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A study on the role of immunosuppressive molecule CD200 in the proliferation and metastasis of gastric cancer cells

조선대학교 대학원

글로벌바이오융합학과

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위암세포의 증식 및 전이 과정에서 면역억제분자 CD200의 역할 연구

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이 논문을 이학 석사학위신청 논문으로 제출함

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위암세포의 증식 및 전이 과정에서 면역억제분자 CD200의 역할 연구

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위암은 전 세계적으로 높은 발병률과 사망률을 차지하고 있으며, 동아시아 특이적으로 발생하는 암종이다. 위암은 전이가 일어나는 경우 생존율이 급격하게 감소하며, 위암의 발병과 진행 원인에는 대표적으로 H. pylori infection, tumor microenvironment가 있다. 하지만 이러한 원인이 위암의 진행 및 전이에 관여하는 기작에 대해서는 명확하지 않다. 따라서 면역반응과 암의 진행에 관여하는 유전자로써 CD200의 역할을 확인하는 것을 목표로 하였다.

CD200은 다양한 암종에서 높은 발현을 보이며, 발현이 높은 경우 예후가 좋지 않다. CD200은 암의 진행, 전이, 암 줄기세포 능력에 대해 연구가 되어있으며, 주요 작용 기전은 면역억제로 알려져 있다. CD200의 주요 작용 기전인 면역억제는 암 세포 외부에서 면역 세포들과의 상호작용에 의해 일어난다. 반면, CD200의 세포 내부에서의 작용 기전은 세포 외부에서의 작용 기전에 비해 연구가 부족하다. 최근 연구에 따르면 CD200의 transmembrane domain이 γ-secretase에 의해 절단되어 CD200 cytoplasmic tail이 생성되고, CD200 cytoplasmic tail이 핵 안으로 전위가 일어나 전사 인자들의 발현을 증가시키는 것으로 보고되었다. 하지만 여전히 세포 내부에서 CD200 분자 기전에 영향을 미치는 신호전달에 대한 연구는 미흡하며, 특히 위암에서 세포 내부의 CD200 작용 기전은 밝혀지지 않았다. 따라서 이

연구는 CD200이 위암 세포 내부에서 작용하는 분자 기전을 밝히는 것에 초점을 두었다.

먼저 public data를 통해서 CD200이 위암 환자에서 높은 발현을 보이며, CD200의 높은 발현이 좋지 않은 예후와 관련이 있음을 확인했다. CD200의 발현이 낮은 위암 세포주 AGS, YCC-2에 CD200을 과발현시키고, CD200이 위암 세포의 진행과 전이에 미치는 영향을 확인하고자 했다. 그 결과 CD200의 과발현은 위암 세포의 증식, 침습, 이동을 증가시켰다. 이러한 표현형의 변화가 세포 내부에서 어떤 신호전달에 의해 일어나는지 확인하고자, 위암에서 대표적으로 전이와 관련 있는 NOTCH, WNT, SHH 신호전달을 선별하여 스크리닝을 진행했다. 그 결과 CD200은 WNT 신호전달과 연관이 있었으며, WNT 신호전달의 핵심 분자인 β-catenin과 직접적으로 상호작용하여 핵 내로 β-catenin의 이동을 증가시키는 것을 보여주었다. 이는 결국 세포 증식 및 이동에 관여하는 WNT/β-catenin 신호전달 표적 유전자들의 발현 증가로 이어지는 것을 검증했다.

이 연구는 CD200이 위암 세포 내부에서 작용하는 분자 기전을 증명했다. 따라서, CD200을 면역억제뿐만 아니라 위암 진행을 조절하는 잠재적인 이중표적 조절자로써 가능성을 제시한다.

ABSTRACT

A Study on the role of immunosuppressive molecule CD200 in the proliferation and metastasis of gastric cancer cells

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Gastric cancer still accounts for a high incidence and mortality worldwide, and is a cancer type that occurs specifically in eastern asia. In addition, gastric cancer is one of the carcinomas in which the survival rate rapidly decreases when metastasis occurs. Representative causes of gastric cancer pathogenesis and progression include *H. Pylori* infection and tumor microenvironment. Therefore, CD200, a gene involved in immune response and cancer progression, was selected as a research subject.

CD200 shows high expression in various carcinomas and has been studied for metastasis and cancer stem cell ability, and its main role is known to be immunosuppressive. The immunosuppressive action of CD200 occurs in extracellular region by interaction with other immune cells. The mechanism of action CD200 intracellular region is less studied than its mechanism of action extracellular region. This is because the CD200 cytoplasmic domain lacks signaling motifs and adapter molecules binding sites. Recently, it has been reported that when CD200 is truncated by γ-secretase and CD200 cytoplasmic tail moves to the nucleus and increases the expression of transcription factors. However, studied on the molecular mechanism of CD200 intracellular region are still insufficient, and the mechanism of CD200 intracellular region in gastric cancer hasn't been elucidated. Therefore, in this study, the

molecular mechanism of CD200 intracellular region in gastric cancer was investigated.

First, through public data, it was confirmed that CD200 is highly expressed in gastric cancer patients, and that CD200 high expression was associated with poor prognosis. CD200 was over-expressed in AGS and YCC-2 cells, gastric cancer cell lines with low CD200 expression, and the effect of CD200 on gastric cancer cell progression was confirmed. As a result, it was verified that the proliferation and motility of gastric cancer cells increased when CD200 was over-expressed. In order to confirm which signaling pathway causes phenotype changes in gastric cancer cells, i screened notch, WNT and sonic hedgehog signaling pathway, which are representative signaling pathway associated with metastasis in gastric cancer. As a result, CD200 was confirmed that it associated WNT signaling pathway and directly bound to β-catenin, a key molecule of WNT signaling pathway, and increased the nuclear translocation of β-catenin. The nuclear translocation of complex enhanced the β-catenin target gene expression.

This study demonstrated the molecular mechanism by which CD200 acts in intracellular region in gastric cancer cells. Therefore, as a dual target, CD200 is a potential target for immunotherapy and as the same time suggests the possibility as a target gene for inhibiting the progression of gastric cancer.

I. INTRODUCTION

Gastric cancer is still an important cancer worldwide, with approximately one million new cases and over 7 hundred thousand deaths in 2020 (1). In addition, gastric cancer is a carcinoma that occurs specifically in eastern asia and is caused by various causes other than *H. Pylori* infection (2, 3). According to the most recent 2019 statistical report from south korea, despite the recent trend of decreasing mortality by cancer in south korea, it is still the number one cause of death. Gastric cancer has a high incidence rate and is one of the five most diagnosed cancers (4). Gastric cancer is one of the cancer types in which the mortality rate drops sharply when metastasis occurs (4). Therefore, gastric cancer is still a global and domestic problem and continuous research is required.

The pathogenesis and progression factor of gastric cancer, *H. Pylori* infection and the tumor microenvironment (TME) are the most representative. *H. Pylori* infection is responsible for environmental factors that induces EMT by generating cancer stem cell (CSC) properties and increases the inflammatory response (5, 6). The TME is a cause of host factor that plays an crucial role in metastasis of gastric cancer (6). Through the secretion of various chemokines and cytokines in the TME, immune cells are introduced into the TME through blood vessels (7). Theses immune cells lose anti-tumor immunity by various factors secreted from the tumor and promoter tumor growth (7). Therefore, CD200, which is related to immune response and tumor progression (8), was selected as a study subject.

CD200 is a type I membrane glycoprotein composed of a short cytoplasmic tail, two extracellular immunoglobulin superfamily domains and a transmembrane region (9, 10). CD200 is mainly expressed in thymocytes, dendritic cells, T and B lymphocytes and endothelial cells (11, 12). A canonical role of CD200 is immunosuppression. CD200 binds to CD200R expressed on monocytes, T lymphocytes, dendritic cells and macrophages (8). The interaction between CD200 and CD200R of various tissues delivers a stop signal to cells of the macrophage lineage (8, 13, 14). These interactions also affect the reduction of Th1 cytokine responses (15), the induction of regulatory T

cells (16) and suppression of tumor-specific T-cell immunity (17). CD200 extracellular domain has been mainly studied in the signaling pathway. The reason is that the CD200 cytoplasmic tail lacks a region where signaling motifs and adapter molecules can bind (18). Recently, other role of CD200 has been reported occasionally. It was reported that the cytoplasmic domain of type I membrane such as CD200 was cleaved by γ-secretase (19). Studies have shown that the truncated cytoplasmic tails undergo nuclear translocation to increase transcription factor expression (18).

