,200 RPM, 4°C for 15 minutes. The separated supernatant was transferred to a new 1.5 ml tube, and the same amount of isopropanol as the supernatant was added, lightly inverted 10-15 times, and reacted at room temperature for 10 minutes. It was then centrifuged at 13,200 RPM, 4°C, for 10 minutes. The supernatant was removed, 75% ethanol was added to the precipitated RNA, and centrifuged at 13,200 RPM, 4°C, for 5 minutes. The supernatant was removed and the remaining solution was removed by air drying for 15 minutes. Afterwards, RNA was dissolved in water containing diethyl pyrocarbonate (DEPC). cDNA was synthesized using cDNA Master Mix (Toyobo. Osaka, Japan). PCR was performed using 2x TOPsimple DyeMIX-nTaq (Enzynomics, Daejeon, Korea), and the primers used for PCR are described in “Table 1”. PCR products were detected by adding Red Safe (iNtRON Biotechnology, Seongnam, Korea) to a 1% agarose gel and performing electrophoresis.

4. Quantitative real-time PCR (qRT-PCR)

Quantitative PCR was performed using TOPreal™ SYBR Green qPCR PreMIX (Enzynomics, Daejeon, Korea), and the mRNA expression level of the target gene was analyzed using the Ct (Cycle threshold) value of GAPDH. The primer list used is described in “Table 1”.

5. Western blot analysis

Cell lysate was extracted using RIPA buffer (Biosesang Inc, Seongnam, Korea) containing phosphatase inhibitor and proteinase inhibitor cocktail (GeneDEPOT, Barker, TX, USA). Lysis buffer was added to the cell lysate and incubated on ice for 10 minutes, followed by sonication in ice. Cell lysate were then incubated on ice for 30 minutes and centrifuged at 13,200 RPM and 4°C for 25 minutes. Protein concentration was measured by absorbance at 660 nm using a 660 nm protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA). The lysate was subjected to SDS-PAGE using an 8% acrylamide gel and then transferred to a PVDF membrane (Millipore, Burlington, MA, USA). The membrane was blocked with 5% skim milk for 90 minutes. Skim milk was used by dissolving it in a mixture of 10% PBS (Dongin Biotech, SEOUL, Korea) and 0.05% Tween20 (Sigma Aldrich, St. Louis, USA) in PBS-T buffer. Afterwards, washing was repeated three times for 10 minutes with PBS-T buffer, and the membrane was incubated with primary antibody for 16 hours at 4°C and washed again. Secondary antibodies were allowed to attach for 90 minutes at RT and the washing process was repeated. Visualization of Western blot data was performed using ECL substrate (Bio-Rad, Hercules, CA, USA). Western blot data quantification was performed using Image J (ver. 1.45), and values were normalized with GAPDH used as a loading control. Information on the antibodies used is written in “Table 2”.

6. Wound healing assay

Human gastric cancer cell lines (SNU-216, NCI-N87, YCC-2 and SNU-668) were transfected with siRNA and Plasmid vector for 24 hours. Afterwards, the cells were trypsinized, and $5x10^5$ - $4x10^6$ cells were seeded in a 6-well plate and cultured in media containing 5% FBS and 1% Antibiotics until the cells reached 80-90%. Then, the bottom of the plate was scraped with 1000 μl pipette tips, the monolayer was carefully washed with DPBS, and images of the cells were taken under a microscope at 0, 12, 24 and 48h.

7. Transwell migration and invasion assay

SNU-216, NCI-N87, YCC-2 and SNU-668 cell lines were transfected with siRNA (scRNA, CD82 siRNA) and plasmid vector (pCMV_3Tag_1A, pCMV_3Tag_1A_CD82). 24h after transfection, $2x10^4$ cells in 200 μl of FBS free medium were added to the top of the transwell chamber (Corning Costar, MA, USA) coated on the filter with 0.5mg/ml collagen type I and Matrigel (1:15) (BD biosciences, Seoul, Korea). Culture medium containing 10% FBS and 1% antibiotics was added to the bottom of the chamber, and the plate was cultured for 16-20 hours. Invaded and migrated cells were visualized by staining with hematoxylin and eosin. After drying the stained chamber, 12 to 15 randomly selected areas were selected through a wide-field microscope (Zeiss, Axio Scope. A1, Oberkochen, Germany), and cells in the area were counted. Data are expressed as mean ± SEM from each independent experiment.

8. DNA demethylation

Human gastric cancer cell lines (AGS, MKN28, NCI-N87, YCC-2, SNU-216, SNU-638 and SNU-668) were treated for 48 hours in RPMI1640 medium containing 10% FBS and 1% antibiotics containing 10uM 5-AZA-2′ deoxycytidine (Sigma Aldrich, St. Louis, MO, USA) dissolved in DMSO (Sigma Aldrich, St. Louis, USA). The control group was treated with the same amount of DMSO. Every 24 hours, the culture medium was removed and replaced with fresh 5-AZA and DMSO-treated medium.

9. Bisulfite modification and methylation specific PCR (MS-PCR)

Genomic DNA (gDNA) of human gastric cancer cell lines, including AGS, YCC-2, SNU-638 and SNU-668, was extracted using the AccuPrep® Genomic DNA extraction Kit (Bioneer, Daejeon, Korea). Afterwards, bisulfite modification was applied to gDNA using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA). To detect methylated genes, the CpG-rich region within the CD82 gene promoter region was amplified from bisulfite modified genomic DNA using the MSP primer set. MSP was performed using the EpiScope® MSP Kit (Takara, Shiga, Japan), and the primer sets used for MSP are described in “Table 1”. MSP products were detected through a 1.5% agrose gel containing Red Safe.

Table 1. Sequence of designed primer

CD82	Forward	5'-AGGATGGGGGCCTATGTCTT-3’
	Reverse	5'-ATGAGCTCAGTCACGATGCC-3’
*CD82*_U	Forward	5'-ATTTGAGGTTGGATTTTGTTTAGT-3'
	Reverse	5'-CAACTCATAAATACTCTCATTACACC-3'
*CD82*_M	Forward	5'-ATTTGAGGTTGGATTTCGTTTAC-3'
	Reverse	5'-CAACTCATAAATACTCTCGTTACGC-3'
GAPDH	Forward	5'-GGCTGCTTTTAACTCTGGTA-3'
	Reverse	5'-ACTTGATTTTGGAGGGATCT-3'

Table 2. Information of primary antibody used in western blot analysis

Primary antibody name	Manufacturer	Product number	Dilution rate
CD82	Santa Cruz	sc-518002	1 : 1000
E-cadherin	Santa Cruz	sc-8426	1 : 1000
N-cadherin	Santa Cruz	sc-59987	1 : 1000
GAPDH	Bioworld Tec.	AP0063	1 : 5000

10. Gene expression profile data and Kaplan-Meier analysis

Available datasets GSE26901 and GSE26899 were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The dataset was normalized using GEO2R and scatterplots were obtained for expression pattern analysis. Survival analyzes for overall survival(OS), first progression survival (FPS), and post progression survival (PPS) of gastric cancer patients were produced using the online resource Kaplan-Meier plotter (http://kmplot.com/analysis).

