2020년 2월

석사학위 논문

The role of up-regulated SPON2 through Notch signaling pathway in gastric cancer progression

조선대학교 대학원

생명과학과

강 현 구

The role of up-regulated SPON2 through Notch signaling pathway in gastric cancer progression

위암에서 Notch 신호전달을 통해 증가하는 SPON2의
역할에 대한 연구

2020년 2월 25일

조선대학교 대학원

생명과학과

강 현 구

The role of up-regulated SPON2 through Notch signaling pathway in gastric cancer progression

지도교수 김 석 준

이 논문을 이학 석사학위신청 논문으로 제출함

2019년 10월

조선대학교 대학원

생명과학과

강 현 구

강현구의 석사학위논문을 인준함

위원장 조선대학교 교 수 전 홍 성 (인)

위 원 조선대학교 교 수 김 정 수 (인)

위 원 조선대학교 교 수 김 석 준 (인)

2019년 11월

조선대학교 대학원

CONTENTS

LIST OF TABLES

LIST OF FIGURES

국 문 초 록

위암에서 Notch 신호전달을 통해 증가하는 SPON2의 역할에 대한 연구

강 현 구
지도교수 : 김석준
생명과학과
조선대학교 대학원

세포외 기질 단백질인 Spondin-2 (SPON2)는 위암 환자의 조직에서 과발현되었으며 발현 수준이 높은 환자는 발현 수준이 낮은 환자에 비해 생존 기간이 짧았다. 하지만 SPON2의 발현과 위암의 진행 간에 상관관계에 대해서 알려진 바가 없다. 따라서 SPON2의 발현이 위암에 미치는 역할에 대해서 연구하고자 하였다.

먼저 일곱 가지의 위암 세포주와 한 가지의 정상 위 내피세포에서 SPON2의 발현량을 확인하였다. 대부분의 위암 세포주는 위 정상 내피세포와 비교하여 높은 SPON2의 발현 수준을 보였다. 그 후 작은 간섭 RNA와 과발현 벡터를 이용해 위암 세포주의 SPON2 발현 수준을 조절한 뒤 위암의 진행과 관련된 다양한 세포 생물학적 기능을 확인하였다. 그 결과 SPON2 발현의 억제는 위암 세포의 세포 증식, 세포 이동 및 침투와 관련된 기능이 유의하게 감소하였으며 반대로 SPON2의 과발현은 세포 증식, 세포 이동 및 침투가 증가하였다. 다음으로 *In vivo*에서 종양 성장에 미치는 SPON2의 역할을 확인했다. 먼저 렌티바이러스를 이용하여 위암 세포주가 SPON2의 발현을 안정적으로 억제하도록 하였다. 그리고 Balb/c 누드 마우스의 피하지방층에 세포주를 주사한 뒤 47일간 관찰하였다. 놀랍게도 SPON2가 억제된 마우스는 대조군과 비교하여 감소한 종양 성장을 보였다.

앞서 SPON2의 발현이 위암의 진행과 상관관계가 있음을 확인하였으며 추가로 위암에서 SPON2가 상향 조절되는 기전에 대해서 연구하고자 하였다. 공개적으로 사용 가

능한 microarray 자료를 이용하여 위암 환자에서 SPON2의 발현 수준과 상관관계가 있는 신호전달을 조사했다. 그 결과 Notch 신호전달 관련 수용체 NOTCH1의 발현이 SPON2 발현 수준과 높은 상관관계가 있음을 확인했다. 다음으로 Notch 신호전달 관련 전사 인자가 SPON2 프로모터 영역에 결합하는 것을 검증했다. 위암 세포주에서 활성화된 NOTCH1의 과발현은 SPON2의 발현 및 세포 증식, 이동 및 침투를 증가시켰으며 SPON2를 억제함으로써 차단되었다. 또한 Notch 신호전달 활성을 억제하는 γ-secretase 억제제를 처리하였을 때 SPON2의 발현은 농도 의존적으로 감소하였다. 모든 연구 결과에 따라 Notch 신호전달이 위암의 진행을 촉진하기 위해 SPON2의 발현을 조절한다고 결정했다.

이 연구는 Notch 신호전달에 의해 증가한 SPON2가 *In vitro* 및 *In vivo*에서 위암의 공격적인 진행과 관련이 있음을 입증했다. 따라서 SPON2 유전자를 위암의 진행을 억제할 수 있는 표적 유전자로써의 가능성을 제시한다.

ABSTRACT

The role of up-regulated SPON2 through Notch signaling pathway in gastric cancer progression

Hyeon-Gu Kang
Advisor : Prof. Seok-Jun Kim. Ph.D.
Department of Life science
Graduate School of Chosun University

Extracellular matrix protein Spondin-2 (SPON2) was over-expressed in the gastric cancer patients, and higher expression levels showed shorter survivals than low expression levels. However, correlation is not known between SPON2 expression and gastric cancer progression. Therefore, the purpose of study was to investigate the role of SPON2 in gastric cancer progression.

At first, SPON2 expression levels were confirmed in seven gastric cancer cell lines and one gastric normal endothelial cell. Most gastric cancer cell lines showed that higher expression level of SPON2 compared to normal gastric endothelial cell. Subsequently, small interfering RNA and over-expression vector were used to regulate SPON2 expression in gastric cancer cell lines, and verified various cell-biological functions related to gastric cancer progression. Silencing of SPON2 expression decreased functions of cell proliferation, migration and invasion. In contrast, SPON2 over-expression increased cell proliferation, migration and invasion. Next, the role of SPON2 in tumour growth was examined. Gastric cancer cell lines were stably suppressed SPON2 expression by lenti-virus infection. The stable cells were xenografted into the subcutaneous layer of Balb/c mice and observes for 47 days. Interestingly, silenced SPON2 expression showed reduced tumour growth in

xenografted mice model.

The determined that SPON2 expression correlates with gastric cancer progression was studied. Second, i studied mechanism of SPON2 up-regulation in gastric cancer. Signaling pathways associated with SPON2 expression levels were investigated using publicly available microarray data. The results showed that Notch signaling associated receptor (NOTCH1) expression was highly correlated with SPON2 expression levels. Notch signaling and SPON2 showed similar expression patterns in seven gastric cancer cells. Next, it was examined whether Notch signaling pathway related transcription factor binds to the promoter region of SPON2 using predictive program and a significant result confirmed through chromatin immunoprecipitation. In addition, over-expression of Notch1 receptor intracellular domain (N1ICD) increased cell proliferation, migration and invasion as well as enhanced expression levels of SPON2. However, these effects were blocked by silencing of SPON2 expression. Inhibition of Notch signaling by γ-secretase inhibitor (GSI) reduced the SPON2 expression and cell viability in gastric cancer cells. All results provided evidence that Notch signaling regulates SPON2 expression to promote gastric cancer progression.

I demonstrated that increased SPON2 expression by Notch signaling is associated with aggressive progression of gastric cancer in *In vitro* and *In vivo*. In conclusion, this study suggests that SPON2 inhibits the progression of gastric cancer as a potential target gene.

I. INTRODUCTION

Cancer is the number one cause of human death in the world and to date has the second highest mortality rate each year (1). Cancer is tissue-specific in various organs of the human, and the regulatory mechanisms are also different. Among them, gastric cancer is the fifth most common type incidence and the second leading cause of cancer related deaths worldwide (2). More than 50% of cases occur in eastern asia and are difficult to therapy (3). The reason for the high death rate compared to the incidence rate is poor prognosis due to high probability of relapse and metastasis. Despite the diagnostic, surgical and pharmacological approaches, the survival rate of gastric cancer patients is still poor (4-8). Recently, many researchers have been studying on metastasis of gastric cancer, but the metastasis mechanism is still unclear (9-11). Therefore, my goal was to focus on improving the prognosis of gastric cancer and understanding the molecular mechanisms for metastasis of gastric cancer.

Spondin-2 (SPON2, also known as Mindin, DIL1 or M-Spondin) is an extracellular matrix protein known to bind to integrin receptors (12). Initially reported as a diagnostic marker specific for prostate cancer (13). Recent studies have shown that up-regulation of various carcinomas, including colorectal carcinoma, hepatocellular carcinoma, laryngeal squamous cell carcinoma, and gastric cancer as well as prostate cancer (14-17). Function of SPON2 regulates M1-like macrophage recruitment, hippo pathway, and is involved in endothelial mesenchymal transition (18). In addition, colorectal cancer has been shown to be up-regulated by the transcription factors MACC1 or Egr-1 to be involved in angiogenesis and metastasis (19-20). According to reports, SPON2 has a positive regulation of cell migration and invasion and also secreted by thyroid hormones (21).

Thus, SPON2 has been shown to be differentially expressed in various carcinomas and involved in the poor prognosis of cancer. In gastric cancer, SPON2 was increased compared to normal gastric tissues, and patients with high expression had poor prognosis (17). However, mechanism responsible for the up-regulation of SPON2 expression or activation needs to be investigated, since the role of SPON2 in gastric cancer remains

unclear. Therefore, i used the SPON2 specific small interfering RNA to confirm the mechanism of SPON2 in gastric cancer cells. I identified genes that were differentially expressed using microarray analysis in SPON2 silencing gastric cancer cells. As a result, the inhibition of SPON2 expression differentially expressed numerous genes, and these genes were shown to have various biological functions. Expression of SPON2 increased the proliferation, migration and invasion in gastric cancer cell lines. In addition, knocked-down of SPON2 expression in xenograft mice models decreased tumor growth.

SPON2 has been shown to correlate with gastric cancer progression. I wanted to know about the upstream regulatory mechanisms of SPON2. To understand the mechanism of SPON2 regulation, i inserted a SPON2 transcriptional regulatory motif into the luciferase reporter plasmid to confirm the promoter activity. I found the position of the active motif and assumed that CBF-1/RBP-Jk, a notch signaling related transcription factor, binds to the motif identified through the TFs prediction program.

Notch signaling were highly expressed in gastric cancer (22). Also, the correlation with SPON2 was very high. Notch signaling is a cascade that plays an important role in development, homeostasis and cell differentiation (23). In mammals there are four receptors (Notch1-4) and five ligands (Delta-like ligand1,3,4 and Jagged1,2) (24). Both receptors and ligands are transmembrane proteins, and signals induce due to cell-to-cell interaction of signal modules. The mechanism of action is the interaction between the receptor and ligand, followed cleave Notch extracellular domain (ECD) by ADAMTS protease (25). The result of the cleaved domain is exposure to the γ-secretase recognition region. Receptors recognized by γ-secretase cleave intracellular domains (ICD) and release domains into cells. The Notch intracellular domains translocate into the nucleus and binds to the CSL domain. The finish of the cascade is the expression of the target gene. Expressions of target genes show a malignant phenotype for cancer involvement in tumor development, metastasis, angiogenesis and epithelial mesenchymal transition (26).

My research indicates that Notch signaling cascade is one of the important mechanisms regulating SPON2 transcription. Over-expression of N1ICD increased the expression of SPON2. In contrast, treatment with γ-secretase inhibitor decreased expression of SPON2. N1ICD induced cell proliferation, migration and invasion was recovered by silencing of SPON2 in gastric cancer cells.

Despite the previous reports that SPON2 is highly expressed in gastric cancer and associated with poor prognosis, its function and mechanism are still unclear. My research provides new insights into the role of SPON2 in the gastric cancer progression, and suggests potential as a targeting molecule in gastric cancer therapy or as a biomarker for prognosis and diagnosis.

Table 1. Sequence of primer design

SPON2	Forward	5'-GGGAGAGTCCATCTGTTCCG-3'
	Reverse	5'-CACAAACGAGACCAGCGAGTG-3'
NOTCH1 (N1ICD)	Forward	5’-GACGAGTACAACCTGGTGCG-3’
	Reverse	5’-AGGTAGCCATGGGGTGACTC-3’
GAPDH	Forward	5’-GGCTGCTTTTAACTCTGGTA-3’
	Reverse	5’-ACTTGATTTTGGAGGGATCT-3’
SPON2 ChIP	Forward	5’-GGCACGGGTGTGAGGAGGGG-3’
	Reverse	5’-AGTGTCTGGCTGCCTCTCAGG-3’
SPON2 -1500bp	Forward	5’-GATCCTCGAGGAGGCCTCTGCTCCCTG CCCTC-3’ (*XhoI*)
SPON2 -1000bp	Forward	5’-GATCCTCGAGTCCCACTCAGCTGGCCT CATTG-3’ (*XhoI*)
SPON2 -500bp	Forward	5’-GATCCTCGAGCCTCACAGGAGCGCCTC TGGTG-3’ (*XhoI*)
SPON2 0bp	Reverse	5’-GATCAAGCTTTCCGACGACACCGACA AAGGAG-3’ (*HindIII*)

II. MATERIALS AND METHODS

II.1. Human gastric cancer tissues microarray and Immunohistochemistry

Tissue microarrays containing a total of 45 patient tissue samples were purchased from US Biomax, Inc. (BS01012c, MD, USA). Tissue-fixed paraffin was removed using xylene and hydrated sequentially with 100%, 95%, 70% ethyl-alcohol. Slides were left to stand in boiling antigen unmasking solution at 100°C for 15 min. Tissue slides were washed and incubated in 1% hydrogen peroxide for 10 min. Next, blocked for 30 min, and SPON2 specific antibodies (sc-166868, 1:200, Santa Cruz, CA, USA) were treated for 90 min. And incubated in HRP-conjugated secondary antibodies for 1 h. ABC kits (Vector Laboratories Inc., CA, USA) were used to amplify the efficiency of HRP and stain the antigen with 3,3'-Diaminobenzidine (DAB). Washed with PBS between all steps of the process. The nucleus of tissues were lightly stained with haematoxylin. Finally stained slides were again dehydrated stepwise with 70%, 95%, 100% ethyl-alcohol. Evaluation of the immunohistochemical expression levels was conducted by professional pathologist.