CD200 is highly expressed in various cancers and is associated with poor prognosis (20-22). Also CD200 has been studied in various cancer types (23-30). In acute myeloid leukemia (AML), CD200 is an immunosuppression and cancer stem cell specific marker through natural killer cell suppression (23, 24). In multiple myeloma (MM), CD200 over-expression affects MM progression by increasing suppressive regulatory T cells and cytokines (25). In breast cancer, regulation by CD200-CD200R is important for controlling solid tumor growth and metastasis identified in mouse models (26, 27). In head and neck squamous cell carcinoma, CD200 is associated with cancer stem cell properties and is involved in regulating chemoradiation responses (28). CD200 expression on melanoma cells suppresses tumorigenesis and lung metastasis through suppression of myeloid cells (29). In pancreatic cancer (PC), CD200 blockade limits PC tumor growth and enhances the efficiency of PD-1 (30). A correlation between CD3+ expression and CD200 on T cells from gastric cancer patients has been reported (31). However, studies on CD200 in gastric cancer are still insufficient and its molecular mechanism is unclear.

In previous studies, CD200 was up-regulated in various carcinomas and has a poor prognosis (20-30). In this study, i aims to confirm the effect of CD200 on gastric cancer progression and metastasis and to identify the molecular mechanism acting in the intracellular region. Therefore, through this study, i would like to suggest CD200 as a potential immunotherapeutic target molecule and a target for inhibiting gastric cancer progression.

II. MATERIALS AND METHODS

II.1. Cell culture

The human gastric cancer cell lines (AGS, MKN-28, SNU-216, SNU-601, SNU-638 and SNU-668) were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). Human normal gastric epithelial cells (GES-1) and YCC-2 cells were obtained from Yonsei Cancer Center (Seoul, Korea). Cell lines were cultured in RPMI1640 (Welgene, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS) (Corning Costar, NY, USA) and 1% antibiotics including streptomycin/penicillin (Glibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37℃ in a humidified incubator with 5% CO_2. Cells were maintained as adherent culture and passaged once every 2-3 days.

II.2. Transfection of construction

Transfection of human plasmid DNA was using Lipofectamine 2000 reagent (Invitrogen, carlsbad, CA, USA). First the cells were seeded in 5% FBS medium without antibiotics and incubated overnight. Transfection reagent, plasmid was diluted in Opti-MEM medium (Glibco, Waltham, MA, USA) for 5 min at room temperature. Diluted materials were mixed and reacted for 15 min at room temperature. Incubated cells were washed with DPBS and diluted materials were treated with Opti-MEM. After 5 h the cells replaced with RPMI1640 culture medium. Human CD200 construct was provided by Seung-Phil Shin.

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

Total RNA from the human gastric cancer cell line was isolated cells using RNAiso reagent (Takara, Shiga, Japan). The cell lysates were resuspended after treatment with 1 ml of RNAiso and added 200 μl of chloroform. After gently inverting 10-15 times, reaction for 15 min at room temperature. Then 16000 ×g for 15 min centrifuged. Separated supernatant was transferred to new ep tube and added 2-propanol. Once again, gently inverting 10-15 times, reaction for 10 min at room temperature and 16000 ×g for 10 min centrifuged. After removing the supernatant, precipitated RNA was washed with 75% EtOH. And washed RNA was dissolved in water containing diethyl pyrocarbonate. cDNA synthesis was carried out using a reverse transcription system (Toyobo, Osada, Japan) and PCR was performed using 2× TOPsimple×™DyeMIX-nTaq (Enzynomics, Daejeon, Korea). Used primers were described in “Table 1”. PCR products were detected by 1.5% agarose gel electrophoresis with redsafe (iNtRON Biotechnology, Seongnam, Korea).

Table 1. Sequence of primer design

OX-2	Forward	5'-CCCATAGTATCCCTTCACTAC-3'
	Reverse	5'-GGAACTGAAAACCAATAGCC-3'
GAPDH	Forward	5'-GGCTGCTTTTAACTCTGGTA-3'
	Reverse	5'-ACTTGATTTTGGAGGGATCT-3'
CDH1	Forward	5'-CGAGAGCTACACGTTCACGG-3'
	Reverse	5'-GGGTGTCGAGGGAAAAATAGG-3'
CDH2	Forward	5'-AGCCAACCTTAACTGAGGAGT-3'
	Reverse	5'-GGCAAGTTGATTGGAGGGATG-3'
VIM	Forward	5'-CCCTCACCTGTGAAGTGGAT-3'
	Reverse	5'-TGACGAGCCATTTCCTCCTT-3'
HES1	Forward	5'-ATGGAGAAAAATTCCTCGTCCC-3'
	Reverse	5'-TTCAGAGCATCCAAAATCAGTGT-3'
HEY1	Forward	5'-GTGCGGACGAGAATGGAAAC-3'
	Reverse	5'-CTCGTCGGCGCTTCTCAATTA-3'
TCF	Forward	5'-TGCTGGTTCTCGTTGAGTGG-3'
	Reverse	5'-AGCACGTCCTTGAACACCAT-3'
CTNNB1	Forward	5'-TGGCAACCAAGAAAGCAAGC-3'
	Reverse	5'-TAGCACCTTCAGCACTCTGC-3'
GLI1	Forward	5'-CTGAGCCTTATGGAGCGAGG-3'
	Reverse	5'-TTCAAGTCGAGGACACTGGC-3'
GLI2	Forward	5'-CCTGTGCAGTGGAATGAGGT-3'
	Reverse	5'-GTAGGCCTTGCGGACTGTAG-3'

AXIN2	Forward	5'-CCGGTGGACCAAGTCCTTAC-3'
	Reverse	5'-CATCACCGACTGGATCTCGG-3'
ZEB1	Forward	5'-TTTCAGCATCACCAGGCAGT-3'
	Reverse	5'-TTGGCAGAACAACAGCTTGC-3'
MYC	Forward	5'-GCTCCTGGCAAAAGGTCAGA-3'
	Reverse	5'-CCAAGACGTTGTGTGTTCGC-3'
MMP9	Forward	5'-GCAATGCTGATGGGAAACCC-3'
	Reverse	5'-AGAAGCCGAAGAGCTTGTCC-3'

II.4. Western blot analysis

Cell lysates were extracted using RIPA buffer (Biosesang Inc., Seongnam, Korea) containing a phosphatase and proteinase inhibitor cocktail (GeneDEPOT, Barker, TX, USA). The cell lysate added lysis buffer and was incubated for 30 min in ice, followed by sonication on ice. The cell lysate was centrifugated at 16000 ×g 30 min at 4℃ and the supernatant collected to new ep tube. Protein concentration was measure by the absorbance at 660 nm using a Pierce™ 660 nm protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA). After protein was seperated by SDS polyacrylamide gel electrophoresis and transfered to polyvinylidene fluoride (PVDF) membrane (Merck, Kenilworth, NJ, USA). After in blocking with 5% skim milk in 1× PBS with 0.05% tween-20 (PBS-T) for 1 h, the membrane was incubated with primary antibodies in 5% bovine serum albumin in 1× PBS with 0.05% tween-20 (PBS-T) overnight at 4℃. The membrane were incubated with HRP-conjugated antibody (1:2500-1:5000) (Bethyl Laboratories, TX, USA) for 2 h at room temperature. PVDF membranes were detected using an ECL solution (Bio-Rad, Hercules, CA, USA) on a Supernova-Q1800 ChemiDoc (Lugen™, Bucheon, Korea).