11. Statistical analysis

Statistical analyzes were performed using GraphPad Prism 8 (GraphPad Software, Inc, San Diego, CA, USA). Statistical analysis was analyzed using Student's t-test and calculated from at least three independent experiments. Statistical significance for Kaplan-Meier estimated survival rates was calculated using publicly available databases. Results were expressed as mean ± SEM. p values < 0.05 were considered to indicate statistical significance.

III. RESULTS

1. Patient survival analysis of CD82 from publicly available clinical data and expression analysis from public GEO dataset and TCGA data

First, I wanted to confirm the role of CD82 in gastric cancer. Kaplan-Meier analysis was performed to confirm the association between CD82 expression and survival rate. in all data from Kaplan-Meier analysis confirmed that lower expression of CD82 was associated with lower overall survival (OS), lower probability of early progression survival (FPS), and lower probability of survival after progression (PPS) (Fig. 1). And to analyze the expression of CD82, I used the public DATA base of two GEO data sets to confirm changes in CD82 expression according to the cancer progression stage. No significant changes in CD82 expression could be confirmed in AJCC stages 1 to 3, where metastasis did not occur. However, it was confirmed that CD82 expression was significantly decreased at stage 4 when metastasis occurred (Fig. 2). TCGA data also confirmed that CD82 expression was significantly decreased in stage 4, when metastasis progresses (Fig. 3). These results indicate that low expression of CD82 is associated with a poor prognosis in gastric cancer patients, and that CD82 expression is reduced when metastasis occurs in gastric cancer.

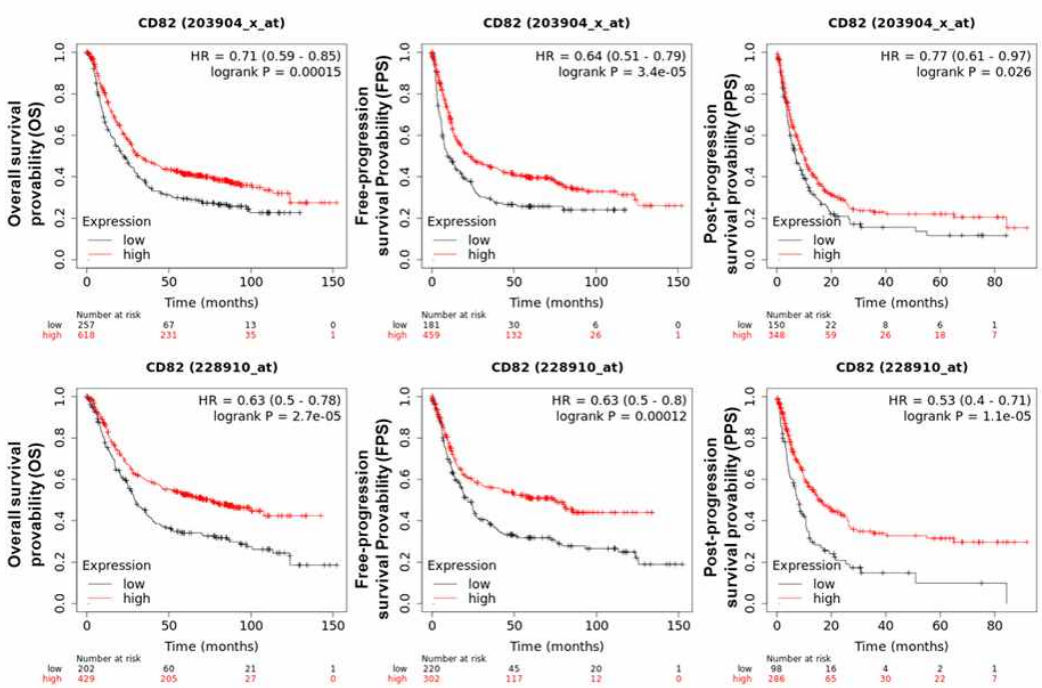

Figure 1. CD82 low expression leads to poor prognosis in gastric cancer patients. Kaplan-Meier survival plot shows the association between CD82 expression and survival in a public database of gastric cancer patients.

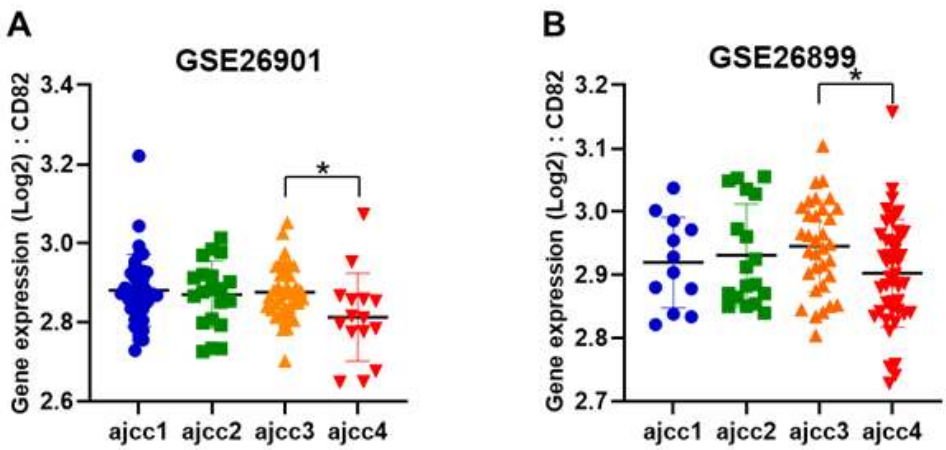

Figure 2. CD82 is down-regulated in metastatic gastric cancer patients. (A-B) CD82 mRNA expression levels in gastric cancer patients confirmed from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). (A) GSE26901 (n=109). (B) GSE26899 (n=108). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (*$p < 0.05$).

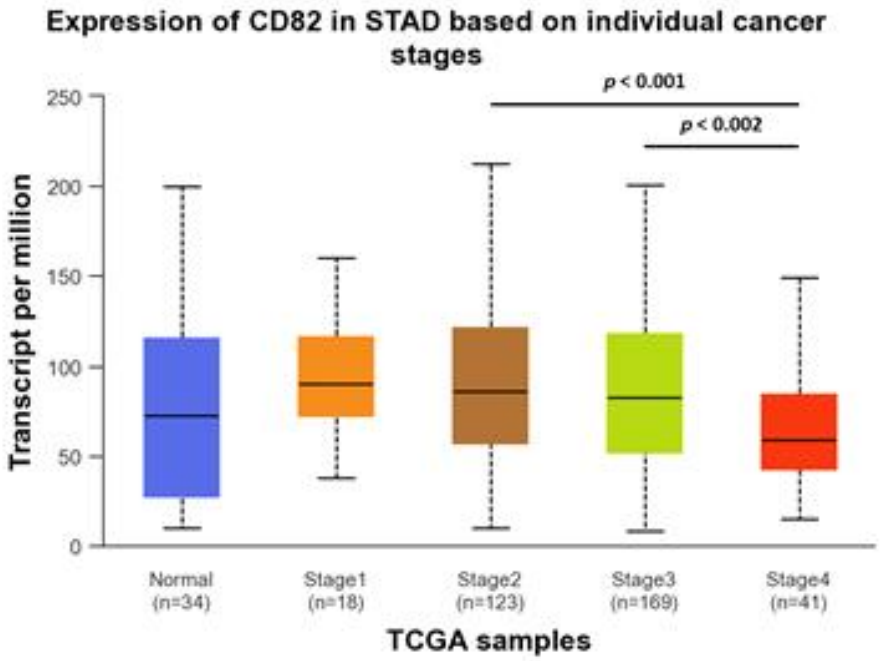

Figure 3. CD82 gene analysis of gastric cancer samples from the TCGA database. Comparison of CD82 mRNA expression individual cancer stages.