II.2. Cell culture

The human gastric cancer cell lines (AGS, MKN28, SNU-216, -601, -638, and -668) were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). YCC-2 cells, HEK293FT cells and human normal gastric epithelial cells (GES-1) were from Yonsei Cancer Center (Seoul, Korea). All cell lines except HEK293FT cells were cultured in RPMI1640 medium (Welgene, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS) (Corning Costar, NY, USA) and 1% antibiotics (including streptomycin/penicillin, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified incubator with 5% CO_2. HEK293FT cells were cultured in high glucose DMEM (Welgene, Gyeongsan, Korea) supplemented with 10% FBS and 1% antibiotics. Cells were maintained as adherent culture and passaged once every 2-3 days.

II.3. Chemical treatment

γ-secretases (GSIs); cbz-IL-CHO (GSI-I, Z-LLNle-CHO) were purchased from Calbiochem (Billerica, MA, USA) for treatment on the cells. GSIs were dissolved 10 mM concentration in dimethyl sulfoxide. Dissolved GSIs were treated 1 μl/ml (1000x) in the medium and serially diluted to adjust the concentration. The GSIs were treated to cells for 24 h. Inhibition of cell viability by GSIs alone was measured using the WST-1 assay.

II.4. Transfection of siRNA and construction

Transfection of human SPON2 siRNA and plasmid DNA were using Lipofectamine RNAiMAX reagent or Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). First the cells were plated in medium without antibiotics and incubated overnight. Transfection reagent, plasmids and siRNA were diluted in Opti-MEM medium (Gibco, Waltham, MA, USA). Diluted materials were mixed and reacted for a while and treated to incubated cells. After 5 h the cells were washed with DPBS and replaced with RPMI1640 culture medium. The coding strands of the SPON2 siRNA purchased from Genolution Inc. (Seoul, Korea), was sequence: 5'-GCGCAUAGCUCCGACUACUU-3'. Human SPON2 clone used pCMV-SPORT6_SPON2 (ID:hMU004103). The human SPON2 construct was provided by the Korea Human Gene Bank (Medical Genomics Research Center, KRIBB, Korea). SPON2 promoter site was cloned into the pGL3-Basic plasmid in the Hind3 and Xho1 restriction enzyme sites, creating the luciferase reporter plasmid construct. The genomic DNA was PCR-amplified using the primer (Table 1). Genomic DNA was isolated from SNU-601 cells.

II.5. 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 reagent and added 200 μl of chloroform. After reaction, centrifuged at 16000 xg for 13 min. Separated supernatant was transferred to new tube and added 2-propanol. Once again, centrifuged at 16000 xg for 10 min. After removing the supernatant, precipitated RNA was dissolved in water containing diethyl pyrocarbonate (DEPC). cDNA synthesis was carried out using a reverse transcription system (Toyobo, Osaka, Japan), and PCR was performed using nTaq DNA polymerase premix (Enzynomics, Daejeon, Korea). Used primers were described in “Table 1”. PCR products were detected by agarose gel electrophoresis with redsafe (iNtRON Biotechnology, Seongnam, Korea).

II.6. Western blot analysis

Briefly, cells were lysed in RIPA buffer (Biosesang Inc, Seongnam, Korea) containing phosphatase and protease inhibitor cocktail (GeneDEPOT, Barker, TX, USA), followed by sonication on ice. The cell lysate was centrifuged at 16000 xg for 20 min, and the supernatant collected. Protein concentration was measured by the absorbance at 660 nm using a Pierce™ 660nm protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA). After protein (20 μg) was separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Merck, Kenilworth, NJ, USA). After blocking with 5% skim milk for 1 h, the membrane was incubated with primary antibodies in 5% bovine serum albumin (BSA) overnight at 4°C. The following antibodies were used: anti-Mindin (SPON2, sc-166867, 1:1000), anti-RBP-jk (sc-271128, 1:1000) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-activated Notch1 (ab8925, 1:2000) (Abcam, CAM, UK) detected cleaved-Notch1 intracellular domain (N1ICD). Anti-β-actin (sc-47778, 1:1000) and anti-GAPDH (AP0063, 1:5000) (Bioworld, OH, USA) were used as the loading control. The membranes were incubated with HRP-conjugated antibody (1:5000) (Bethyl Laboratories, TX, USA) for 90 min, followed by detection with an ECL kit (Bio-Rad, Hercules, CA, USA) using Supernova-Q1800.

II.7. Cell proliferation assay

Human gastric cancer cells (AGS, MKN28, SNU-601, and SNU-668) were seeded in 96-well plates (4×10^3 cells per well). The following morning, cells were transfected with siRNA (scRNA or SPON2 siRNA) and vector plasmid (pCMV-SPORT6_Empty (E.V) or pCMV-SPORT6_SPON2). Cell proliferation assays were performed using the cell permeable tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). WST-1 assay solution (Daeil Lab Services Co., Ltd, Seoul, Korea) was added to each well 48 h after transfection. The plates were incubated for another 1-2 h and gently shaken and the absorbance measured at 450 nm.

II.8. Transwell migration and invasion assays

AGS, MKN28, SNU-601, and SNU-668 cells were transfected with siRNA (scRNA or SPON2 siRNA) and plasmid vector (pCMV-SPORT6-Empty or pCMV-SPORT6-SPON2). After 24 h transfection, 2×10^4 cells in 200 μl FBS free medium, were isolated and added to the upper transwell chamber (Corning Costar, MA, USA) on a filter coated with 0.5 mg/ml collagen type I (BD Biosciences, Seoul, Korea) for the migration assay and Matrigel (1:15) (BD Biosciences) coated filters for the invasion assay. RPMI 1640 containing 10% FBS and 1% antibiotics was added to the lower chamber, and the plates incubated for 20 h. Cells that migrated and invaded were visualized and quantified after haematoxylin and eosin staining. For quantification, cells were counted from five randomly selected areas in each well using wide-field microscopy. Data expressed as mean ± SEM from three independent experiments.

II.9. Lenti-virus production and generation of stable cell lines

HEK293FT cells were transfected with 6 μg of pLKO1 plasmid vector (empty, SPON2 shRNA#1, SPON2 shRNA#2), 3 μg of PMDG, 3 μg of RSV/REV and 3 μg of PMDLG PPRE using lipofectamine 2000. The condition medium were collected at 72 h

post-transfection and centrifuged at 350 xg for 3 min. Once again supernatants were collected and filtered through a 0.45 μm filter. To generate stable cell lines, lenti-viral particles were treated directly on SNU-601 cells. After 24 h, infected cells were selected by 10 μg/ml of puromycin. Antibiotics were treated once every 3 days for 2 weeks. Expression levels of stable cell lines were determined by RT-PCR and western blot analysis.

II.10. Xenograft mice model

All animal experiments were approved by the Institutional Review Board of the National Cancer Center (NCC Korea) and performed in specific pathogen-free facilities and conditions following the guidelines for the care and use of laboratory animals of NCC (NCC-11-034D). Six-week-old female specific pathogen-free Balb/c nude mice were purchased from OrientBio (Seungnam, Korea). Mice were inoculated subcutaneously into both flanks with 2×10^6 stable SNU-601 cells in each flank under 20 μl of ketamine/rompun (9:1) anesthesia. From palpable tumor formation until termination, tumor sizes were measured every 2 or 3 days using calipers, and tumor volume was calculated according to the formula, length×width^2×0.5236. Mice were killed in 7.5% CO_2 chamber, and tumors were harvested for other analysis.

II.11. Luciferase reporter assay

MKN28 and SNU-601 cells were seeded in 6-well plates (5×10^4 cells per well). After overnight incubation, cells were transfected with 2 μg of pGL3-basic luciferase reporter plasmid and 0.25 μg of β-galactosidase expression plasmid vector. After incubation for 24 h, cells were harvested and luciferase assay was performed using luciferase assay system kit (Promega, Madison, WI, USA). After lysis the cells, cell lysates and luciferin were reacted in a light-blocked 96-well plate. Luminescences were measured for 10 sec for each well. β-galactosidase enzyme assay (Promega) was used as control to evaluate transfection efficiency.

II.12. Chromatin immunoprecipitation assay

Gastric cancer cell lines were cultured in a 150-mm dish (2×10^5 cells). After incubation for 48 h, cells were treated with 1% formaldehyde for cross-linking. Chromatin immunoprecipitation (ChIP) assays were performed using Pierce™ Agarose ChIP kit (Thermo Fisher Scientific, Waltham, MA, USA). After harvesting the cells, the cell membranes were lysed using membrane lysis buffer. Separated nuclei were treated micrococcal nuclease (MNase) for DNA digestion. The nuclear membrane were dissolved using a nuclei lysis buffer. Supernatants were subjected to immunoprecipitation using antibodies for overnight. Immunoprecipitated complexes were washed three times with wash buffer. Add the elution buffer to release the binding between the antibody and the beads. The proteinase K was treated to remove the protein. Eluted samples were passed through clean-up column to purify only DNA. Antibodies used for IP were: anti-Cleaved Notch1 (N1ICD) and anti-RBP-jk same as the antibody used in western blot analysis. Anti-rabbit immunoglobulinG (IgG) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit IgG was used as negative control. Primers were designed with SPON2 promoter binding sites and RT-PCR was performed. Used primers were discribed in “Table 1”. The primers with the GAPDH promoter site were obtained from the ChIP kits. RT-PCR was performed with Ex Taq DNA polymerase (Takara, Shiga, Japan).

II.13. Gene expression profile data and Kaplan-Meier plot analysis

The available datasets GSE13861, GSE30727, GSE27342 and GSE63089 were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). These three datasets were normalized using GEO2R and a scatter plot was obtained for the expression pattern analysis. 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) (32).

II.14. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (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, unless otherwise specified. Data from public databases were used to determine differences in patient 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 SPON2 expression from cDNA microarray data and publicly available data on survival rate in gastric cancer patients

To study the role of SPON2 in gastric cancer, i first need to identify the level of SPON2 expression in patients with gastric cancer. SPON2 expression levels were determined form gastric cancer patient data, available in the GEO database (Fig. 1A-C). SPON2 mRNA expression were significantly increased in tissues of gastric cancer patients compared to normal tissues in GSE13861 (n=90, Fig. 1A), GSE30727 (n=60, Fig. 1B), and GSE27342 (n=160, Fig. 1C). Also, SPON2 protein levels were confirmed by immunohistochemistry (IHC) (Fig. 2A). Tissue microarrays containing 45 gastric cancer tissues were obtained from US Biomax (BS01012c). Assessment of IHC was conducted by professional pathologist. According to the expression intensity of SPON2, that was divided into two groups, high and low. Expression intensities of SPON2 were significantly related with poorly differentiation (Fig. 2B, Table 2). Kaplan-Meier analysis was performed to generate survival curves for gastric cancer patient data (Fig. 3). Overall survival rate over five years was poor in the SPON2 high groups (n=545) compared to the SPON2 low groups (n=331). In addition, the survival rates in the high SPON2 expression group were same-like poor in the variables of progression free survival rate (n=641) and post progression survival (n=499). In all data indicate that SPON2 adversely affects for gastric cancer. To subdivide the role of SPON2, i silenced the SPON2 using small interfering RNA (siRNA) in gastric cancer cells (MKN28) and performed microarray analysis. 1519 genes showed differential expression in SPON2 silenced MKN28 cells (FC>1.5, Fig. 4). Among them, 156 genes showed strong differential level (FC>2). The Gene ontology terminology of these differentially expressed genes were investigated (Fig. 5). The terms related to the genes in the order of significance were immune response, innate immune response, and defense response. Immunity is a defense mechanisms against disease, but it can sometimes cause disease (26-28). These results strongly support that increased

expression of SPON2 in gastric cancer tissues compared to normal tissues, directly correlated with reduced survival rate.