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

Primary antibody name	Manufacturer	Product number	Dilution rate
OX-2	Santa Cruz	sc-53100	1 : 1000
CD200	R&D system	AF2724	1 : 2000
E-cadherin	Santa Cruz	sc-8426	1 : 1000
N-cadherin	Santa Cruz	sc-59987	1 : 1000
Vimentin	Santa Cruz	sc-6269	1 : 1000
β-catenin	Santa Cruz	sc-7963	1 : 1000
Lamin A/C	Santa Cruz	sc-376248	1 : 2000
DDDDK	MBL Intreanational	M185-3L	1 : 2000
GAPDH	Bioworld Tec.	AP0063	1 : 2000
β-actin	Santa Cruz	sc-47778	1 : 2000

II.5. Cell proliferation assay

Human gastric cancer cells (AGS and YCC-2) were seeded in 96-well plates (5×10^3 cells per well) and incubated at 37℃ in a humidified incubator with 5% CO_2 for overnight. Cells were transfected with vector plasmid (pCMV-3Tag-3A_Empty (E.V.) or pCMV-3Tag-3A_CD200). After transfection for 48 h, cell proliferation assay was performed using the Quanti-Max™ WST-8 cell viability assay solution (Daeil, Seoul, Korea). WST-8 solution was added to each well, the plates were incubated for another 1-2 h. After reaction, the plates were gently shaken, and the absorbance was measured at 450 nm using a microplate reader (TECAN, Spark, Männedorf, Switzerland).

II.6. Transwell invasion and migration assay

Human gastric cancer cells (AGS and YCC-2) were transfected with vector plasmid (pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200). After 24 h transfection, 2×10^4 cells in 200 μl FBS free medium and added to the upper transwell chamber (Corning Costar, MA, USA) on a filter coated with Matrigel (1:15) (BD Biosciences) coated filters for the invasion assay and 0.5 mg/ml collagen type I (BD Biosciences, Seoul, Korea) for the migration assay. RPMI1640 containing 10% FBS and 1% antibiotics was added to the lower chamber and the plates incubated for 20 h. Cells that invaded and migrated were visualized and quantified after hematoxylin and eosin staining. For quantification, cells were counted from ten randomly selected areas in each well using wide-field microscopy (Zeiss, Axio Scope.A1, Oberkochen, Germany). Data expressed as mean ± SEM from 10 independent experiments.

II.7. Wound healing assay

Human gastric cancer cells (AGS and YCC-2) were transfected with vector plasmid (pCMV-3Tag-3A_E.V. Or pCMV-3Tag-3A_CD200). After 24 h transfection, scrape cell layer by 1000 µl pipette tips in a straight line. After scratch, gently wash cell monolayer using PBS to remove detached cells. Immediately after making the scratch, a cell images is obtained using a microscope. Place in image using phase contrast microscope. Cells were incubated untill cells are confluent in the medium (0-48 h) and image the cells under a microscope every 24 h.

II.8. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using TOPreal™ SYBR Green qPCR PreMIX (Enzynomics, Daejeon, Korea). Used primers were described in “Table 1”. cDNA was amplified using Rotor-Gene 3000 real-time PCR system (Corbett Research, USA).

II.9. Luciferase reporter assay

AGS cells were seeded in 60 mm dishes. After overnight incubation, cells were transfected with 1.5 µg of the CSL, FOP-Flash and TOP-Flash Luciferase reporter vectors together 0.5 µg of expression vector plasmid (pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200). After incubation for 24 h, cells were harvest and luciferase assay was performed using luciferase assay system kit (Promega, Madison, WI, USA). After lysis the cells, cell lysates were reacted with substrate (luciferin) in a light-blocked 96 well plate. Luminescence were measured for 10 sec for each well.

II.10. Immunoprecipitation (IP)

AGS cells were transfected with vector plasmid (pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200). After 48 h transfection, transfected cells were lysed in IP lysis buffer containing a phosphatase and proteinase inhibitor cocktail (GeneDEPOT, Barker, TX, USA) on ice for 30 min, followed by sonication on ice. The cell lysate was centrifuged at 16000 ×g for 30 min at 4℃, and the supernatant collected. Cell lysates (1 mg) were incubated with 2 μg of anti-DDDDK antibody at 4℃ overnight with rotary agitation. Protein A/G PLUS-Agarose-beads (Santa Cruz Biotechnology, CA, USA) were added to lystaes and incubated for 2 h at 4℃ with rotary agitation. Beads were washed three times with cold 20% IP lysis buffer for 5 min at 4℃ and boiled for 5 min in 2× SDS sample loading buffer. Immunoprecipitates and whole-cell lysates (Input) were separated by SDS polyacrylamide gel electrophoresis and analyzed by western blot analysis.

II.11. Nuclear cytoplasmic fraction assay

AGS cells were transfected with vector plasmid (pCMU-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200). After 48 h transfection, Buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 20 mmol/L KCl, 1mmol/L DTT, 0.2 mmol/L PMSF and 0.1% NP-40] was added to the total cell lysates of AGS to isolate the cytosolic proteins. After incubation for 15 min on ice, cell lysates were centrifuged at 900 ×g for 10 min and the supernatant collected to new ep tube. The cytosolic proteins were resuspended using buffer A' (buffer A without NP-40) and centrifuged at 1500 ×g for 10 min. Once again cytosolic proteins in supernatant were collected. The nucleic proteins were extracted by adding buffer C [20 mmol/L HEPES (pH 7.9), 25% glycerol, 0.42 mol/L NaCl, 0.2 mmol EDTA, 1.5 mmol/L $MgCl_2$, 1 mmol/L DTT, and 0.2 mmol/L PMSF] to remaining pellet. Then nucleic proteins were incubated on ice for 30 min with vortexing every 10min and centrifuged at 16000 ×g for 10 min. The supernatant collected to the other new ep tube. Protein expression levels were analyzed by Western blot analysis.

II.12. Gene expression profile data and Kanplan-Meier analysis

The available datasets GSE19826, GSE54129, GSE56807, GSE63089 and GSE64951 were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). These five datasets were normalized using GEO2R and a scatter plot was obtained for the expression pattern analysis. The Cancer Genome Atlas (TCGA) evaluated the mRNA expression level of CD200 in gastric cancer from various aspect such as sample types (normal and primary tumor), individual cancer stages (normal, stages 1, 2, 3 and 4) and nodal metastasis status (normal, N0, 1, 2 and 3). Kaplan-Meier curves for overall survival, progression-free survival and post-progression survival in gastric cancer patients were generated using the online resource Kaplan-Meier Plotter (http://kmplot.com/analysis).

II.13. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc, San Diego, Ca, USA). Statistical analysis were calculated from at least three independent experiments. The data were analyzed using Student's *t*-test. Data from public database were used to determine differences in patients survival using the Kaplan-Meier plotter. Statistical significance for Kaplan-Meier estimated survival were calculated through a publicly available database. Results were presented as mean ± SEM. p values < 0.05 were considered to indicate statistical significance.

III. RESULTS

III.1. Analysis of CD200 expression from public the GEO datasets, TCGA data and publicly available data on survival probability in gastric cancer patients.

First, i confirmed the CD200 expression in gastric cancer patients to study the role of CD200 in gastric cancer. I compared the CD200 gene expression between normal tissue and gastric cancer tissue using the public database of five GEO datasets (Fig. 1). It was confirmed that CD200 mRNA was expressed higher in tissue of gastric cancer patient than in normal tissue. In addition, the expression level of CD200 according the cancer progression was confirmed in TCGA data (Fig. 2). In TCGA data, it was confirmed that the CD200 expression in the primary tumor was higher than normal tissue (Fig. 2A). It was confirmed that the expression level of CD200 increased with cancer progression. (Fig. 2B) And as nodal metastasis progressed, the CD200 expression level increased compared to that of normal (Fig. 2C). Next, to confirm the association between survival provability and CD200 expression level, Kaplan-Meier analysis and TCGA data were used in gastric cancer patients (Fig. 3). In all data of Kaplan-Meier analysis, it was confirmed that low overall survival provability (OS), low first progression survival provability (FPS) and low post progression survival provability (PPS) were shown when CD200 expression was high (Fig. 3A). Also, through TCGA data, it was confirmed that CD200 high expression showed a poor survival provability that the low and medium expression in gastric cancer patients (Fig, 3B). Therefore, CD200 showed high expression in gastric cancer patients and CD200 high expression was associated with poor survival provability.