2. CD82 silencing increase invasion and migration in gastric cancer cells

I confirmed the basal level of CD82 protein expression in normal gastric epithelial cells and 10 gastric cancer lines through western blot analysis. The results show that the expression of CD82 is down-regulated in most gastric cancer cell lines compared to normal gastric epithelial cells. (Fig. 4). Among them, SNU-216 and NCI-N87 cell lines with high expression, and YCC-2 and SNU-668 cell lines with low expression were selected and conducted additional experiments.

First, I used SNU-216 and NCI-N87 cell lines, which have high expression of CD82, to determine the effect of inhibiting CD82 expression. Expression of CD82 was silenced through specific siRNA in SNU-216 and NCI-N87 cell lines. The level of inhibition of CD82 expression by siRNAs was confirmed through RT-PCR, qRT-PCR, and western blot analysis. SNU-216 and NCI-N87 cell lines were transfected with scRNA, CD82 siRNA#1, and siRNA#2 for 48 hours (Fig. 5). Cell mobility regulated by CD82 knockdown was investigated through wound healing assay. The results showed that wound closure increased faster when CD82 was knockdown (Fig. 6). In addition, Transwell assay was performed to determine whether it affected cell invasion and mobility. When CD82 was knocked down, invaded cells were increased by 247% for siRNA #1 and 146% for siRNA #2 in the SNU-216 cell line and by 139% and 115% in the NCI-N87 cell line compared to the control group (Fig. 7). Additionally, When CD82 was knocked down, the migrated cells showed increases of 231% and 144% for siRNA #1 and 144% for siRNA #2 in the SNU-216 cell line, and 133% and 110% increases in the NCI-N87 cell line compared to control (Fig. 8).

These results confirmed that CD82 silencing increases invasion and migration in gastric cancer cells.

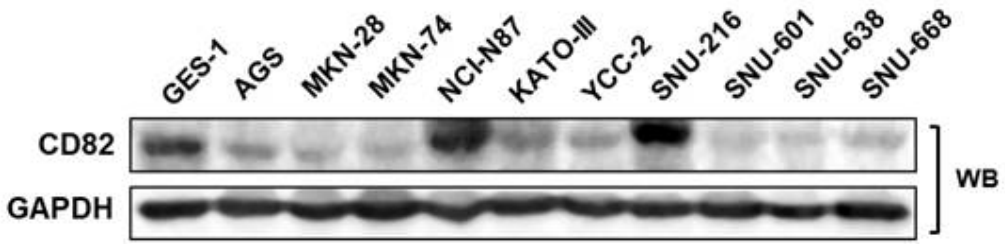

Figure 4. Protein expression level of CD82 in gastric cancer cell lines. CD82 expression levels in normal gastric epithelial cells (GES-1) and ten human gastric cancer cell lines (AGS, MKN-28, MKN-74, NCI-N87, KATO-III, YCC-2, SNU-216, SNU-601, SNU-638 and SNU-668) were measured by western blot.

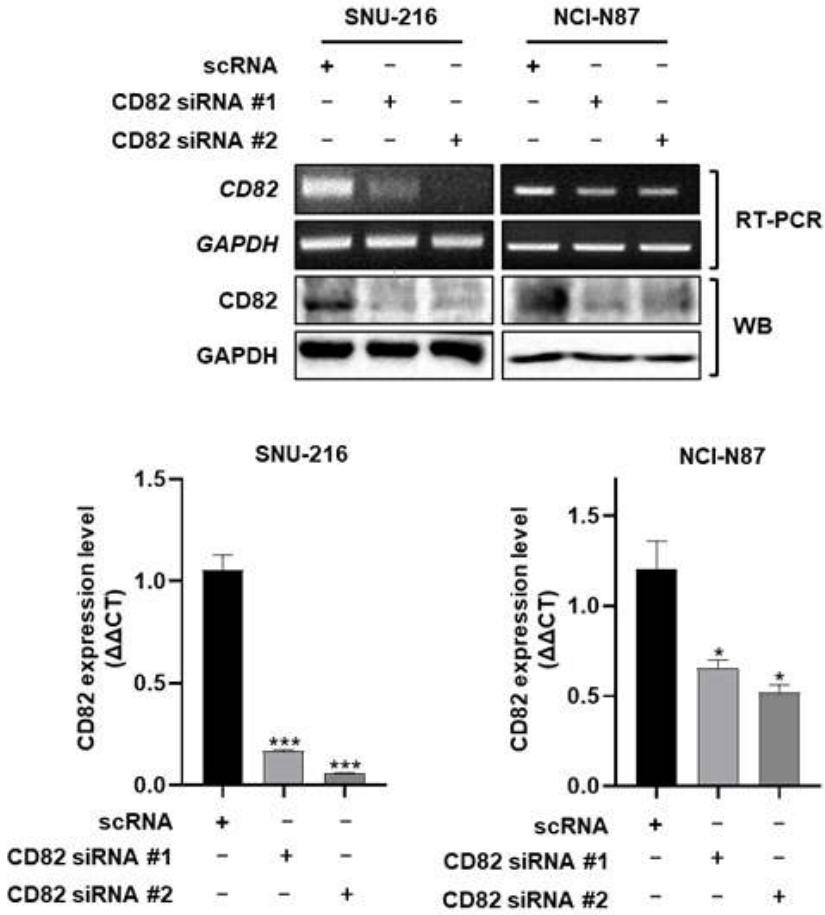

Figure 5. Silencing of CD82 expression by small interfering RNA. SNU-216 and NCI-N87 cell lines were transfected with scrambled siRNA (scRNA) or CD82-specific small interfering RNA #1, #2 (CD82 siRNA #1, #2). CD82 expression levels were performed by RT-PCR, qRT-PCR, and Western blot analysis. GAPDH was used as a loading control. The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (*$p < 0.05$ and ***$p < 0.001$).