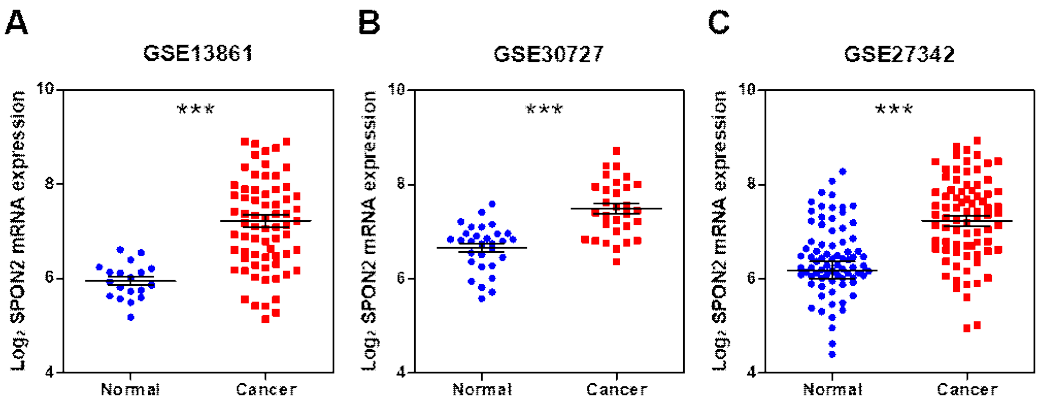

Figure 1. SPON2 is up-regulated in gastric cancer patients. (A-C) SPON2 mRNA expression levels in gastric cancer patients confirmed from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). (A) GSE13861 (n=90), (B) GSE30727 (n=60), (C) GSE27342 (n=160). The *p*-values calculated using Student's *t*-test (*** $p < 0.001$).

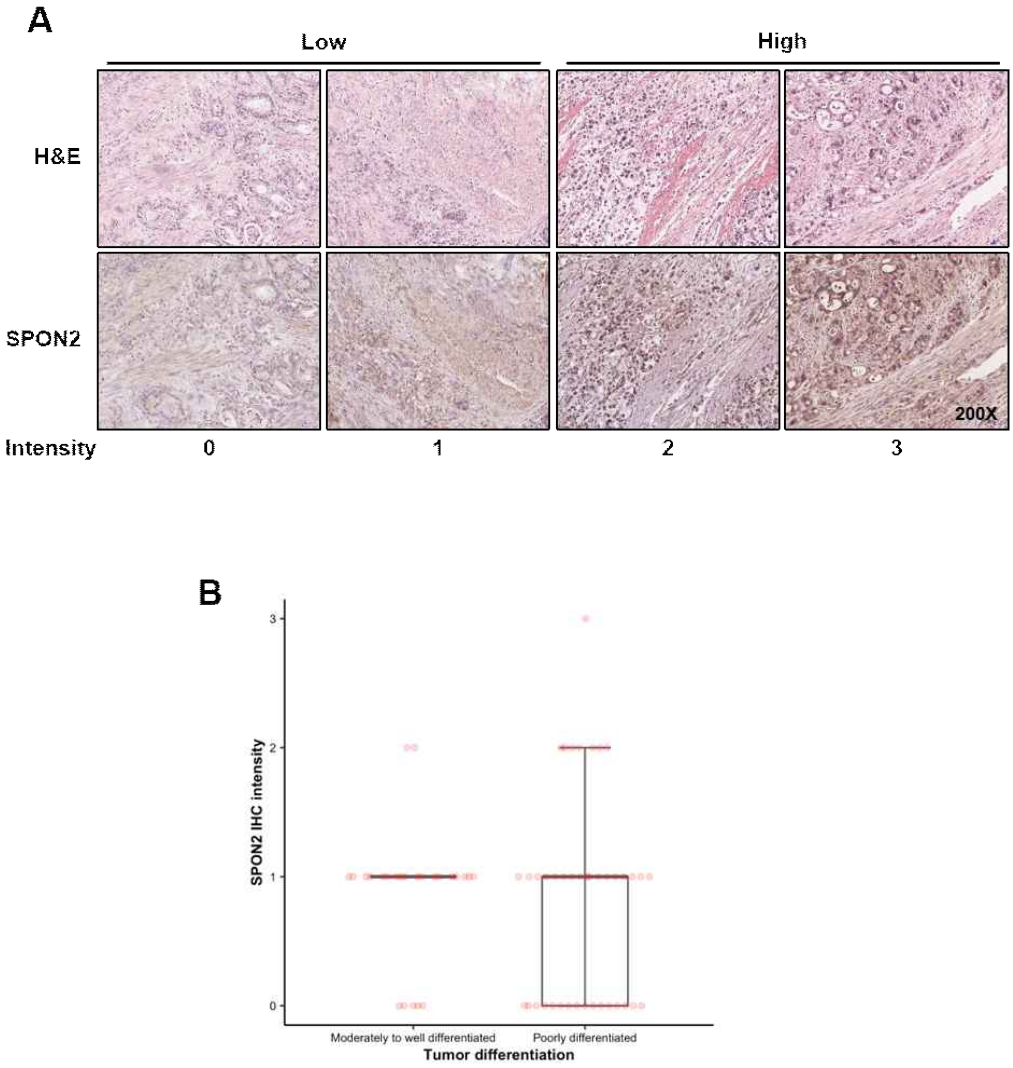

Figure 2. SPON2 expression levels in gastric cancer tissues. SPON2 expression levels were evaluated in tissues of 45 patient by immunohistochemistry. The expression levels was divided into two groups (High or Low) by intensity.

Table 2. Immunohistochemical expression of SPON2 in tissue microarray slides (n=45)

	No. of cases	Low SPON2 expression	High SPON2 expression	*p*-value
Tumor differentiation				
Moderate to well differentiation	20	14	6	0.023
Poor differentiation	25	9	16	

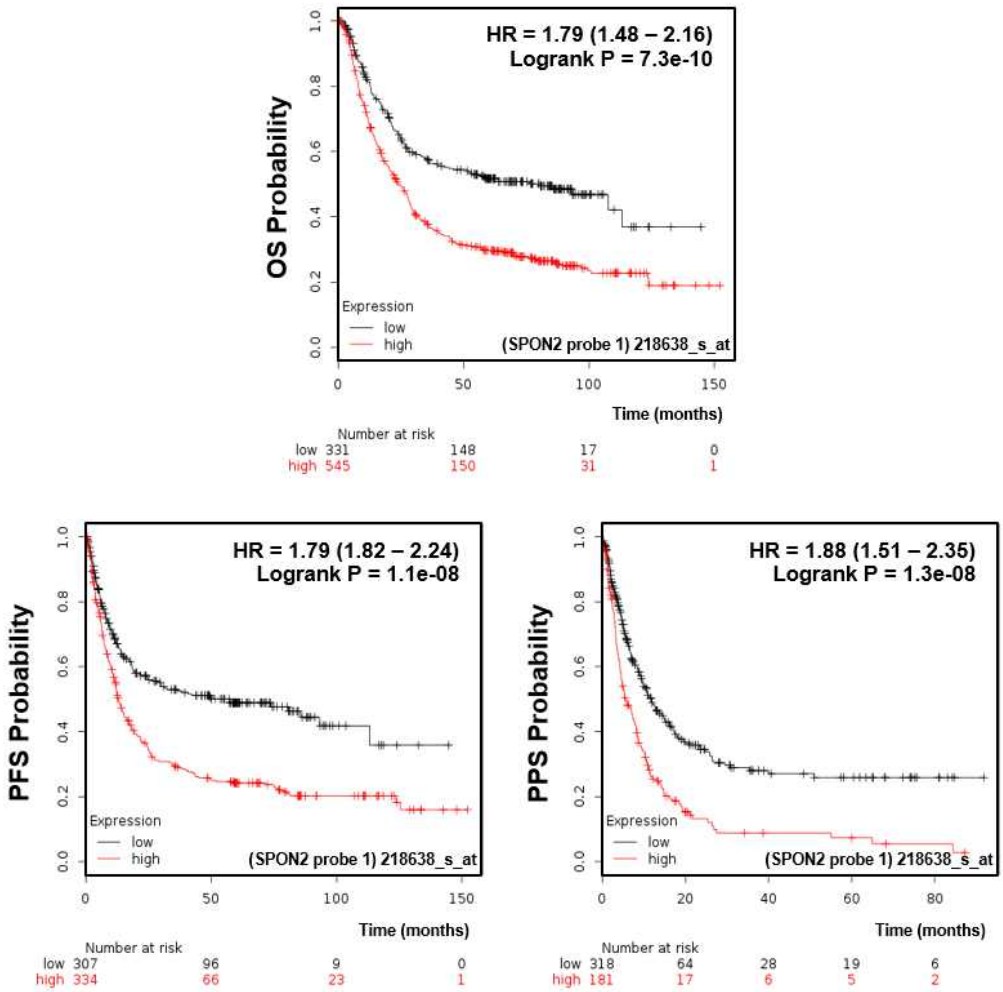

Figure 3. Gastric cancer patients survival rate according to SPON2 expression levels. Kaplan-Meier survival plots showed a association between SPON2 expression levels and poor survival rates in the public database of gastric cancer patients. Three variables were analyzed: overall survival (OS), progression-free survival (PFS), and post-progression survival (PPS).

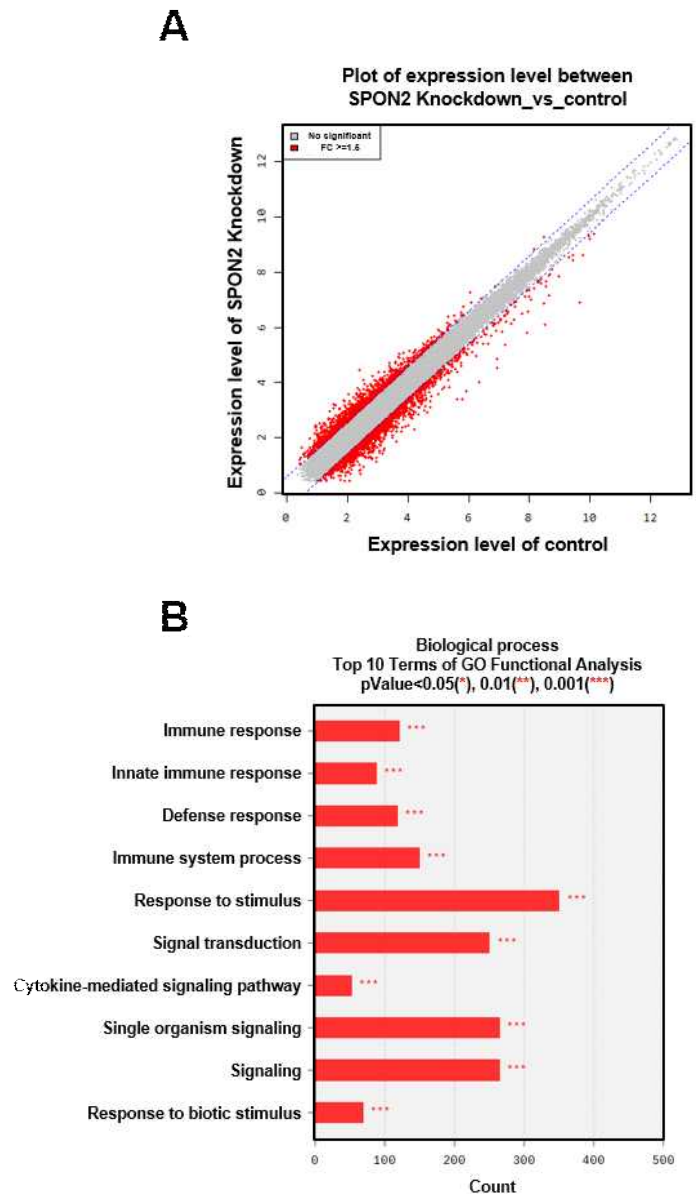

Figure 4. Expression of various genes by SPON2 knock-down in gastric cancer cells. (A-B) Gene expression in SPON2 silenced MKN28 cells was analyzed by microarray analysis. (A) Plot showed differentially expressed genes (Red dot = FC>1.5, grey dot = FC<1.5). (B) Gene ontology terms related to biological processes for differentially expressed genes (FC>1.5). The graphs are listed in order of significance (up). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

III.2. SPON2 knocked-down decreases cell proliferation, migration, and invasion of gastric cancer cells

SPON2 expression level confirmed by RT-PCR in normal gastric epithelial cell line and seven gastric cancer cell lines (Fig. 5). The data showed that most gastric cancer cell lines had high SPON2 expression levels compared to the GES-1 normal gastric epithelial cells. Among these, i selected AGS, SNU-668 (with relatively low expression) and MKN28, SNU-601 (relatively high expression) cells for further experiments. SPON2 expression was silenced using specific siRNAs and the interference efficiency was confirmed by RT-PCR and western blot analyses (Fig. 6). After SPON2 knock-down, cell proliferation was analyzed by WST-1 assay. MKN28 cells showed a 0.72-fold decrease and SNU-601 cells showed a 0.74-fold decrease in cell proliferation. The results showed that knockdown of SPON2 significantly reduced the proliferation of gastric cancer cells (Fig. 7). Moreover, cell migration and invasion assays showed that knockdown of SPON2 inhibited the migratory and invasive abilities of gastric cancer cells (Fig. 8 and 9). Knocked-down of SPON2 showed decreased cell migrative ability to 63% in MKN28 cells and 63% in SNU-601 cells (Fig. 8). In addition, invasive ability reduced to 50% in MKN28 cells and 54% in SNU-601 (Fig. 9).