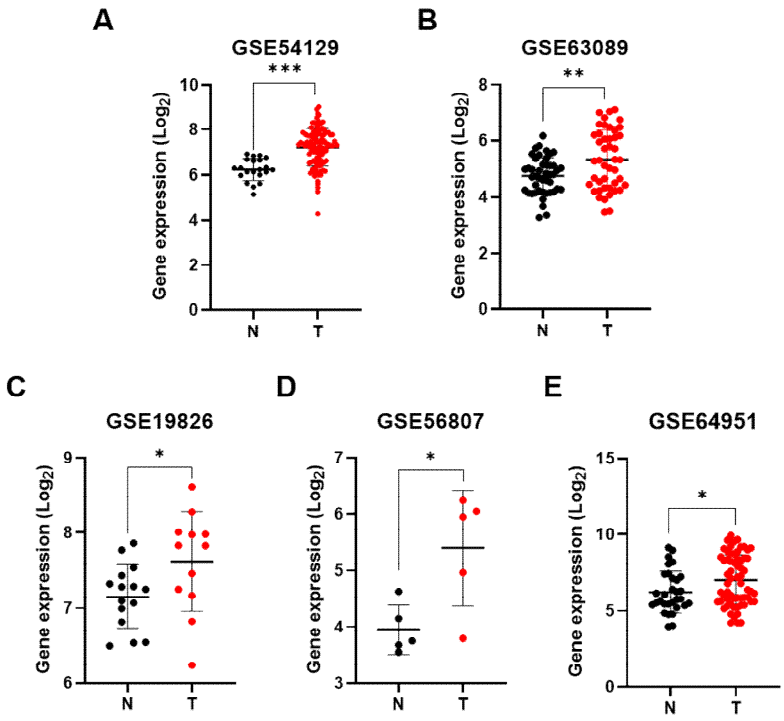

Figure 1. CD200 is up-regulated in gastric cancer patients. (A-E) CD200 mRNA expression levels in gastric cancer patients confirmed from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). (A) GSE54129 (n=132) (B) GSE63089 (n=90) (C) GSE19826 (n=27) (D) GSE56807 (n=10) (E) GSE64951 (n=94) The *p*-values evaluated using Student's *t*-test and significant differences are showed by * (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$).

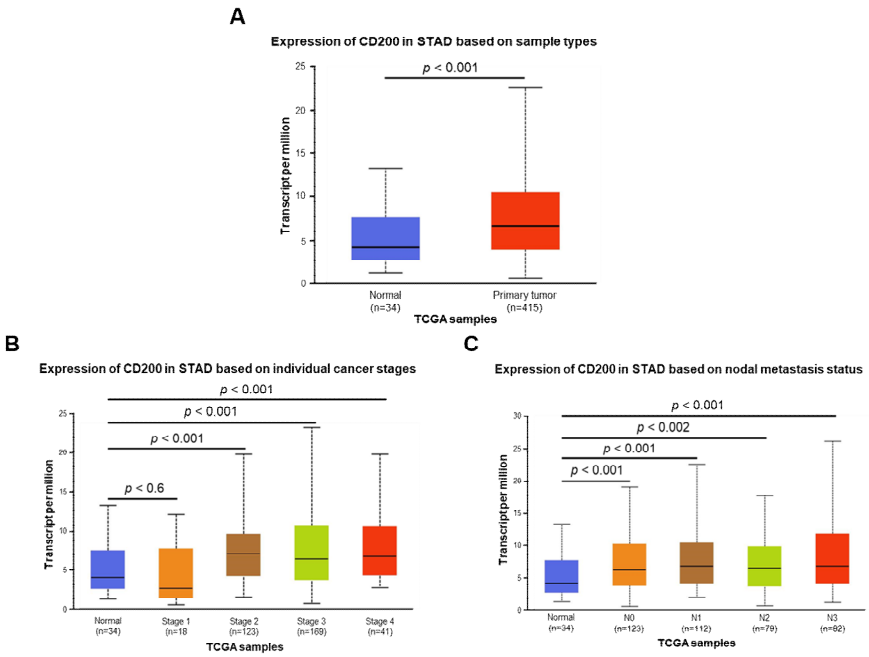

Figure 2. CD200 gene analysis of gastric cancer samples from TCGA database. Comparison of CD200 mRNA expression (A) between normal and gastric cancer samples, (B) individual cancer stages and (C) nodal metastasis status.

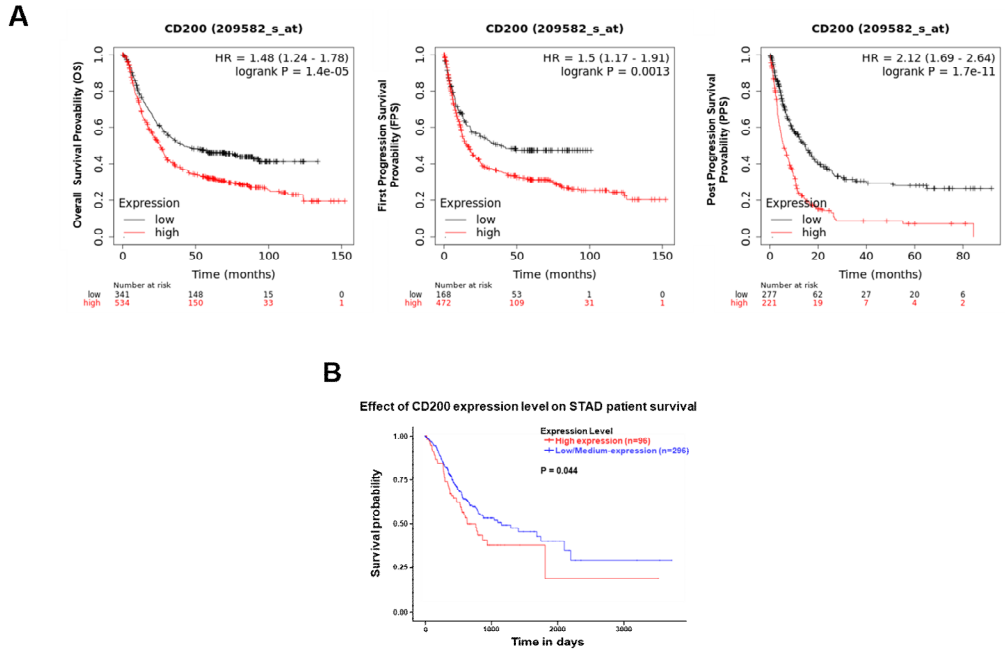

Figure 3. CD200 high expression leads to poor prognosis in gastric cancer patients. (A) Kaplan-Meier survival plots showed an associated between CD200 expression levels and poor survival rates in the public database of gastric cancer patients. (B) Survival curves of patients with high and low/medium-expression of CD200 in gastric cancer patients.

III.2. CD200 over-expression increases cell proliferation, invasion and migration in gastric cancer cells.

I confirmed the expression of CD200 in 7 cell lines of gastric cancer and a normal gastric epithelial cell (GES-1) using RT-PCR and western blot analysis (Fig. 4). The results confirmed that the expression level of CD200 was significantly higher in gastric cancer cells compared to normal gastric epithelial cells. Among them, i conducted subsequent experiment using low-expression AGS and YCC-2 cells.

First, the effect of over-expression of CD200 was investigated using AGS and YCC-2 cells with low CD200 expression levels. AGS and YCC-2 cells were transiently transfected with empty-vector (pCMV-3Tag-3A_E.V.) or CD200 over-expression vector (pCMV-3Tag-3A_CD200) for 48 h. The CD200 over-expression was confirmed by RT-PCR and western blot analysis (Fig. 5). Cell proliferation according to CD200 over-expression was confirmed by performing WST-8 assay (Fig. 6). When CD200 was over-expressed, it was confirmed that AGS cells increased 1.43-fold and YCC-2 cells 1.14-fold increased cell proliferation. Invaded and Migrated cells were confirmed using a transwell assay. When CD200 was over-expressed, invaded cells were enhanced by 136% in AGS cells and 147% in YCC-2 cells compared to control (Fig. 7). In addition when CD200 was over-expressed, migrated cells were confirmed to enhanced 143% in AGS cells and 134% in YCC-2 cells compared to control (Fig. 8). In addition, through wound healing assay performed to confirm the wound closure ability. When CD200 was over-expressed, it was confirmed that wound closure was increased more quickly (Fig. 9). Therefore it was confirmed that CD200 increased the cell proliferation and motility of gastric cancer cells. To determine whether CD200 over-expression affects epithelial-mesenchymal transition (EMT) related genes. Protein expression of EMT markers was analyzed by western blot analysis (Fig. 10). When CD200 was over-expressed, it was confirmed that increased mesenchymal markers N-cadherin and vimentin, while the epithelial marker E-cadherin decreased in protein expression.