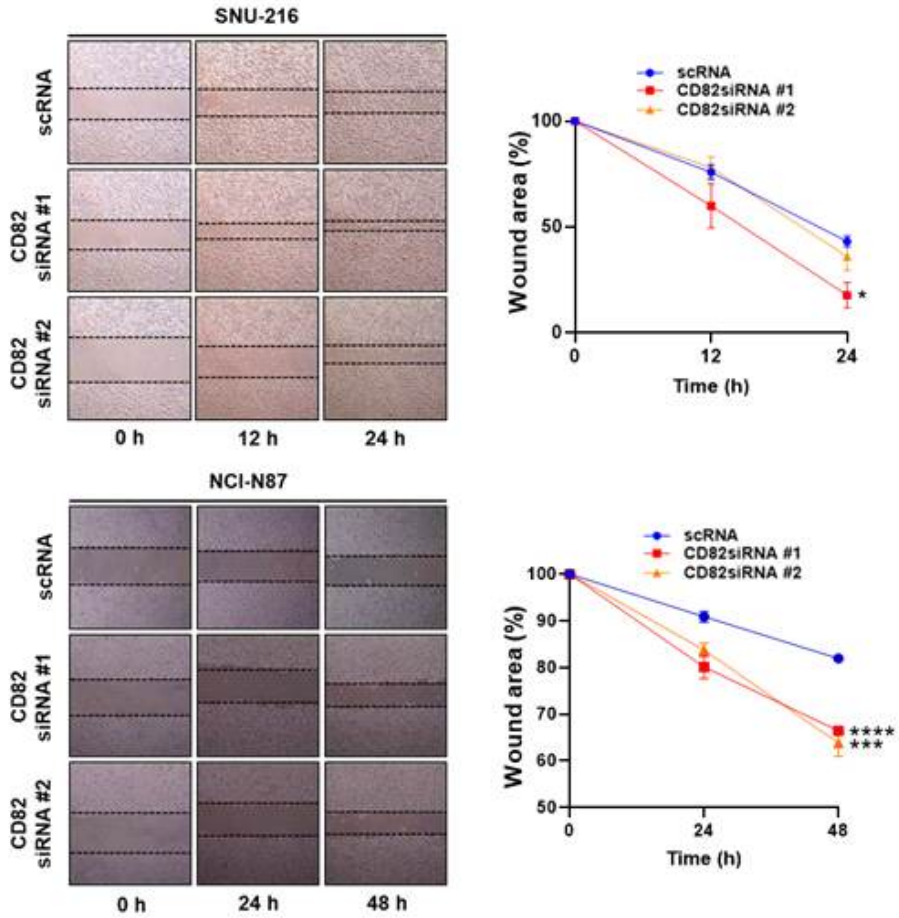

Figure 6. Silencing CD82 leads to rapid closure of the wound area. Wound closure measured by wound healing assay. Cell images were visualized and the wound area was measured using image J. Data are presented as mean ± SEM (n=3). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (*$p < 0.05$; ***$p < 0.001$ and ****$p < 0.0001$).

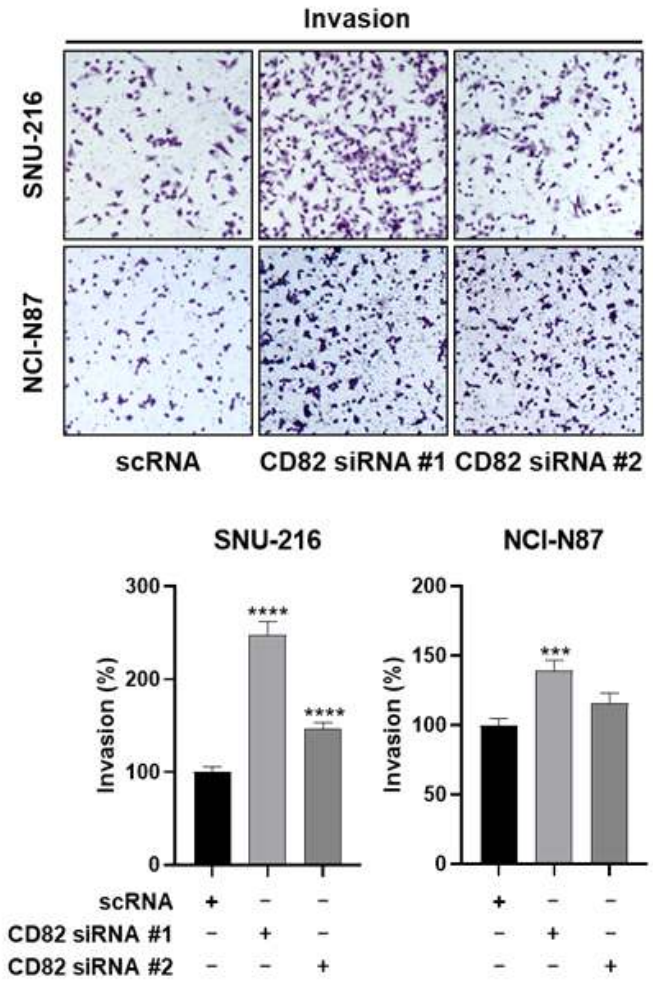

Figure 7. Silencing of CD82 decreases cell invasion in gastric cancer cells. Invaded cells were detected through transwell assay. Random areas were acquired under a light microscope and the number of cells was counted. Invaded cells were visualized. Data are expressed as mean ± SEM (n=15). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (***$p < 0.001$ and ****$p < 0.0001$).

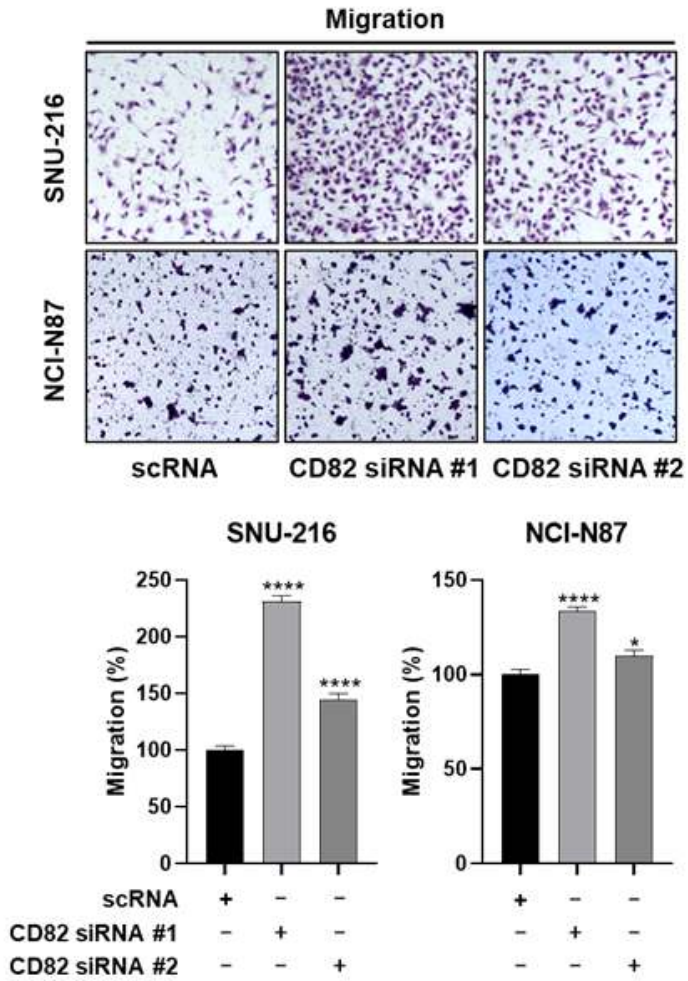

Figure 8. Silencing of CD82 decreases cell migration in gastric cancer cells. migrated cells were detected through transwell assay. Random areas were acquired under a light microscope and the number of cells was counted. Invaded cells were visualized. Data are expressed as mean ± SEM (n=15). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (*$p < 0.05$; ***$p < 0.001$ and ****$p < 0.0001$).