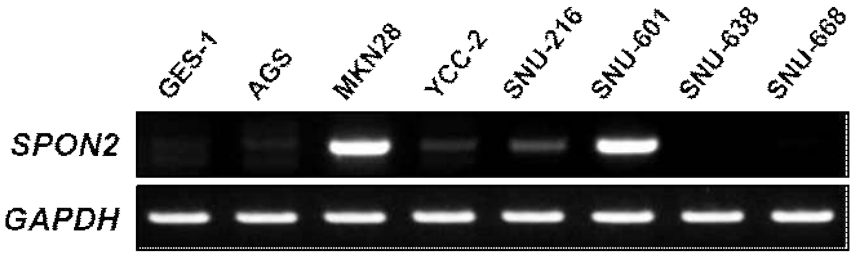

Figure 5. Endogenous mRNA expression level of SPON2 in the gastric cancer cell lines. SPON2 mRNA expression levels in normal gastric epithelial cell (GES-1) and seven human gastric cancer cell lines (AGS, MKN28, YCC2, SNU-216, SNU-601, SNU-638, and SNU-668) were evaluated by RT-PCR. GAPDH was used loading control.

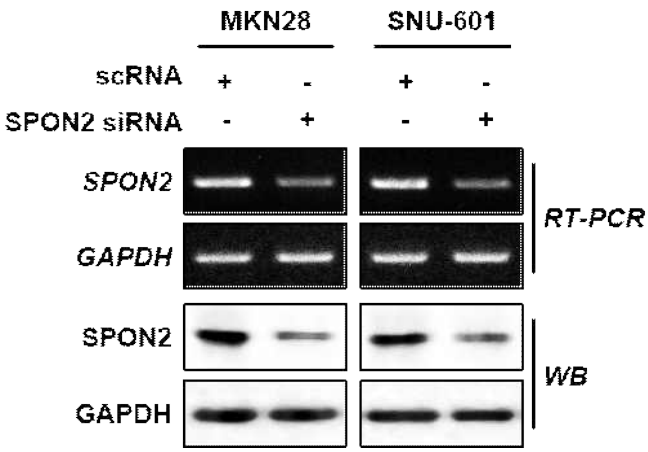

Figure 6. Inhibition of SPON2 expression by RNA interfering. MKN28 and SNU-601 cell lines were transfected with scrambled siRNA (scRNA) or SPON2 specific small interfering RNA (SPON2 siRNA). SPON2 expression levels were detected by RT-PCR and western blot analysis. GAPDH was used as the loading control.

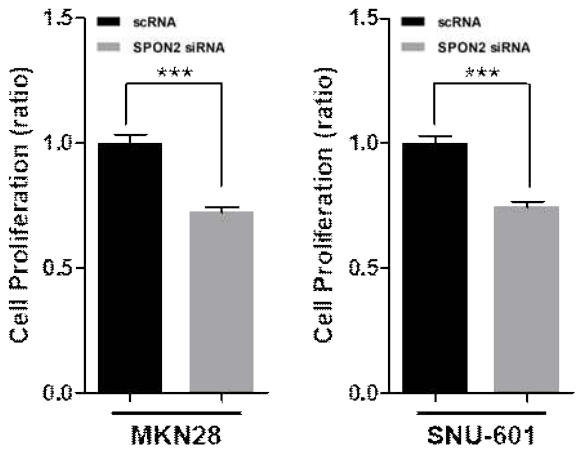

Figure 7. Silencing of SPON2 decreases cell proliferation in gastric cancer cells. Cell proliferation was detected by WST-1 assays performed. Results were measured 48 h after transfection. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

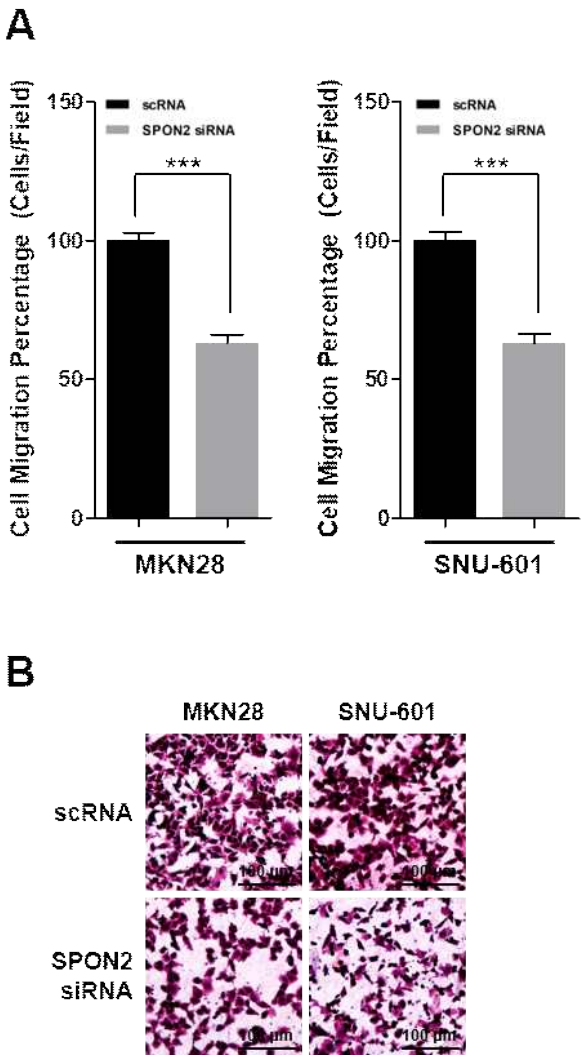

Figure 8. Silencing of SPON2 decreases cell migration in gastric cancer cells. Cell migrative ability was confirmed by transwell assay. (A) Random fields were taken by optical microscopy and cell numbers were quantified. (B) Migrative cells were visualized. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

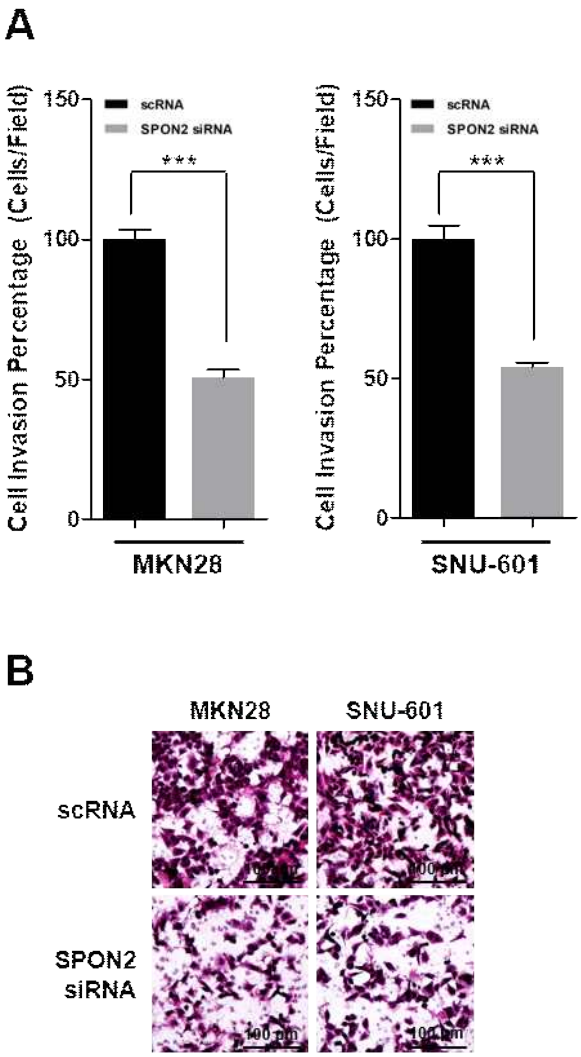

Figure 9. Silencing of SPON2 decreases cell invasive ability in gastric cancer cells. Cell invasive ability detected by transwell assay. (A) Random fields were taken by optical microscopy and cell numbers were quantified. (B) Invasive cells were visualized. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

III.3. SPON2 over-expression increases cell proliferation, migration, and invasion of gastric cancer cells.

The effect of SPON2 expression in gastric cancer cells was examined. AGS and SNU-668 cell lines with low endogenous expression were transiently transfected with the SPON2 over-expression vector (pCMV-SPORT6_SPON2) or empty-vector (pCMV-SPORT6_E.V) for 48 h. The Over-expression efficiency of SPON2 was confirmed by RT-PCR and western blot analysis (Fig. 10). Enhanced SPON2 expression significantly increased cell proliferation (Fig. 11). There was 1.5-fold improvement in AGS cells and 1.37-fold improvement in SNU-668 cells. Cell migration and invasion were confirmed using transwell assay. Over-expression of SPON2 showed enhanced cell migrative ability to 170% in AGS cells and 140% in SNU-668 cells compared to control (Fig. 12). In addition, invasive ability increased to 190% in AGS and 173% in SNU-668 (Fig. 13). These findings suggested that SPON2 was involved in gastric cancer cell proliferation and motility.

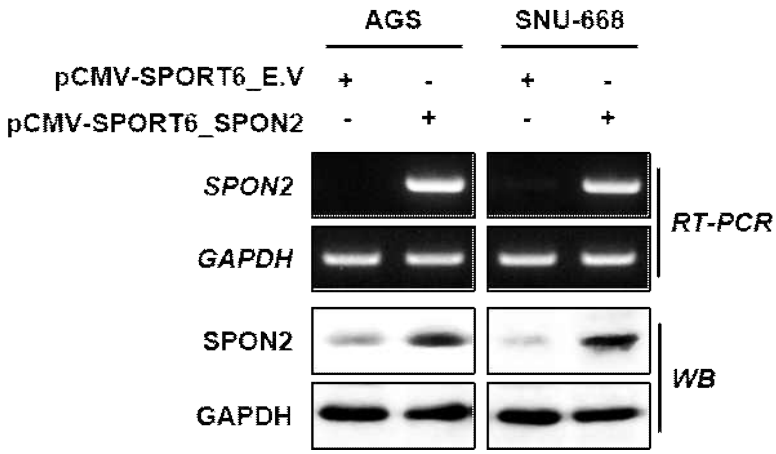

Figure 10. Increased SPON2 expression levels by plasmid vector system. Over-expression of SPON2 in AGS and SNU-668 cells was achieved by transfection with an empty vector (pCMV-SPORT6_E.V) or SPON2 over-expression vector (pCMV-SPORT6_SPON2). SPON2 expression levels were detected by RT-PCR and western blot analysis. GAPDH was used loading control.