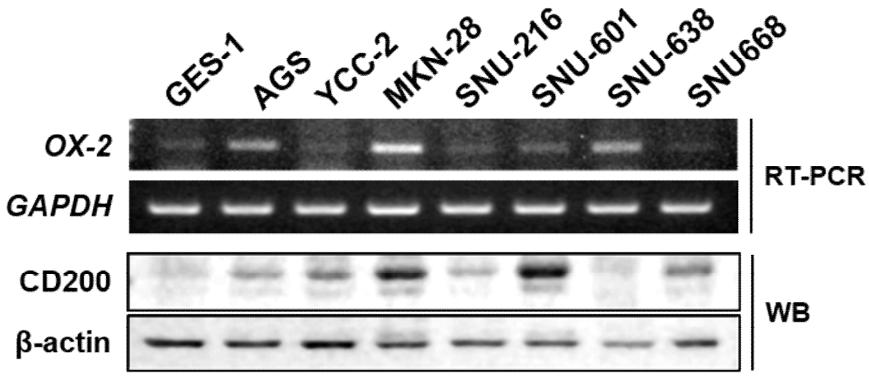

Figure 4. Endogenous mRNA and protein expression level of CD200 in gastric cancer cell lines. CD200 expression level in normal gastric epithelial cell (GES-1) and seven human gastric cancer cell lines (AGS, YCC-2, MKN-28, SNU-216, SNU-601, SNU-638 and SNU-668) were measured by RT-PCR and western blot. *GAPDH* and β–actin were used positive control.

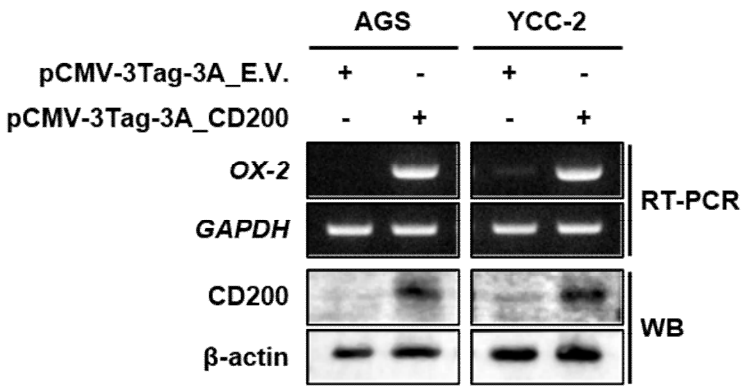

Figure 5. Increased CD200 expression levels by plasmid vector system. AGS and YCC-2 cells were transfected with pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200. CD200 over-expression levels were detected by RT-PCR and western blot. *GAPDH* and β-actin were used positive control.

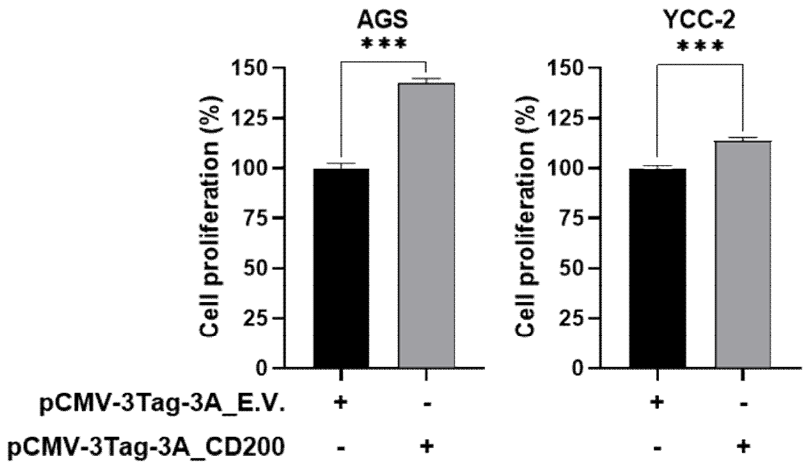

Figure 6. CD200 over-expression increases cell proliferation in gastric cancer cells. Cell proliferation was measured by WST-8 assay. The results were measured 48 h after transfection. Data is presented as mean ± SEM (n=12). The *p*-values are evaluated using Student's t-test and significant differences are showed by * (*** $p < 0.001$).

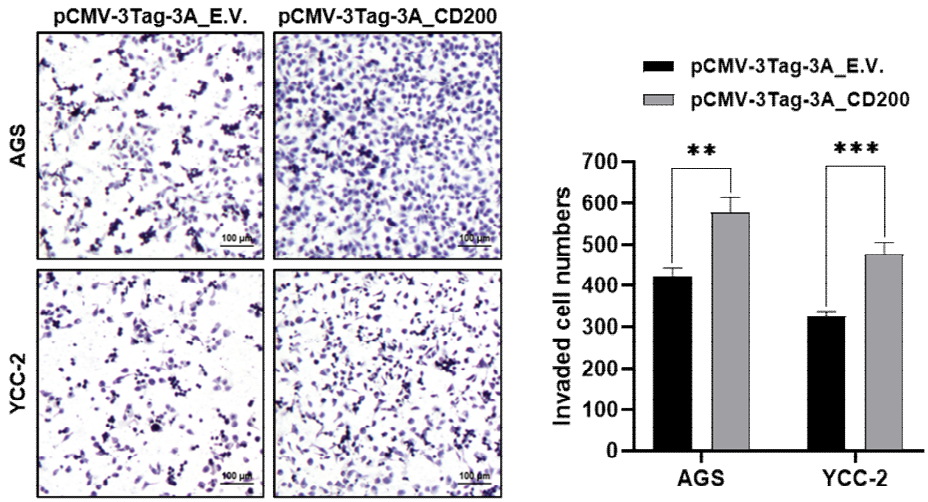

Figure 7. CD200 over-expression increases invaded cells in gastric cancer cells. Invaded cells detected by transwell assay. Random fields were taken by optical microscopy and cell numbers were counted. Invaded cells were visualized. Data is presented as mean ± SEM (n=10). The *p*-values are evaluated using Student's t-test and significant differences are showed by * (**$p < 0.01$; *** $p < 0.001$).

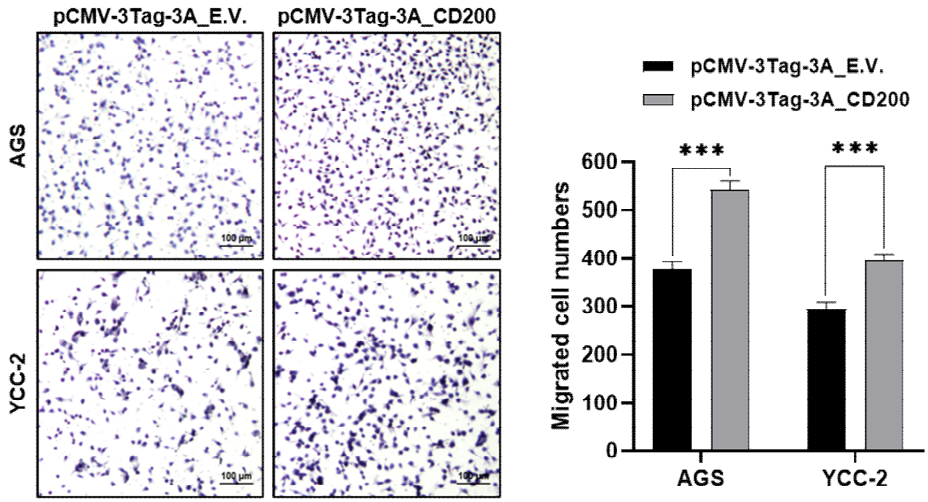

Figure 8. CD200 over-expression increases migrated cells in gastric cancer cells. Migrated cells detected by transwell assay. Random fields were taken by optical microscopy and cell numbers were counted. Migrated cells were visualized. Data is presented as mean ± SEM (n=10). The *p*-values are evaluated using Student's t-test and significant differences are showed by * (*** $p < 0.001$).