3. CD82 over-expression decrease invasion and migration in gastric cancer cells

The effect of over-expression of CD82 was investigated in YCC-2 and SNU-668 cell lines, which have relatively low CD82 expression. YCC-2 and SNU-668 cell lines were transiently transfected with empty vector (pCMV_3Tag_1A_E.V.) and CD82 over-expression vector (pCMV_3Tag_1A_CD82) for 48 hours. Over-expression of CD82 was confirmed using RT-PCR, qRT-PCR, and western blot analysis (Fig. 9). Wound healing assay was performed to investigate cell mobility regulated by CD82 over-expression. Wound healing assay was performed to investigate cell mobility regulated by CD82 over-expression. The results showed that when CD82 was over-expressed, wound closure was delayed (Fig. 10). Evaluation of the invasion and migration ability of gastric cancer cells by over-expression of CD82 was confirmed through trasnwell assay. The invaded cells in over-expressed CD82 were reduced to 51.6% in the YCC-2 cell line and 70% in the SNU-668 cell line compared to control (Fig. 11). Additionally, over-expression of CD82 reduced migrating cells to 64% in YCC-2 cell line and 82% in SNU-668 cells compared to control (Fig. 12). The results show that over-expression of CD82 reduces cell invasion and migration.

Additionally, I investigated whether changes in CD82 expression affected epithelial-mesenchymal transition (EMT) related genes. First, TCGA RNAseq data was used to confirm co-relation between CD82 and EMT markers. E-cadherin (CDH1) and CD82 showed a positive correlation, N-cadherin (CDH2) and CD82 showed a negative correlation. Expression of EMT markers in gastric cancer cells was confirmed through western blot analysis. Decreased expression of CD82 showed decreased protein expression of E-Cadherin, and it was shown that N-Cadherin also increased with increased protein expression of CD82. (Fig. 13).

Overall, these results indicate that CD82 is involved in mobility and metastasis in gastric cancer cells.

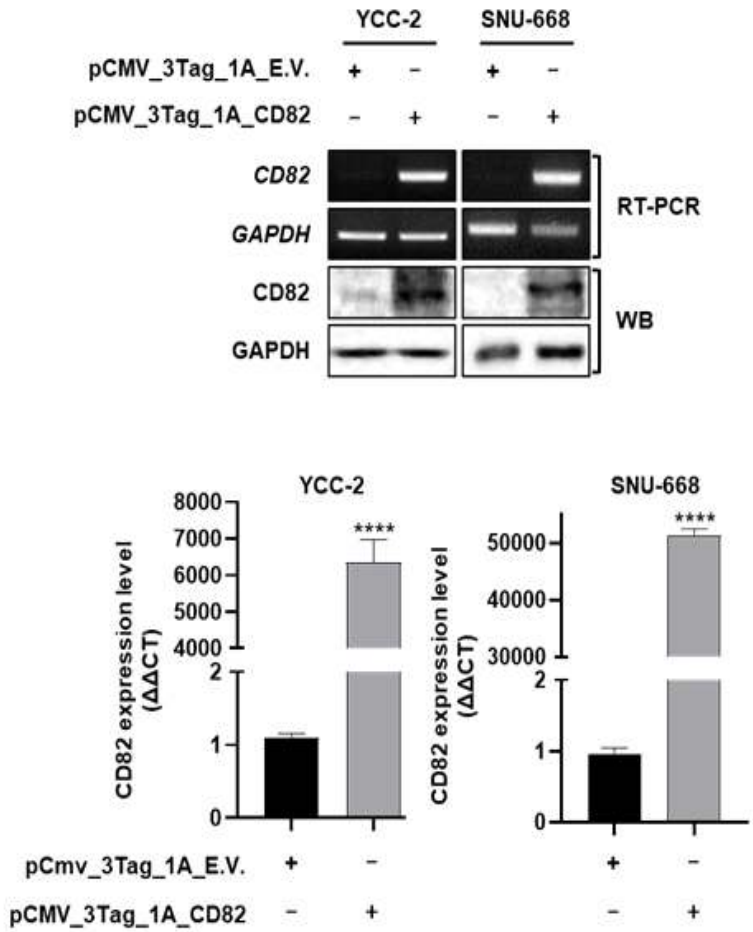

Figure 9. Over-expression of CD82 by plasmid vector system. YCC-2 and SNU-668 cells were transfected with pCMV_3Tag_1A_E.V. or transfected with pCMV_3Tag_1A_CD82. CD82 over-expression levels were detected by RT-PCR, qRT-PCR, and western blot. GAPDH was used as a loading control. Data are presented as mean ± SEM (n=3). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (****$p < 0.0001$).

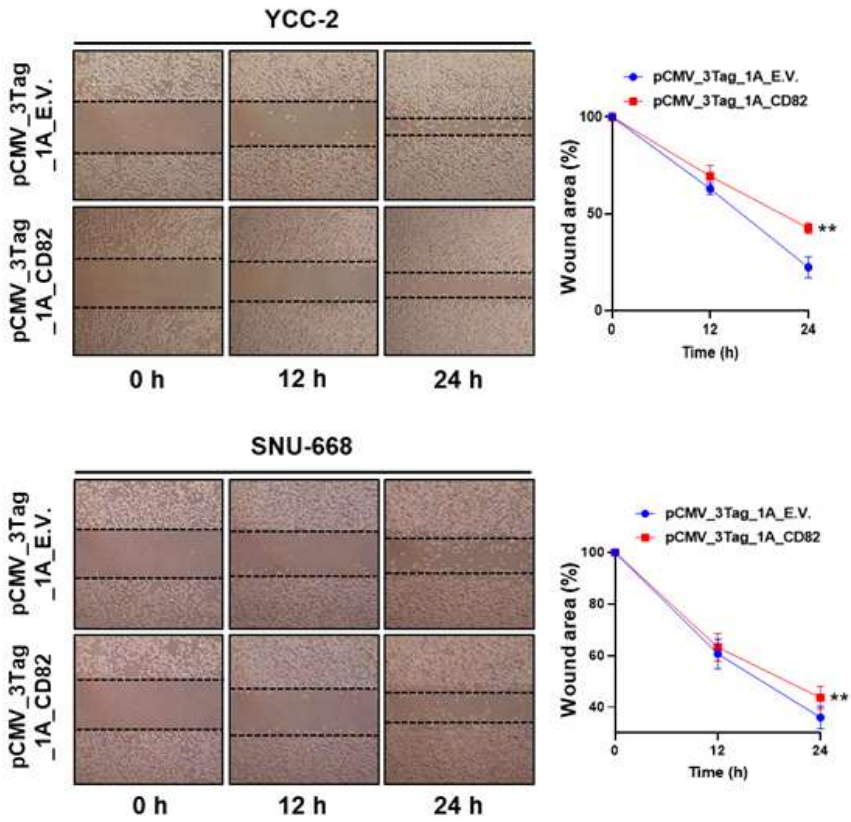

Figure 10. Over-expression of CD82 delays closure of the wound area. Wound closure measured by wound healing assay. Cell images were visualized and the wound area was measured using image J. Data are presented as mean ± SEM (n=3). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (**$p < 0.01$).