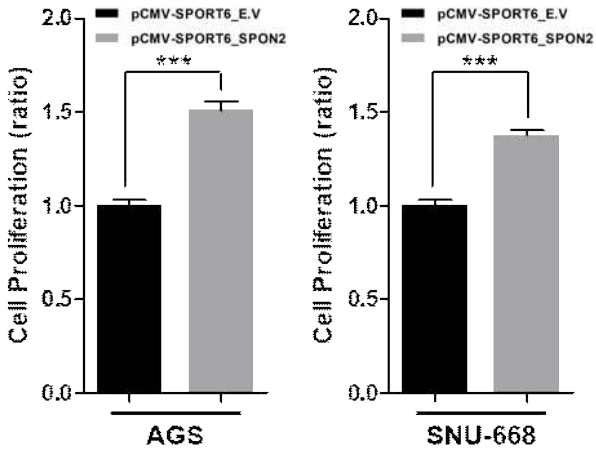

Figure 11. Over-expression of SPON2 increases cell proliferation in gastric cancer cells. Cell proliferation was detected by WST-1 assays. The results were measured 48 h after transfection. Data are presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

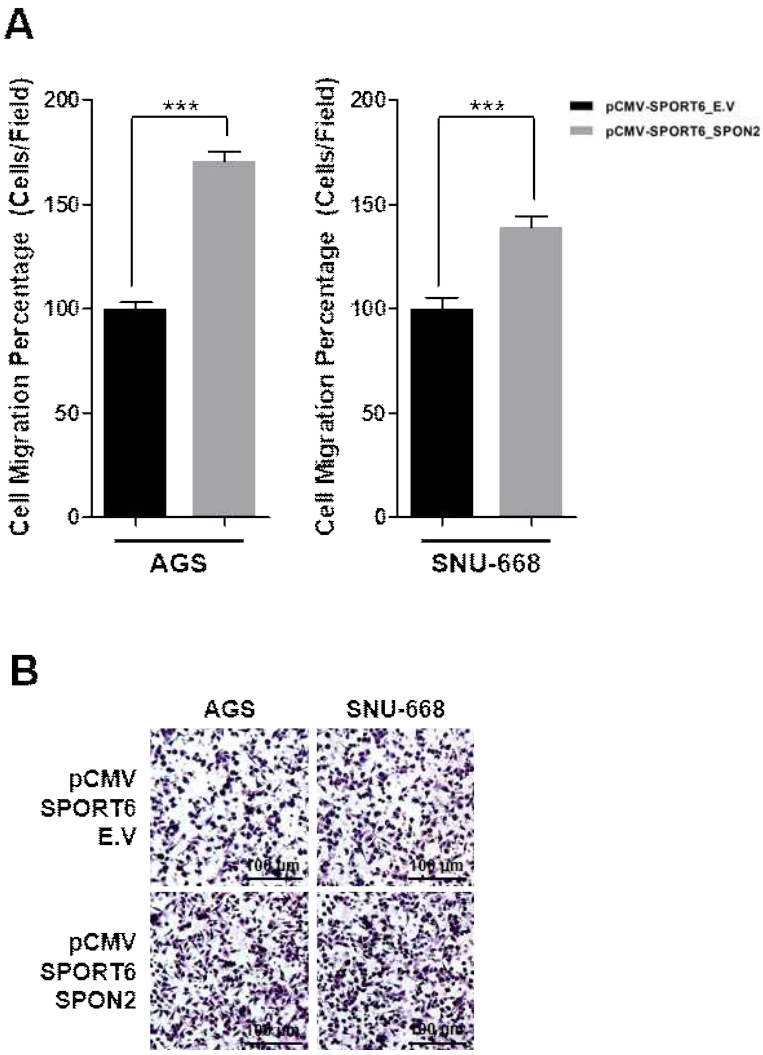

Figure 12. Over-expression of SPON2 increases cell migration in gastric cancer cells. Cell migration detected by transwell assay. **(A)** Random fields were taken by optical microscopy and cell numbers were quantified. (B) Migrative cells were visualized. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

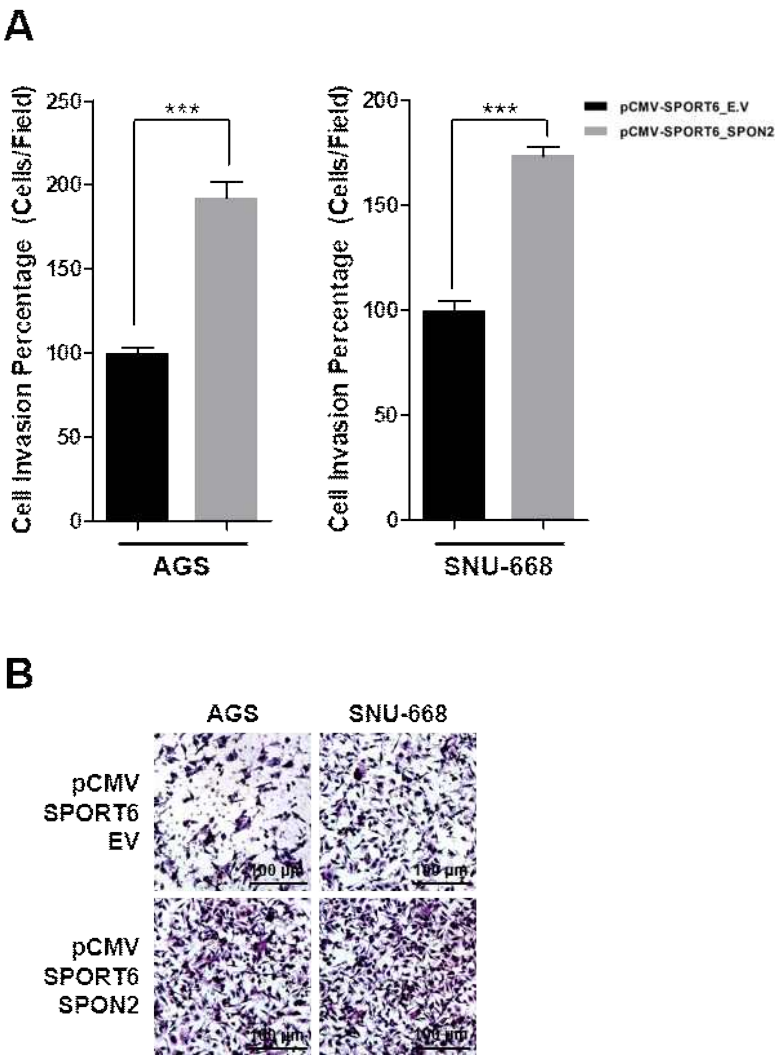

Figure 13. Over-expression of SPON2 increases cell invasion in gastric cancer cells. Cell invasive ability was detected by transwell assay. **(A)** Random fields were taken by optical microscopy and cell numbers were quantified. (B) Invasive cells were visualized. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

III.4. *In vivo* effect of SPON2 inhibition in xenograft mice model

Investigated that the effect of SPON2 inhibition on tumorigenic ability in a mice xenograft model was studied. I generated two stable SNU-601 cell lines that were silenced for SPON2 shRNA#1. Stable suppression of SPON2 expression levels in SNU-601 cells was confirmed by RT-PCR and western blot analysis (Fig. 14). SPON2 shRNA#2 was added to reduce errors for non-target effects and increase the confidence of the results. These cell lines were injected subcutaneously into nude mice (Fig. 15). Compared to tumors derived from negative control pLKO 0.1-expressing SNU601 cells at 18 days post-injection, the SPON2 knock-down group of mice showed reduced tumor growth rate. Mice were sacrificed before the tumour size reached 1500 mm^3 according to the law of the research ethics committee. Tumours were separated and visualized in mice (Fig. 16) In addition, tumour weight was shown by quantification that was significantly reduced (Fig. 17). These data strongly suggest that silencing of SPON2 decelerates gastric cancer progression *in vivo*.

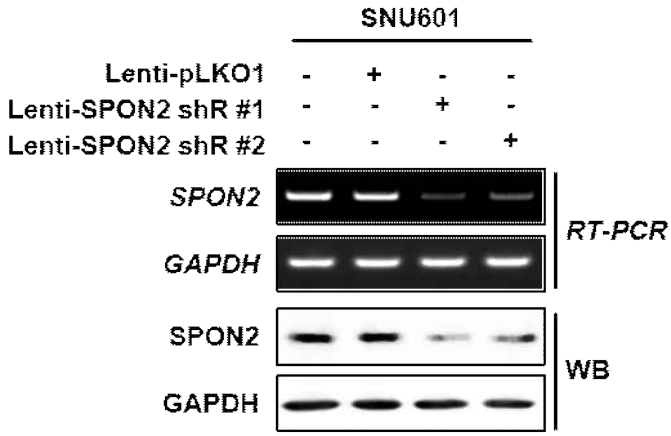

Figure 14. Constant inhibition of SPON2 through lenti-viral infection. SPON2 silencing SNU-601 cells were generated by lenti-virus infection containing shRNA#1 or shRNA#2. Infected cells were selected for 2 weeks by antibiotics. Decreased SPON2 expression levels were detected by RT-PCR and western blot analysis. GAPDH was used loading control.

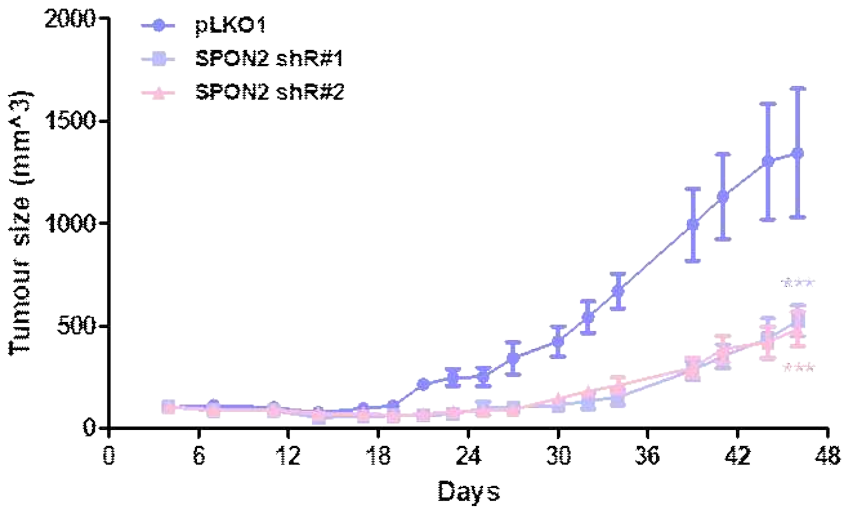

Figure 15. Effect of SPON2 inhibition on tumour growth in xenograft mice model. SPON2 silencing SNU-601 cells (50 µl, 2×10^6) with matrigel were implanted into Balb/c nude mice to form xenografted mice model. Tumour sizes of xenografted mice were measured three times per week and sacrificed at 47 days. Mice xenografted with pLKO1 expressing SNU-601 cells were used as a control. The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

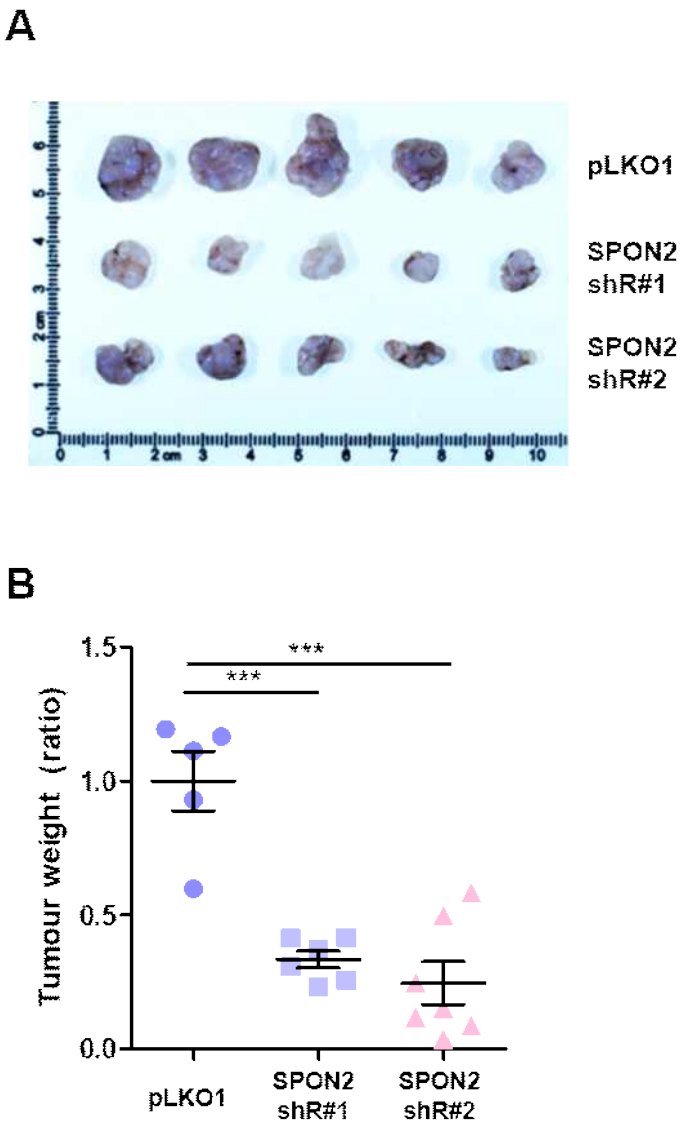

Figure 16. Silencing of SPON2 expression reduces tumour volume in xenograft mice model. (A-B) Xenografted tumours were isolated from the sacrificed mice at 47 days. (A) Resected tumour fixed with formaldehyde and visualized with the documentary system. (B) Tumours were quantified using a microbalance. Data is presented as mean ± SEM (n≥5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