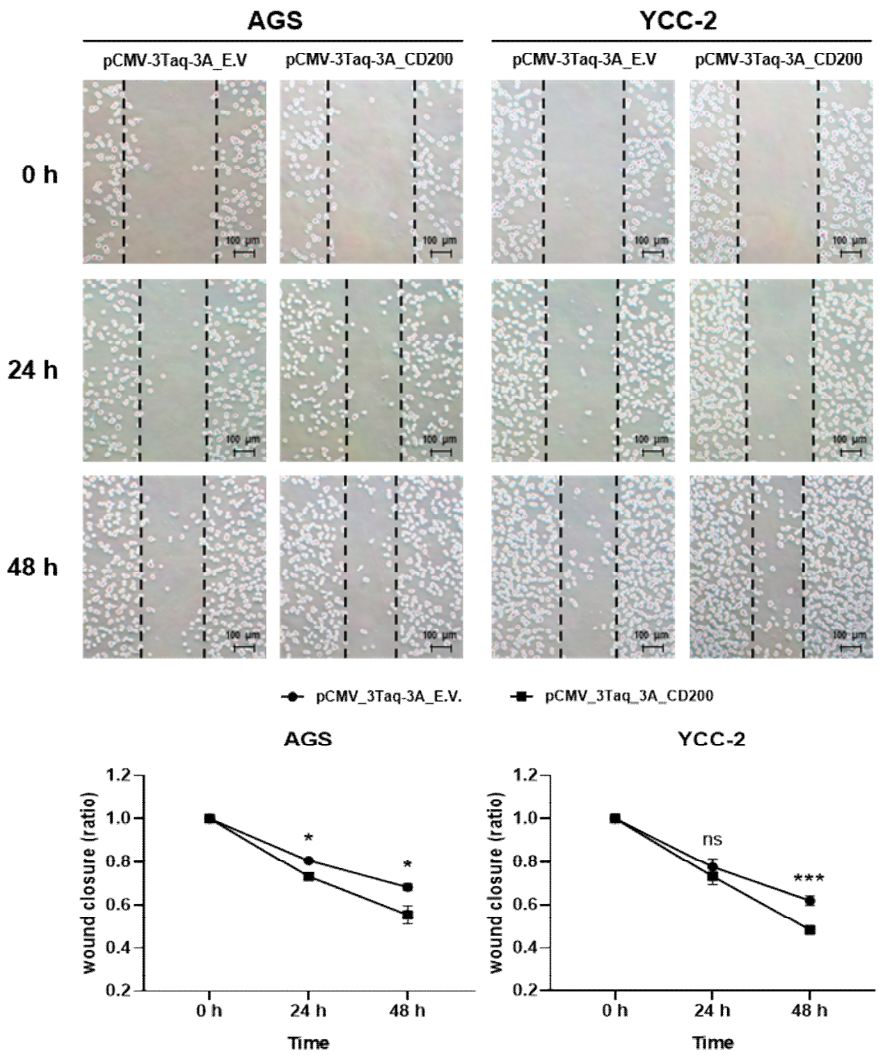

Figure 9. CD200 over-expression increases wound closure more rapidly. Wound closure measured by wound healing assay. Cell images were visualized. Data is presented as mean ± SEM (n=4). The *p*-values are evaluated using Student's t-test and significant differences are showed by * (ns: not significant; *$p<$ 0.05; ***$p<$ 0.001)

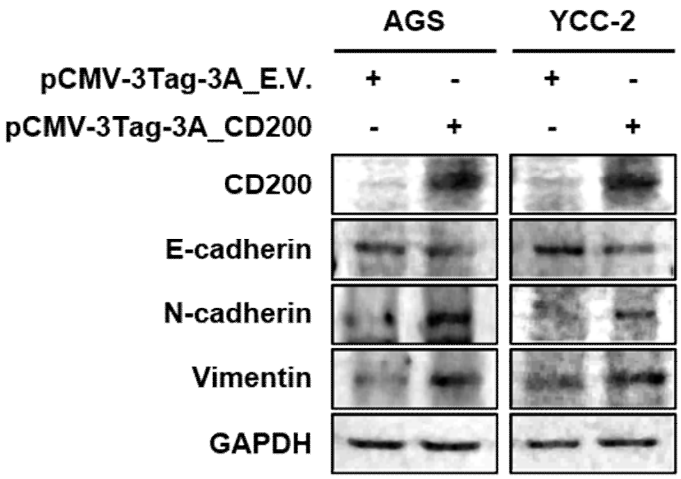

Figure 10. CD200 over-expression affects gene expression related to EMT. AGS and YCC-2 cells were transfected with pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200. Protein expression of EMT related gene was detected by western blot. GAPDH was used positive control.

III.3. CD200 is associated with WNT signaling pathway in gastric cancer cells

When CD200 is over-expressed, i tried to identify which signaling pathway causes phenotype changes such as increased proliferation and motility. Screening was performed on notch, WNT and sonic hedgehog signaling pathway, which are representative signaling pathways related to EMT in gastric cancer. Prior to screening, CD200 should be truncated to generate a CD200 cytoplasmic tail in order for CD200 to function in the intracellular region. It was confirmed that CD200 was truncated to generate a CD200 cytoplasmic tail in gastric cancer cells (Fig. 11). First, the expression of transcription factors acting in the three signaling pathways mentioned above was confirmed through qRT-PCR (Fig. 12). As a result, the mRNA expression of transcriptional factors in the WNT and sonic hedgehog signaling pathway was increased, but no significant difference was found in the notch signaling pathway. Then a luciferase assay was performed to measure the activity of transcriptional factors (Fig. 13). It was confirmed that the CSL transcriptional activity of the notch signaling pathway decreased (Fig. 13A) and the TCF4 transcriptional activity of WNT signaling pathway increased (Fig. 13B). Thus, i found that CD200 is related to the WNT signaling pathway.

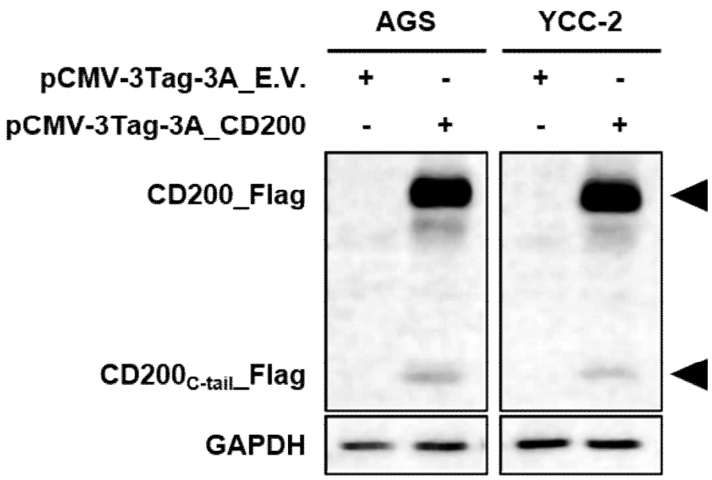

Figure 11. CD200 is truncated to generate a CD200 cytoplasmic tail in gastric cancer cells. AGS and YCC-2 cells were transfected with pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200. Protein expression of truncated CD200 form was detected by western blot. GAPDH was used positive control.

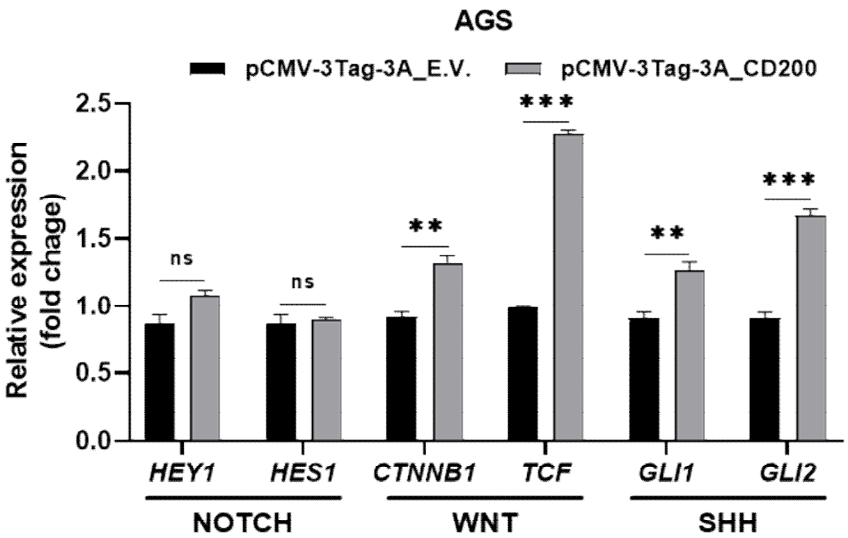

Figure 12. CD200 affects the transcription factor of WNT and sonic hedgehog signaling pathway in gastric cancer cells. AGS cells were transfected with pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200. mRNA expression of transcription factors were detected by qRT-PCR. Data is presented as mean ± SEM (n=3). The *p*-values are evaluated using Student's t-test and significant differences are showed by * (ns: not significant; ** $p < 0.01$; *** $p < 0.001$).