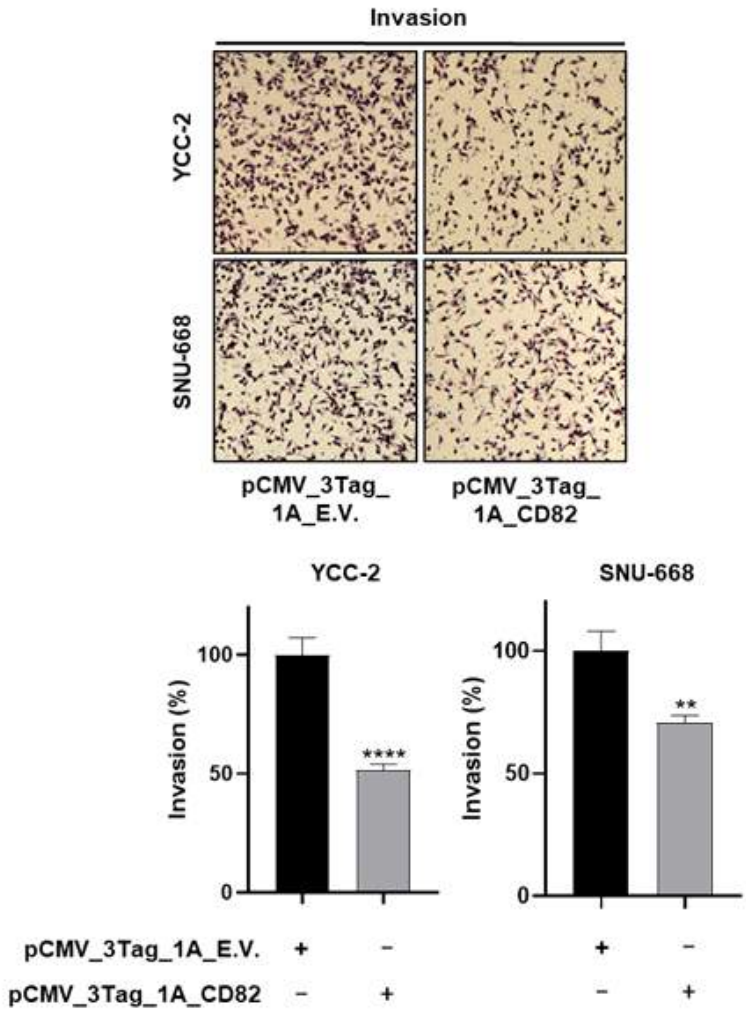

Figure 11. Over-expressed CD82 decrease cell invasion in gastric cancer cells. Invaded cells were detected through transwell assay. Random areas were acquired under a light microscope and the number of cells was counted. Invaded cells were visualized. Data are expressed as mean ± SEM (n=12). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (**$p < 0.01$ and ****$p < 0.0001$).

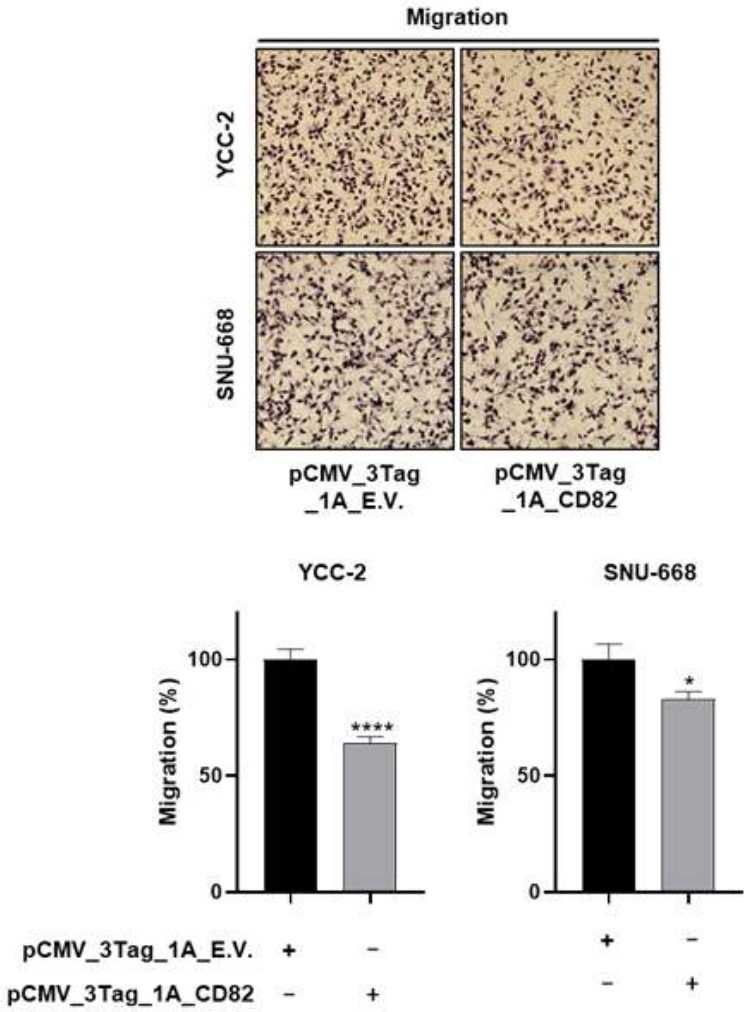

Figure 12. Over-expressed CD82 decrease cell migration in gastric cancer cells. migrated cells were detected through transwell assay. Random areas were acquired under a light microscope and the number of cells was counted. Invaded cells were visualized. Data are expressed as mean ± SEM (n=12). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (*$p < 0.05$ and ****$p < 0.0001$).

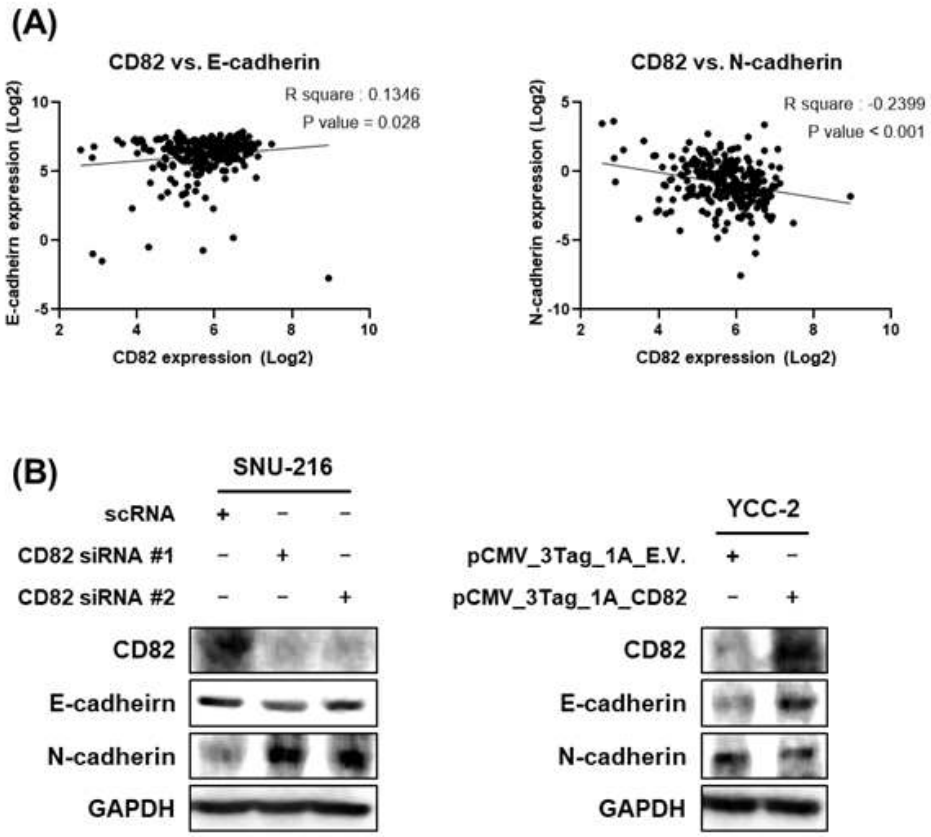

Figure 13. CD82 affects the expression of EMT related markers. (A) Spearman's correlation tests showing the relationship between CD82 and E-cadherin and CD82 and N-cadherin in gastric cancer patient mRNA expression data obtained from a TCGA (Nature 2014) RNA seq V2 RSEM (n=265). (B) SNU-216 cell lines were transfected with scrambled siRNA (scRNA) or CD82-specific small interfering RNA #1, #2 (CD82 siRNA#1, #2). SNU-668 cells were transfected with pCMV_3Tag_1A_E.V. or transfected with pCMV_3Tag_1A_CD82. E-cadherin, N-cadherin protein expression levels in SNU-216 and YCC-2 were evaluated by Western blot analysis. GAPDH was used as loading control.