III.5. Regulation of SPON2 expression depends on Notch signaling pathway

Described the oncogenic role of SPON2 in gastric cancer. The mechanism of regulation of SPON2 expression was traced to provide clear evidence for promoting gastric cancer. First, i inserted a promoter binding region shared with SPON2 in the luciferase reporter vector. Transcriptional activity was confirmed at motifs up to -1500bp by Luciferase assay (Fig. 17). The results showed a significant increase between 0bp and -500bp or between -1000bp and -1500bp. I assumed that exist various transcriptional regulatory motifs in the region between 0bp and -500bp. But difficult to subdivide and focused on the activity between -1000bp and -1500bp. Transcriptional actors expected to bind to sequences between -1000bp and -1500bp were investigated using prediction program (Fig. 18). The binding motif of RBP-Jk was identified in the sequence. RBP-Jk is a DNA binding protein that interacts with the Notch intracellular domain and is involved in transcriptional regulation. Also published data were indicated the correlation between NOTCH1 and SPON2 expression (Fig. 19). My data is spearman's correlation R=0.8553 show that a highly correlates between NOTCH1 and SPON2. In addition, the protein expression levels of SPON2, RBP-Jk and activated Notch1 (N1ICD) were compared in gastric cancer cell lines (Fig. 20). The expression levels of each the proteins in seven gastric cancer cells showed a fairly similar pattern. Therefore, i assumed that expression of SPON2 is regulated by notch signaling. I investigated whether Notch signaling transcription factors actually combinate to the SPON2 promoter binding region. ChIP assays were performed to identify DNA fragments for N1ICD and RBP-Jk (Fig. 21). The result confirmed a 289bp PCR product and indicates that N1ICD binds between SPON2 promoter region -1319bp to -1030bp. Over-expression of N1ICD showed increased expression levels of SPON2 compared to negative control (pcDNA4_DNNI) (Fig. 22). These results suggest that enhanced signaling increases expression by transcriptional regulation of SPON2.

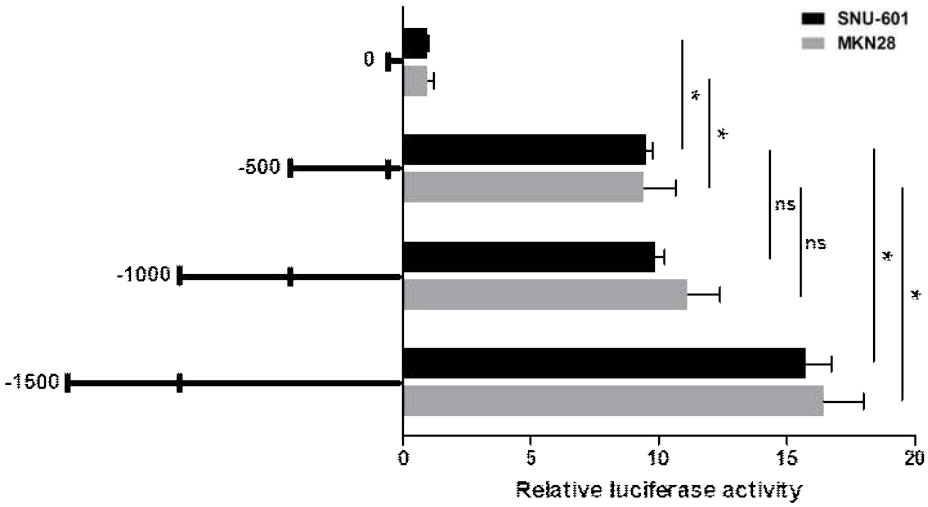

Figure 17. Transcriptional activity that regulates SPON2 expression in gastric cancer cells. MKN28 and SNU-601 cells were transfected with pGL3-luciferase vector containing the SPON2 promoter binding motifs. Activity was confirmed motifs of extending from position -500bp by luciferase reporter assays. The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (* $p < 0.05$).

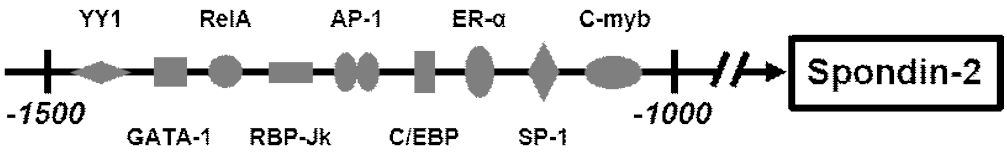

Figure 18. Prediction of transcription factors that bind to SPON2 transcriptional active region. SPON2 promoter motifs -1500bp to -1000bp Identified where transcriptional factors were expected to bind. Predicted transcription factor was confirmed using promo3.0 (http://alggen.lsi.upc.es).

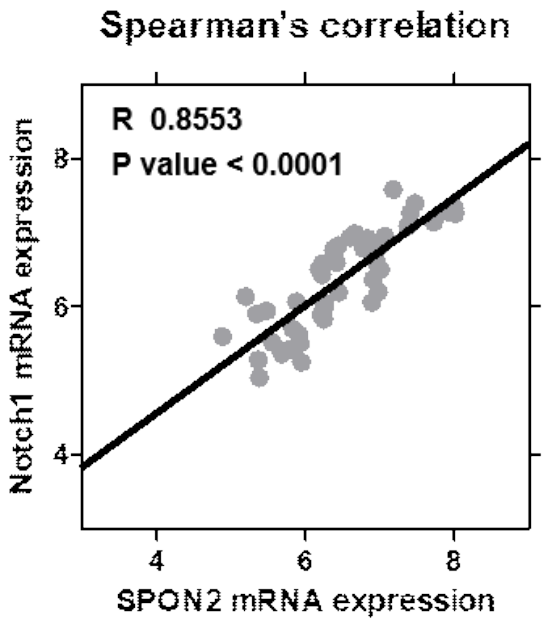

Figure 19. Correlation between SPON2 and Notch signaling in gastric cancer. Spearman's correlation tests showing the relationship between SPON2 and NOTCH1 in gastric cancer patient data obtained from a public database such as GSE63089 (n=45).

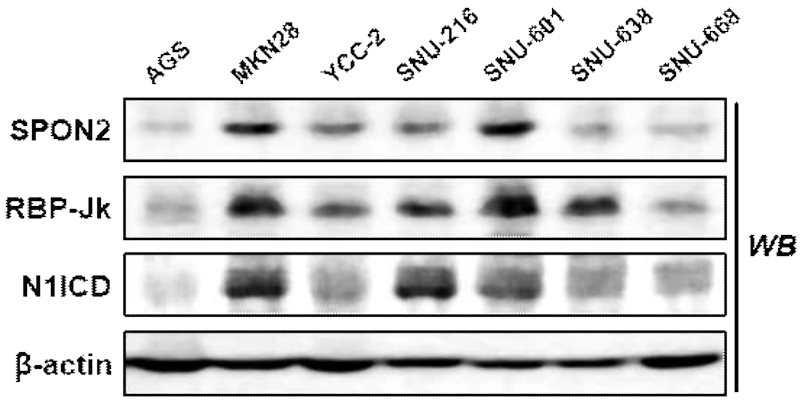

Figure 20. Endogenous expression levels of SPON2 and Notch associated protein in the seven gastric cancer cell lines. SPON2, RBP-jk and N1ICD expression levels in seven human gastric cancer cell lines (AGS, MKN28, YCC2, SNU-216, SNU-601, SNU-638, and SNU-668) were evaluated by western blot analysis. β-actin was used loading control.

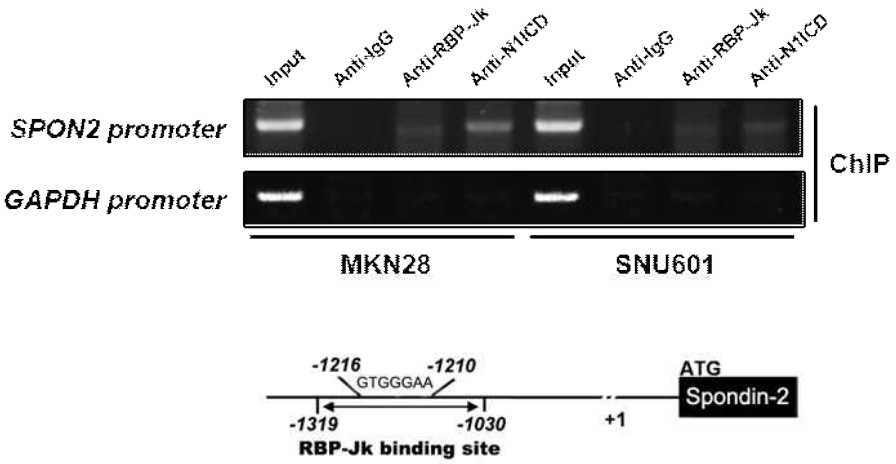

Figure 21. Interaction between SPON2 promoter region and Notch signaling associated transcription factor. Immunoprecipitation performed with anti-RBP-Jk and anti-N1ICD, and interacting -1319bp to -1030bp DNA was detected by RT-PCR. Total genomic DNA in ther input lane was used as a control for the PCR. GAPDH promoter region was used negative control for non-specific binding.

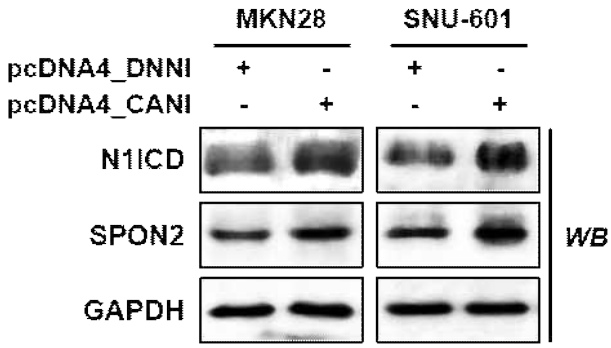

Figure 22. SPON2 expression levels were regulated by Notch signaling pathway. MKN28 and SNU-601 cell lines were transfected with pcDNA4_CANI vector (N1ICD). pcDNA4_DNNI is dominant negative control for pcDNA4_CANI. Expression levels were detected by western blot analysis. GAPDH was used loading control.

III.6. Notch signaling pathway up-regulates SPON2 expression to increases cell proliferation and motility in gastric cancer cells

I examined whether over-expression of N1ICD increased cell proliferation, migration and invasion in gastric cancer cells. The AGS (low SPON2 expression) cells and SNU-601 (high SPON2 expression) cells were used to effectively confirm that changes occurred by modulation of SPON2 expression. Cells were co-transfected with N1ICD expression vector and SPON2 siRNA for 48 h. The expression levels of N1ICD and SPON2 confirmed by RT-PCR and western blot analysis (Fig. 23). Over-expressed N1ICD showed enhancement of SPON2 expression in AGS cells and SNU-601 cells. Also, additional treatment of SPON2 siRNA improved this response. Over-expressed N1ICD enhanced cell proliferation, and SPON2 silencing significantly reduced the induced cell proliferation caused by N1ICD (Fig. 24). In addition, over-expression of N1ICD increased cell migration (Fig. 25) and cell invasion (Fig. 26). The increased migration and invasive abilities were diminished upon SPON2 silencing, similar to that on decreased cell proliferation. Interestingly, Knockdown of SPON2 in AGS cells slightly reduced cell proliferation, migration and invasion, but there was no significant difference. This is expected to be due to the low SPON2 expression levels in AGS cells. These results suggested that Notch signaling promotes cell proliferation, migration and invasion by modulating SPON2 expression.