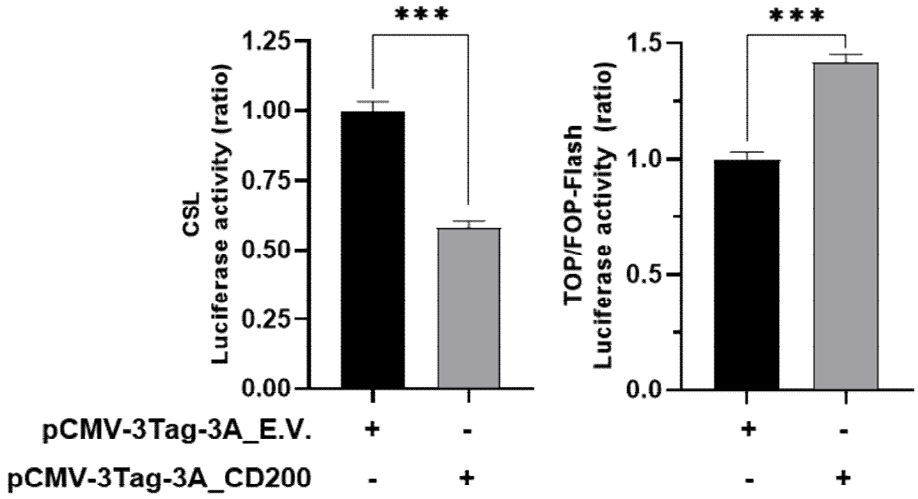

Figure 13. CD200 activates TCF4 transcriptional activity in gastric cancer cells. (A) AGS cells were transfected with CSL luciferase reporter vector. (B) AGS cells were transfected with TOP-Flash or FOP-Flash Luciferase reporter vector. Data is presented as mean ± SEM (n=6). The *p*-values are evaluated using Student's t-test and significant differences are showed by * (*** $p < 0.001$)

III.4. $CD200_{C\text{-}tail}$ directly interaction with β-catenin in gastric cancer cells

Based on the previous research that γ-secretase cleavages immune ligands and receptors and the cytoplasmic domain of CD200 is truncated by γ-secretase, nuclear translocation occurs and expression of transcription factor increases. I want to confirm the mechanism of CD200 in the cytoplasm. Therefore, IP assay was performed to confirm the interaction between β-catenin, a key molecule related to EMT in the WNT signaling pathway, and $CD200_{C\text{-}tail}$ (Fig. 14). As a result, it was confirmed that $CD200_{C\text{-}tail}$ and β-catenin were directly bound. Next, nuclear fraction assay was performed to confirm whether direct binding of $CD200_{C\text{-}tail}$ and β-catenin to nuclear translocation occurs (Fig. 15). It was confirmed that when $CD200_{C\text{-}tail}$ bind to β-catenin, total β-catenin expression and β-catenin nuclear translocation were increased. Therefore, when the cytoplasmic domain is truncated, $CD200_{C\text{-}tail}$ directly binds to β-catenin and increases β-catenin nuclear translocation.

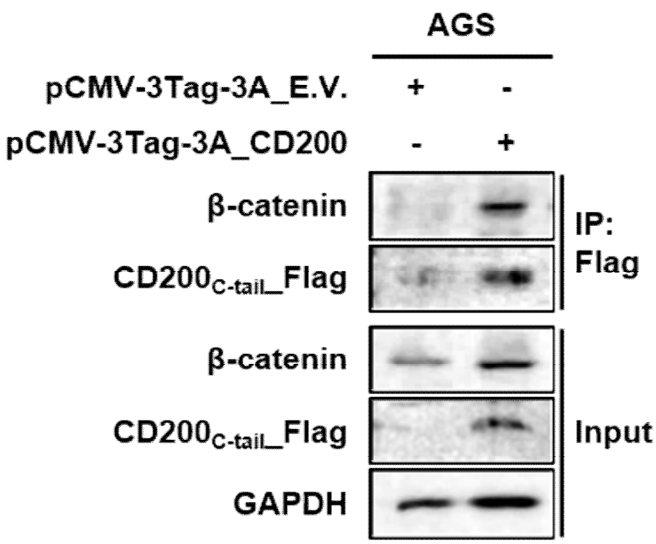

Figure 14. Direct interaction between $CD200_{C\text{-}tail}$ and β-catenin in gastric cancer cells. IP analysis with an anti-DDDDK antibody to detect $CD200_{C\text{-}tail}$ interaction with β-catenin in AGS cells. The amount of protein in input lanes are 15 μg. The protein interaction of β-catenin and $CD200_{C\text{-}tail}$ was detected by western blot. GAPDH was used positive control.

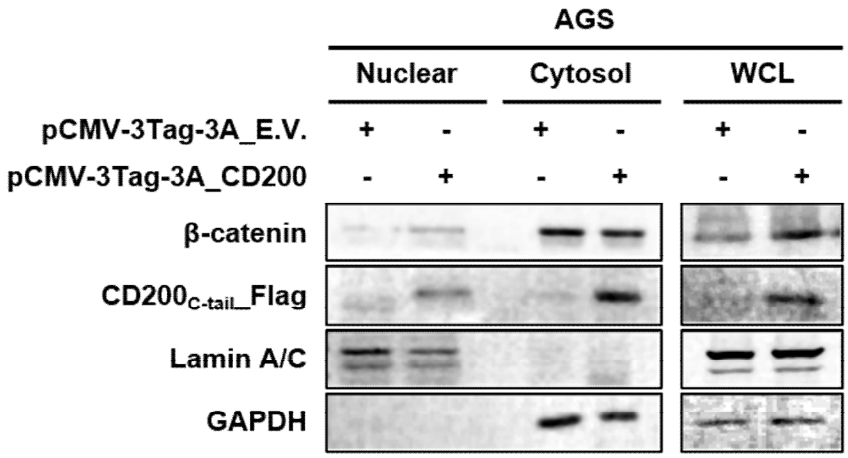

Figure 15. Increases total β-catenin expression and β-catenin nuclear translocation when $CD200_{C\text{-}tail}$ is generated. AGS cells were transfected with pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200. The protein expressions of β-catenin and $CD200_{C\text{-}tail}$ in nuclear, cytosol and whole-cell lysate (WCL) were detected by western blot. Lamin A/C and GAPDH were used positive control.

III.5. $CD200_{C\text{-}tail}$/β-catenin complex affects the β-catenin target gene expression

It was confirmed that nuclear translocated $CD200_{C\text{-}tail}$/β-catenin complex acts as a transcription factor and enhances the β-catenin target gene expression. qRT-PCR was performed to confirm the β-catenin target gene expression involved in cancer progression and metastasis (Fig. 16). When $CD200_{C\text{-}tail}$/β-catenin complex translocated to the nucleus, it was confirmed that the expression of *ZEB1* and *MMP9* involved in cancer metastasis increased.

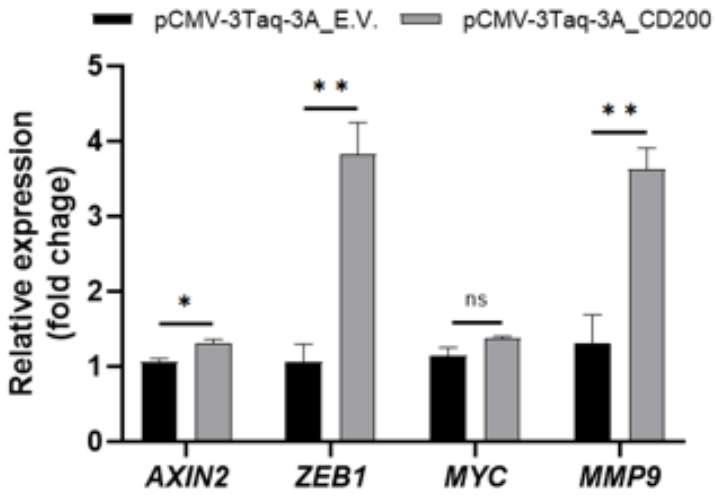

Figure 16. $CD200_{C\text{-}tail}$/β-catenin complex affects the downstream target genes of WNT/β-catenin signaling pathway. AGS cells were transfected with pCMV-3Tag-3A_E.V. or pCMV-3Tag-3A_CD200. β-catenin target gene mRNA expression was detected by qRT-PCR. Data is presented as mean ± SEM (n=3). The *p*-values are evaluated using Student's t-test and significant differences are showed by * (ns: not significant; *$p < 0.05$; **$p < 0.01$).