4. Expression of CD82 is regulated by DNA methylation

I investigated what factors change the expression of CD82 in gastric cancer cells. Abnormal hypermethylation and gene suppression in tumor suppressor genes are known to be important early events in malignant tumors [20]. To determine whether CD82 is hypermethylated in gastric cancer, human gastric cancer cell lines (AGS, MKN-28, NCI-N87, YCC-2, SNU-216, SNU-638 and SNU-668) were treated with the DNA methyltransferase inhibitor 5-AZA-2′-deoxycytidine (5-AZA). After 48 hours, the cells were harvested, and changes in CD82 expression as DNA methylation was released were confirmed through RT-PCR and qRT-PCR. Treatment with 5-AZA increased the expression of CD82 in AGS, YCC-2, SNU-216 and SNU-668 cell lines, which had low CD82 expression (Fig. 14). Afterward, I wanted to confirm whether CD82 expression was truly suppressed by methylation. The promoter sequence of CD82 contains a CpG-rich region, and we expected that some residues within this region would be methylated. To determine the CD82 promoter methylation status in gastric cancer cell lines, methylation-specific PCR was performed after bisulfite modification of genomic DNA in the corresponding region. In NCI-N87 and SNU-216 cell lines, which showed no change in CD82 expression despite treatment with 5-AZA, more MSP products were detected using unmethylation-specific primers. However, in cell lines whose expression was significantly changed by 5-AZA, more MSP products were detected from methylation-specific primers. These results show that the CD82 gene contains methylated residues within the promoter region (Fig. 15). Additionally, since treatment with 5-AZA increased the expression of CD82, we sought to determine whether it exhibited the same phenotype as up-regulated CD82. A wound healing assay was performed on YCC-2 and SNU-668 cell lines in which CD82 expression increased due to treatment with 5-AZA. The results confirmed that wound closure was delayed in the 5-AZA treatment group (Fig. 16).

This shows that the expression of CD82 is suppressed by methylation in gastric cancer cells, and that inhibiting DNA methylation can increase the inhibitory effect on cell motility, which is a function of CD82.

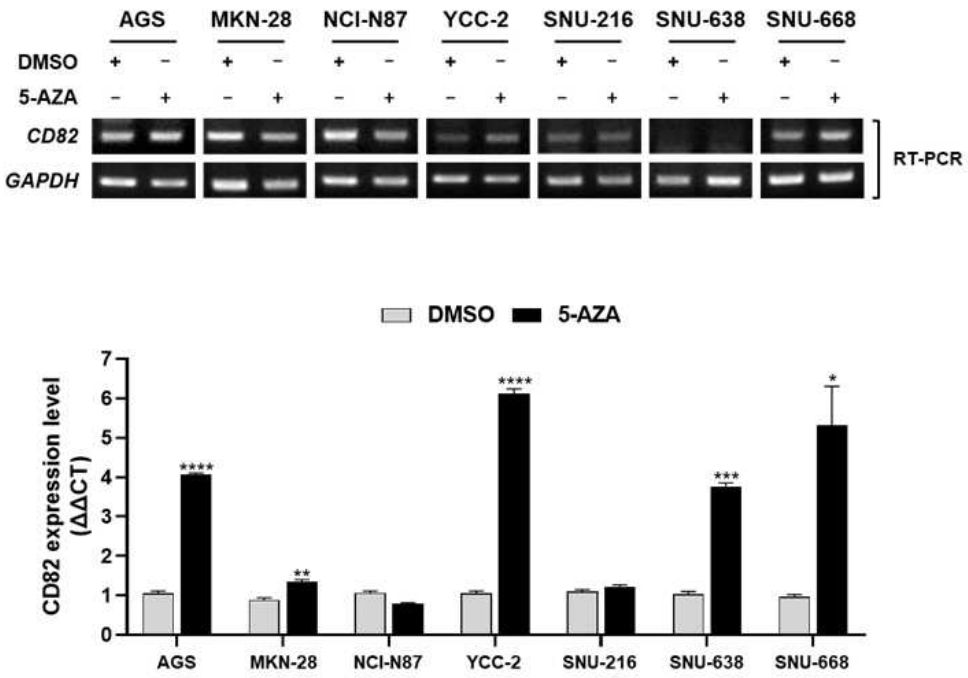

Figure 14. CD82 expression is suppressed by DNA methylation. AGS, MKN-28, NCI-N87, YCC-2, SNU-216, SNU-638 and SNU-668 cells were treated with 5-AZA-2′-deoxycytidine for 48 hours. The mRNA expression level of CD82 was detected by RT-PCR and qRT-PCR. GAPDH was used as a loading control. The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (*p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001).

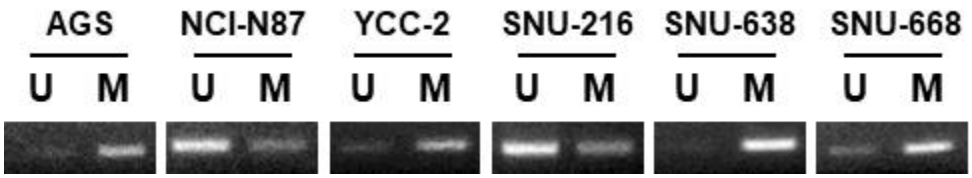

Figure 15. CD82 is hypermethylated at the CpG island. gDNA extracted from gastric cancer cell lines was modified with bisulfite, and methylation specific-PCR was performed using primers specific for unmethylation (U) or methylation (M).