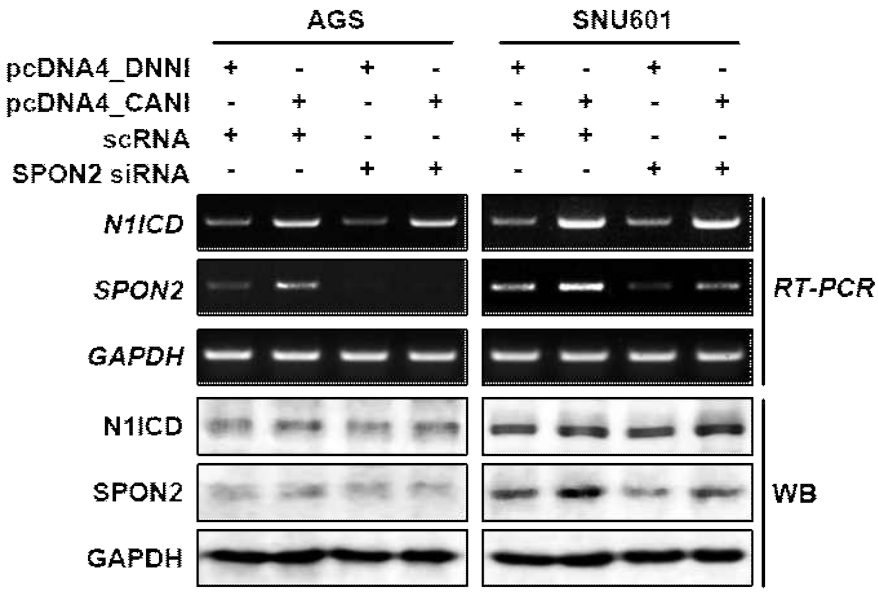

Figure 23. Silencing of SPON2 expression induced by over-expression of N1ICD. mRNA and protein expression levels of N1ICD and SPON2 detected by RT-PCR and western blot analysis. AGS and SNU-601 cell lines were co-transfected with pcDNA4_CANI vector or pcDNA4_DNNI and SPON2 siRNA or scRNA. GAPDH was used as a loading control.

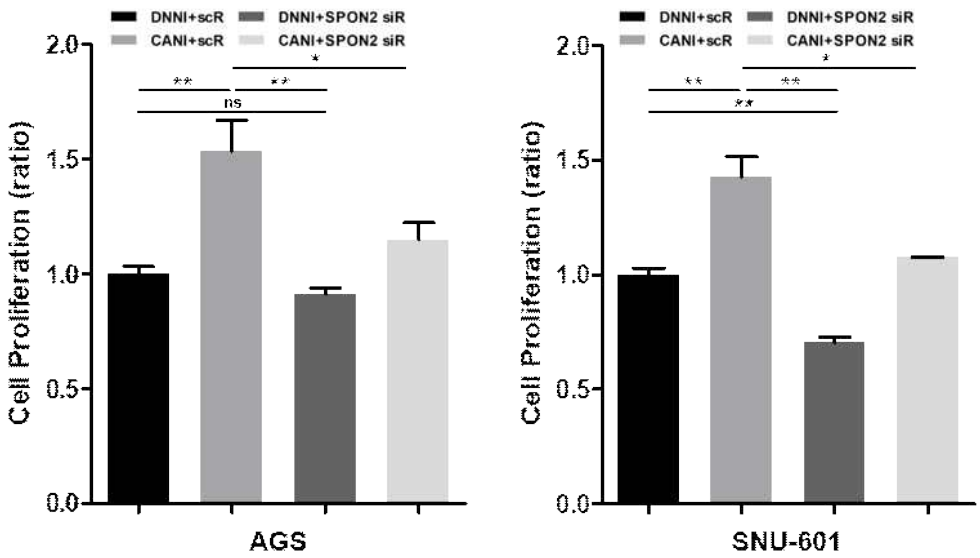

Figure 24. Over-expressed N1ICD mediated SPON2 expression promotes proliferation of gastric cancer cells. Cell proliferation was detected by WST-1 assays performed. Results were measured 48 h after transfection. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (* $p < 0.05$, ** $p < 0.01$).

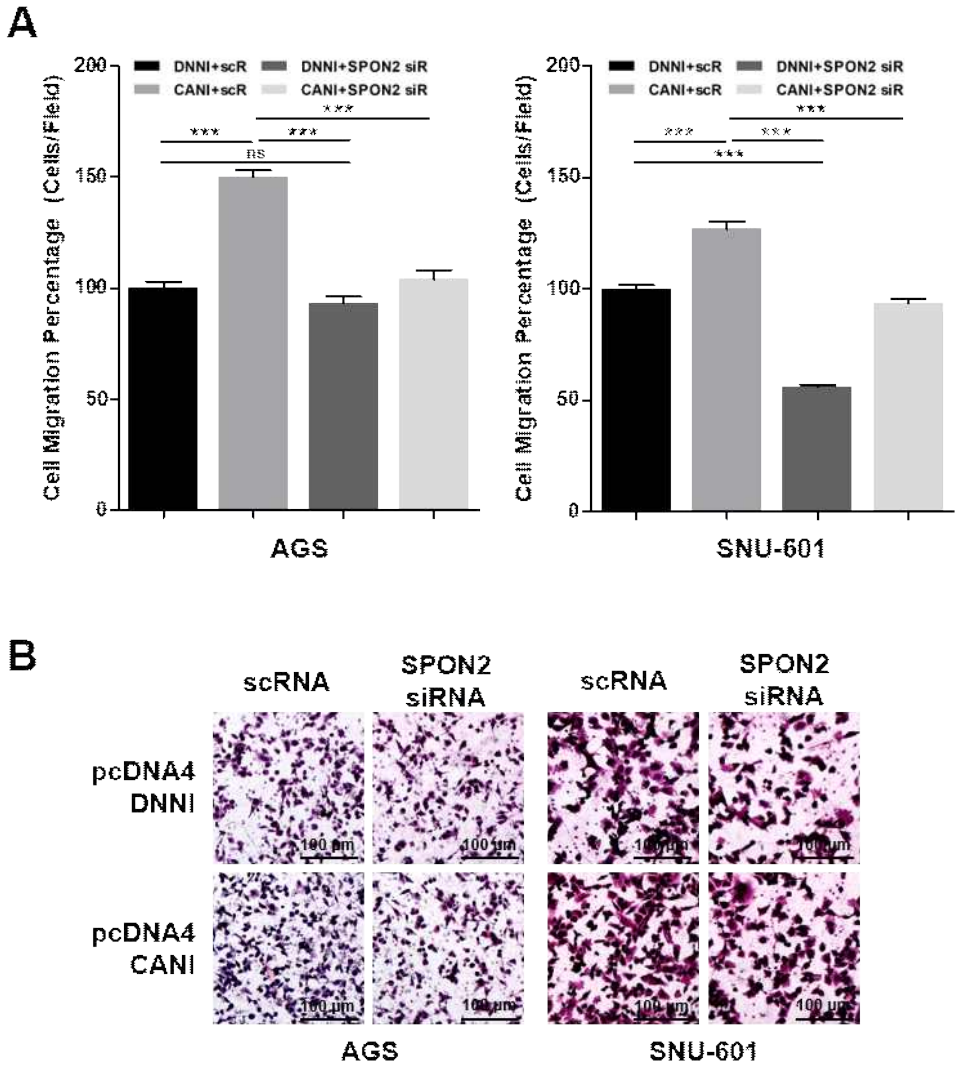

Figure 25. Over-expressed N1ICD mediated SPON2 expression promotes cell migration in gastric cancer cells. Cell migrative ability was confirmed by transwell assay. (A) Random fields were taken by optical microscopy and cell numbers were quantified. (B) Migrative cells were visualized. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

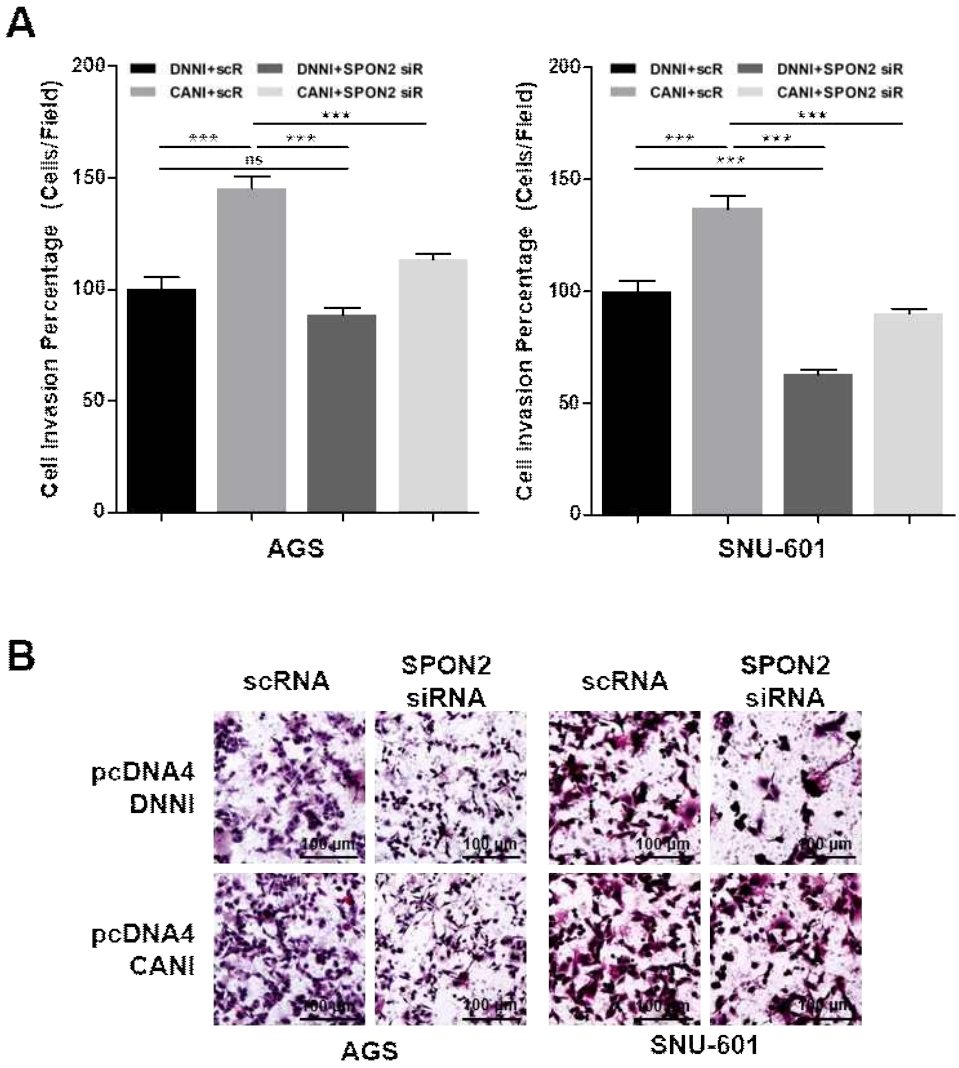

Figure 26. Over-expressed N1ICD mediated SPON2 expression promotes cell invasion in gastric cancer cells. Cell invasive ability detected by transwell assay. (A) Random fields were taken by optical microscopy and cell numbers were quantified. (B) Invasive cells were visualized. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (*** $p < 0.001$).

III.7. Inactivation of Notch signaling by GSI decreases SPON2 expression levels in gastric cancer cells

The mechanism of Notch signaling begins with cleavage of the Notch1 receptor. Notch signaling was inactivated using an γ-secretase inhibitor (GSI) that inhibits an γ-secretase that cleaves the receptor. Therefore, GSIs treatment showed high toxicity in gastric cancer cells (Fig. 27). To verify if the Notch signaling inactivated, N1ICD expression levels were checked after GSIs treatment using RT-PCR and western blot analysis (Fig. 28). Expression levels of cleaved N1ICD decreased in a dose-dependent without variation of the mRNA levels. Inactivation of Notch signaling was resulted in a decrease of SPON2 mRNA and protein expression levels. AGS cells with lower basal levels of NOTCH1 were showed that high toxicity compared to SNU-601 cells (relatively high expression levels). γ-secretases were involved in various signaling in mammalian cells. This is expected to be due to various signaling transductions involved in γ-secretase. Thus, inhibition of Notch signaling by GSIs treatment suggests that it may have a therapeutic effect on SPON2 expression.