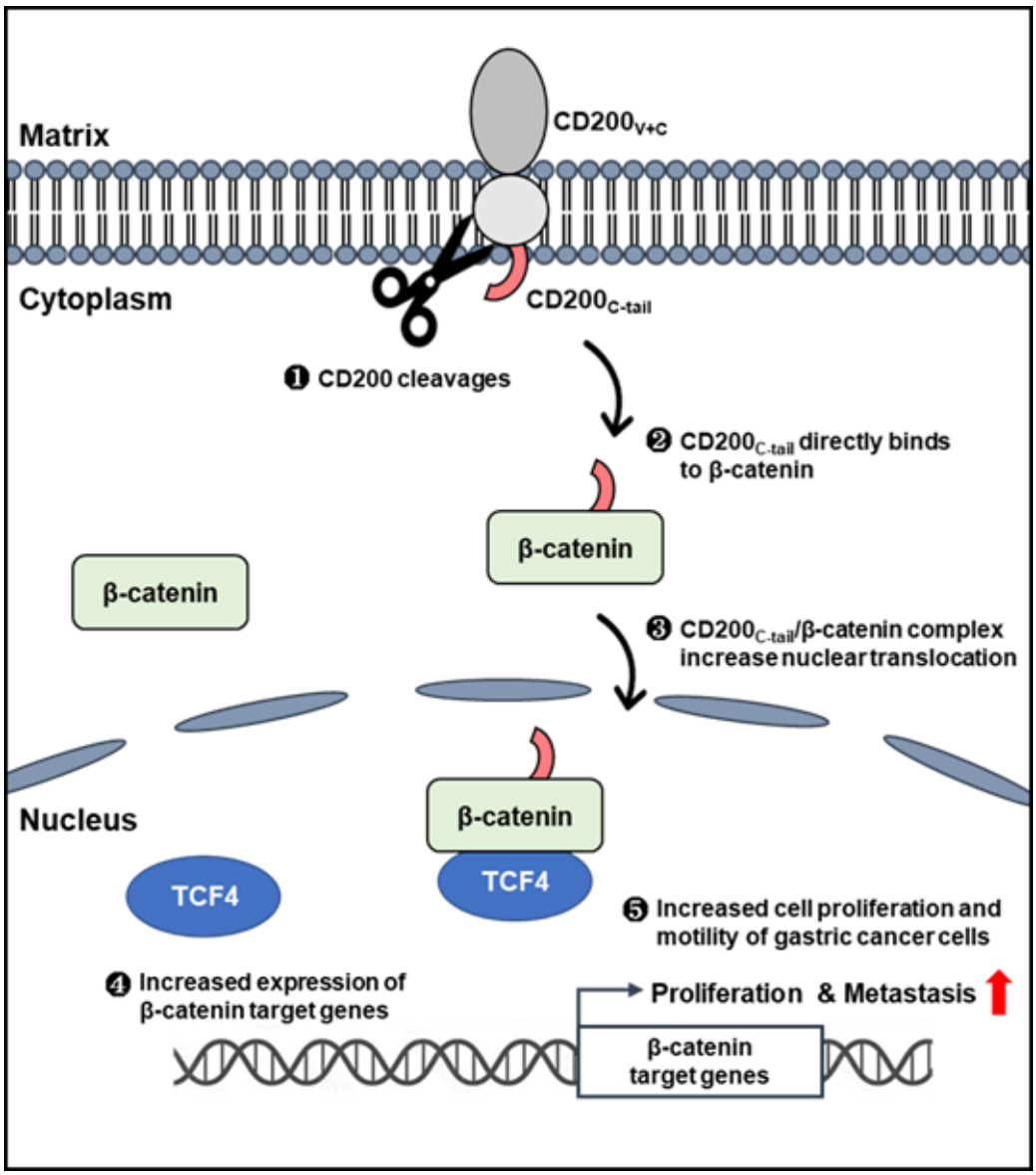

Figure 17. Schematic diagram explaining the molecular mechanism that CD200 is involved in the progression and metastasis of gastric cancer by regulating the WNT/ β-catenin signaling pathway.

IV. DISCUSSION

Currently, as the importance of precision medicine increases in cancer treatment, trend of cancer treatment are shifting toward targeted therapy and immunotherapy. Due to the chemoresistance responses seen in chemotherapy, targeted therapy is becoming a necessity rather than an option, despite some limitations (32). Immunotherapy is developing centering on T cell such as CTLA-4 and PD-1/L1 (33). However, despite steady research, there are very few molecules that can be used as target (33). Therefore, continuous discovery and research of new target molecules based on molecules are required (34). CD200 is attracting attention as a new target molecule for immunotherapeutic agents (35). In fact, when CD200 was blocked using a CD200 antibody, it was verified that anti-tumor response was improved in solid tumor and mouse models (36, 37). Through this study, i would like to propose CD200, which is involved in innate immune and cancer progression, as a new potential immunotherapeutic target involved in innate immunity, unlike conventional adaptive immune.

High expression of CD200 and poor prognosis were confirmed in various carcinomas (20-30). First, i analyzed using public data to confirm the CD200 expression in gastric cancer patients. It was confirmed that the CD200 expression level was high in mRNA obtained from gastric tumor tissue compared to normal in GEO datasets (Fig. 1). It was confirmed that the CD200 expression level was higher in gastric primary tumors compared to normal and it was confirmed that the expression of CD200 increased as gastric cancer stages and metastasis progressed in TCGA data (Fig. 2). As a result of confirming survival probability using Kaplan-Meier plot and TCGA data, it was confirmed that high expression CD200 showed low survival probability (Fig. 3). Therefore, it was confirmed that CD200 showed high expression in gastric cancer patients and CD200 high expression was associated with poor prognosis.

Second, based on the study that CD200 affects cancer stem cell, metastasis and progression in various cancer types (23-30), i verified *in vitro* whether CD200 affects progression and metastasis in gastric cancer cell lines. When CD200 was

over-expressed, cell proliferation, invasion, migration and wound closure increased (Fig. 6-9). Additionally, i confirmed the expression of EMT-related marker that affect cancer metastasis (38). As a result, it was confirmed that increased the mesenchymal markers N-cadherin and vimentin, while the epithelial cell marker E-cadherin decreased (Fig. 10).

Finally, to identify which signaling pathways causes these phenotype and gene expression changes, three representative signaling pathway related to EMT in gastric cancer, notch, WNT and sonic hedgehog, were selected (39-41). In gastric cancer cells, CD200 truncated to confirmed the formation of a cytoplasmic tail that precedes the signaling pathway activation in the intracellular region (Fig. 11). When the expression of each transcription factor of each signaling pathway was confirmed, increased expression was confirmed in WNT and sonic hedgehog (Fig. 12). Through luciferase assay, it was confirmed that the transcriptional activity of TCF4 in WNT signaling was increased (Fig. 13). Therefore, it was confirmed that CD200 is related to the WNT signaling pathway. I wanted to confirm which molecules the cytoplasmic tail of CD200 interacts with inside cells. Therefore, it was confirmed that it affects metastasis and interact with β–catenin, a key molecule in the WNT signaling pathway (42). Direct binding between β–catenin and CD200 cytoplasmic tail was confirmed (Fig. 14), and β–catenin nuclear translocation increased when CD200 cytoplasmic tail was generated (Fig. 15). When nuclear translocation of CD200 cytoplasmic tail/β-catenin complex increased, expression of genes related to metastasis among WNT/β–catenin target genes was increased (Fig. 16).

In conclusion, in this study, i verified the molecular mechanism of CD200 in the intracellular region in gastric cancer cells. It was revealed that the CD200 cytoplasmic tail directly binds to β–catenin, increases β–catenin nuclear translocation and increases the expression of β–catenin target genes, thereby increasing gastric cancer cell progression and metastasis (Fig. 17). I suggest that CD200, as a dual target, is a potential target for immunotherapy and target for inhibiting the progression of gastric cancer.

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