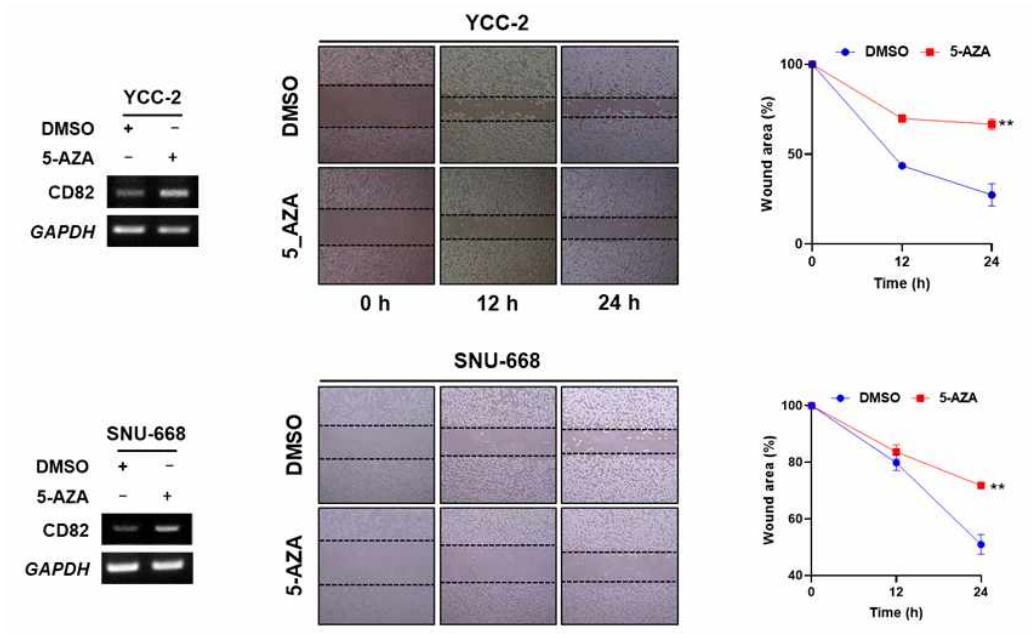

Figure 16. Demethylation of DNA delays wound closure in gastric cancer cells. Wound closure measured by wound healing assay. YCC-2 and SNU-668 cells were treated with 5-AZA-2′-deoxycytidine for 48 hours. Cell images were visualized and the wound area was measured using image J. Data are presented as mean ± SEM (n=3). The significance of the data was evaluated using Student's *t*-test, and significant differences are indicated by * (**p < 0.01).

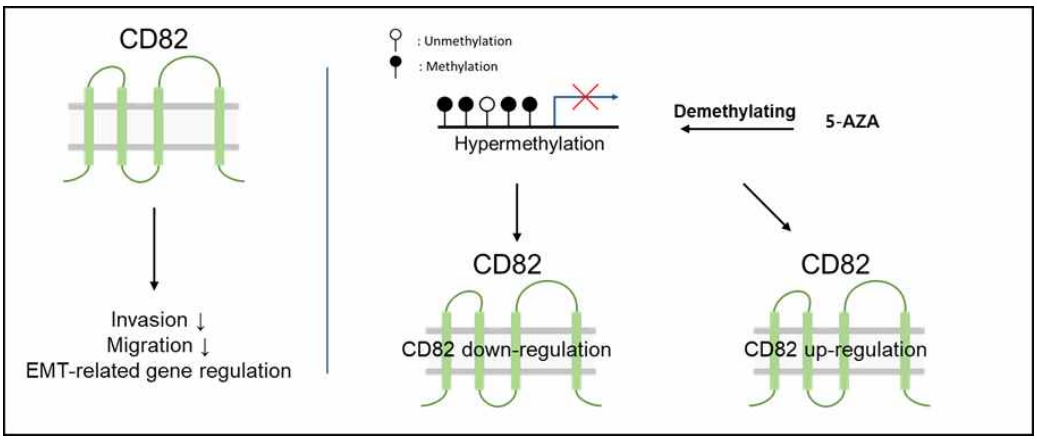

Figure 17. Schematic diagram showing that CD82 reduces invasion and migration in gastric cancer regulates EMT-related genes, and is actually down-regulated by DNA methylation.

IV. DISCUSSION

Gastric cancer is one of the cancer types that still shows high mortality and incidence despite progress in the development of treatments. Before gastric cancer develops, it progresses through the stages of gastritis, intestinal metaplasia, gastric epithelial dysplasia, and finally carcinogenesis [22]. If gastric cancer can be detected before or at an early stage, the 5-year survival rate can be greatly increased [2]. However, since most gastric cancer does not show symptoms until it progresses and diagnosis relies heavily on endoscopy, it is important to discover biomarkers for the diagnosis of gastric cancer.

CD82, also known as KAI1, is known to be a cancer metastasis suppressor gene in several studies. However, the expression of CD82 is down-regulated during cancer progression. Through this study, I attempted to verify whether CD82 actually functions as a suppressor and to determine whether CD82 expression is regulated by DNA methylation. DNA methylation is an epigenetic modification that occurs during development by adding a methyl group to the cytosine-C5 position to form 5-methyl cytosine [23]. CpG dinucleotide clusters in GC-rich regions are called CpG islands and contribute to cancer development by affecting the methylation status of CpG sites in gene promoters [24]. It is known that hypermethylation of CpG sites in gene promoter regions can act as a mechanism to inactivate tumor suppressor genes without gene mutation [25]. However, this mechanism in gastric cancer is not well known, so I designed an experiment to find out.

First, i sought to determine the expression and prognosis of CD82 in actual gastric cancer patients. As a result of confirmation through Kaplan-Meir plot and public data, it was confirmed that CD82 showed lower expression in gastric cancer and that this was related to the patient's poor prognosis (Fig. 1-3).

Afterwards, based on research showing that CD82 is down-regulated in various carcinomas and affects metastasis and invasion [26-28], i sought to determine whether CD82 functions as a suppressor in actual gastric cancer cell lines. Compared to normal epithelial cells, CD82 was down-regulated on most gastric cancer cells (Fig. 4).

Silencing of CD82 increased the invasion and migration of gastric cancer cells and increased the wound closure rate, while over-expressed CD82 decreased invasion and migration and delayed the wound closure rate (Fig. 5-12). Additionally, the expression of EMT-related genes, which play an important role in the early stage of cancer metastasis, was also confirmed. When the correlation with CD82 was confirmed through TCGA data, it was confirmed that there was a positive correlation with E-cadherin and a negative correlation with N-cadherin. In addition, changes in markers due to changes in CD82 expression were confirmed in actual gastric cancer cells (Fig. 13).

Lastly, i sought to determine what factors change CD82, which exhibits this phenotype in gastric cancer and regulates gene expression changes. Previous studies have shown that CD82 down-regulation is not caused by loss of heterozygosity or hyper-methylation of the promoter region [29]. 5-AZA, a demethylating agent, is known to irreversibly bind to DNA and lose the activity of methyltransferase [30]. Treatment with 5-AZA resulted in increased expression of CD82 in cell lines where CD82 expression was low (Fig. 14). Afterwards, to confirm whether CD82 was actually methylated, the sequence of the CpG island region within the promoter region of CD82 was confirmed through MSP analysis, and a higher MSP product was obtained from the methylation-specific primer (Fig. 15). Since the expression of CD82 was increased by 5-AZA treatment, it was confirmed that demethylation showed the same phenotype as over-expression of CD82, and it actually delayed wound closure (Fig. 16).

This study has several limitations. First, the increase in CD82 expression due to 5-AZA treatment was confirmed only at the mRNA level. Additionally, it is unknown whether the phenotype of inhibiting mobility in gastric cancer cells changed by 5-AZA treatment is due to modification of CD82 alone. Therefore, I would like to verify changes in the expression level of CD82 protein and check whether CD82 downstream signal regulators identified through previous research show changes when DNA methylation is suppressed [31].

In conclusion, this study verified the function of CD82 as a metastasis suppressor gene in gastric cancer cells. In addition, CD82 is highly likely to be modified by methylation in the promoter region during the progression of gastric cancer, which may result in a decrease in expression (Fig. 17). These results are expected to help reveal the mechanism of epigenetic modification of CD82.

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