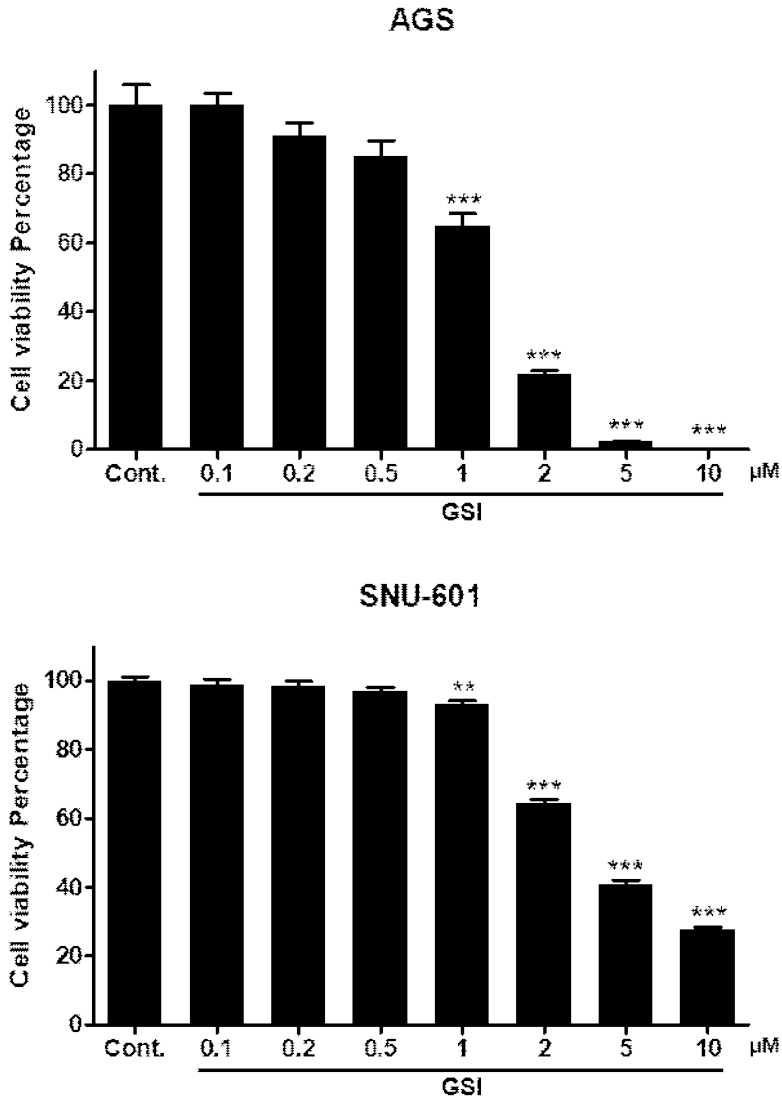

Figure 27. Effect of GSI treatment on cell viability in AGS and SNU-601 cells. AGS and SNU-601 cell lines were treated with γ-secretase inhibitor (GSI-I). After 24 h, cells were detected cell viability by WST-1 assays. The control was treated with 0.1% DMSO. Data is presented as mean ± SEM (n=5). The *p*-values are calculated using Student's *t*-test and significant differences are indicated by * (** $p < 0.01$, *** $p < 0.001$).

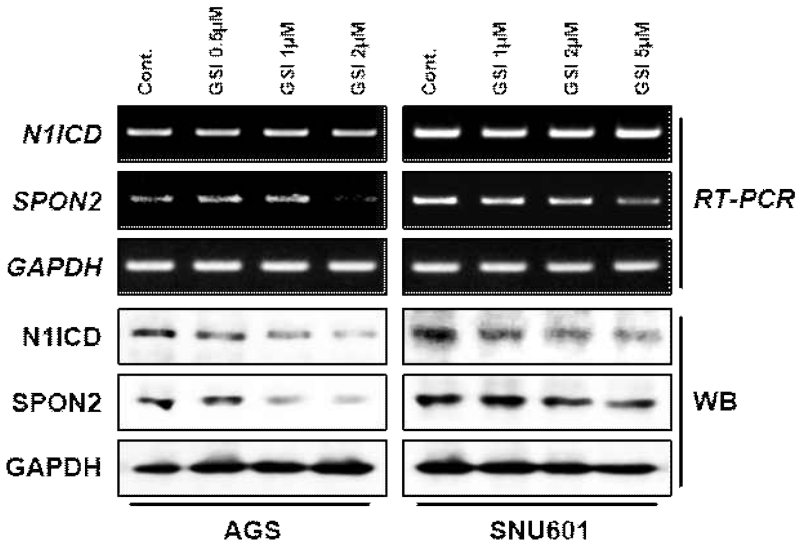

Figure 28. Expression levels of SPON2 and N1ICD by GSIs treatment. AGS and SNU-601 cell lines were treated with GSIs of each concentration for 24 h. The control was treated with 0.1% DMSO. Expression levels were detected by RT-PCR and western blot analysis. GAPDH was used loading control.

IV. DISCUSSION

Although studies on gastric cancer continue to progress, there is still a lack of understanding of gastric cancer. Thus, the incidence of gastric cancer and survival rate of gastric cancer patients and still poor. Efforts to identify targets for gastric cancer therapy and to understand the mechanisms of gastric cancer progression and metastasis are necessary. Previous reports have indicated the possibility for SPON2 as a potential target in gastric cancer (17). However, the mechanism by which SPON2 plays a role in gastric cancer is unknown. Therefore, i focused on the mechanism of SPON2 in gastric cancer. Experiments have been carefully designed, performed, and validated by linking SPON2 with the cellular functions involved in gastric cancer progression and metastasis.

In the first, i investigated the expression level of SPON2 in gastric cancer patients. The expression level of SPON2 increased in gastric cancer patients of three GEO dataset provided by the National Cancer Center. Also, Kaplan Meier-plot indicates that high expression of SPON2 correlates with poor survival rate. All patient data showed that SPON2 is associated with a malignant phenotype of gastric cancer. Next, i knocked down SPON2 in gastric cancer cell lines and performed cDNA microarray analysis. The results were the regulation of the immune response. Immune responses sometimes cause attack of cancer (27-29). Consistent with patients data, SPON2 knock-down significantly repressed cell proliferation, migration and invasion in MKN28 and SNU-601 cells, whereas SPON2 up-regulation enhanced the cell proliferation, migration and invasive abilities of AGS and SNU-668 cells. Also, silencing of SPON2 decreased tumour growth in xenograft mice models. All results strongly support the important role of SPON2 in gastric cancer cell proliferation and motility.

In the second, the expression of SPON2 regulated by Notch signaling were investigated. I constructed a plasmid that shares a promoter region for study the mechanisms of transcriptional regulation of SPON2. Next, i found a promoter region that regulates SPON2 transcription through luciferase assays. This region was confirmed that the region binds CBF-1/RBP-Jk using a prediction program. The correlation between NOTCH1 expression level and SPON2 expression level is the evidence for the theory. SPON2 transcriptional

regulation was confirmed using over-expression vector of N1ICD by performed luciferase assay and western blot analysis. As expected, enhanced notch signaling induced up-regulation of SPON2. Notch signaling is highly expressed in gastric cancer, and is involved not only developments and differentiations, but also gastric cancer progression and metastasis (23-26). Therefore, the role of Notch signaling and SPON2 is important in the gastric cancer progression.

This paper is the second report of SPON2 in gastric cancer. The first report correlates the expression of SPON2 with the progression and prognosis of gastric cancer (17). My research is consistent with first study. Nevertheless, SPON2 expression may cause a contradictory result in cancer. Initially, expression of SPON2 by MACC1 regulation was reported to promote colorectal cancer (19). However, other studies has showed that SPON2 suppressed colon cancer by blocking angiogenesis (20). SPON2 also recruited M1-like macrophage and inhibited hepatocellular carcinoma, but the expression levels were increased in malignant hepatocellular carcinoma (15,18). This suggests that there may be differences races and regions. The opposite reporting requires ongoing research. There have been no reports of conflicting in gastric cancer.

The causes of gastric cancer are vary including gastritis, Helicobacter pylori (H. pylori) infection, dietary factors, genetic factors and other environmental factors (30,31). My microarray data show that inhibition of SPON2 regulates a variety of immune response associated genes. Gastric cancer can sometimes have a poor prognosis due to excessive and abnormal immune responses. For these reasons, SPON2 is expected to play an important role in the occurrence of gastric cancer with inflammation and H. pylori infection. Therefore, i need further studies on it.

In conclusion, this study suggests that increased SPON2 expression by Notch signaling promotes cell proliferation and motility in gastric cancer progression. I purpose SPON2 as a potential therapeutic target for regulating the progression and metastasis of gastric cancer.

V. REFERENCES

[1] GBD 2017 Causes of Death Collaborators: Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018;392(10159):1736-88.

[2] F Bray, J Ferlay, I Soerjomataram, et al: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for Clinicians. 2018;0:1-31.

[3] Rahman R, Asombang AW, Ibdah JA: Characteristics of gastric cancer in Asia. World J Gastroenterol. 2014;20(16):4483-90.

[4] Catalano V, Labianca R, Beretta GD, et al: Gastric cancer. Crit Rev Oncol Hematol. 2009;71(2):127-64.

[5] Aoyama T, Yoshikawa T: Adjuvant therapy for locally advanced gastric cancer. Surg Today. 2017;47(11):1295-302.

[6] Kanat O, O'Neil B, Shahda S: Targeted therapy for advanced gastric cancer: A review of current status and future prospects. World J Gastrointest Oncol. 2015;7(12):401-10.

[7] Kanda M, Kodera Y, Sakamoto J: Updated evidence on adjuvant treatments for gastric cancer. Expert Rev Gastroenterol Hepatol. 2015;9(12):1549-60.

[8] Yoo C, Park YS: Companion diagnostics for the targeted therapy of gastric cancer. World J Gastroenterol. 2015;21(39):10948-55.

[9] Peng L, Yu K, Li Y, et al: Gastric metastasis of recurrent hepatocellular carcinoma: A case report and literature review. J Cancer Res Ther. 2018;14.

[10] Zhou Y, Zhang GJ, Wang J, et al: Current status of lymph node micrometastasis in gastric cancer. Oncotarget. 2017;8(31):51963-9.

[11] Jin X, Zhu Z, Shi Y: Metastasis mechanism and gene/protein expression in gastric cancer with distant organs metastasis. Bull Cancer. 2014.

[12] Li Y, Cao C, Jia W, et al: Structure of the F-spondin domain of mindin, an integrin ligand and pattern recognition molecule. EMBO J. 2009;28:286-97.

[13] Qian X, Li C, Pang B, et al: Spondin-2 (SPON2), a more prostate-cancer-specific diagnostic biomarker. PLoS One. 2012;7:e37225.

[14] Ni H, Ni T, Feng J, et al: Spondin-2 is a novel diagnostic biomarker for laryngeal squamous cell carcinoma. Pathol Res Pract. 2019;215(2):286-291.

[15] Feng Y, Hu Y, Mao Q, et al: Upregulation of Spondin-2 protein expression correlates with poor prognosis in hepatocellular carcinoma. J Int Med Res. 2019;47(2):569‑579.

[16] Zhang Q, Wang XQ, Wang J, et al: Upregulation of spondin-2 predicts poor survival of colorectal carcinoma patients. Oncotarget. 2015;6(17):15095-110.

[17] Jin C, Lin JR, Ma L, et al: Elevated spondin-2 expression correlates with progression and prognosis in gastric cancer. Oncotarget. 2017;8(6):10416‑10424.

[18] Zhang YL, Li Q, Yang XM, et al: SPON2 Promotes M1-like Macrophage Recruitment and Inhibits Hepatocellular Carcinoma Metastasis by Distinct Integrin-Rho GTPase-Hippo Pathways. Cancer Res. 2018;78(9):2305‑2317.

[19] Schmid F, Wang Q, Huska MR, et al: SPON2, a newly identified target gene of MACC1, drives colorectal cancer metastasis in mice and is prognostic for colorectal cancer patient survival. Oncogene. 2016;35(46):5942-5952.

[20] Wang LF, Liu YS, Yang B, et al: The extracellular matrix protein mindin attenuates colon cancer progression by blocking angiogenesis via Egr-1-mediated regulation. Oncogene. 2018;37(5):601-615.

[21] Liao CH, Yeh SC, Huang YH, et al: Positive regulation of spondin 2 by thyroid hormone is associated with cell migration and invasion. Endocr Relat Cancer. 2010;17(1):99-111.

[22] Huang T, Zhou Y, Cheng AS, et al: NOTCH receptors in gastric and other gastrointestinal cancers: oncogenes or tumor suppressors? Mol Cancer. 2016;15(1):80.

[23] Artavanis-Tsakonas S, Rand MD, Lake RJ: Notch signaling: cell fate control and signal integration in development. Science. 1999;284(5415):770-6.

[24] Hori K, Sen A, Artavanis-Tsakonas S: Notch signaling at a glance. J Cell Sci. 2013;126(Pt 10):2135-40.

[25] Yuan X, Wu H, Xu H, et al: Notch signaling: An emerging therapeutic target for cancer treatment. Cancer Lett. 2015;369(1):20-7.

[26] Allenspach EJ, Maillard I, Aster JC, et al: Notch signaling in cancer. Cancer Biol Ther. 2002;1(5):466-76.

[27] Hussain SP, Amstad P, Raja K, et al: Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. Cancer Res. 2000;60:3333‑3337.

[28] Coussens LM, Werb Z: Inflammation and cancer. Nature. 2002;420:860-867.

[29] Beaugerie L, Svrcek M, Seksik P, et al: Risk of colorectal high-grade dysplasia and cancer in a prospective observational cohort of patients with inflammatory bowel disease. Gastroenterology. 2013;145:166-175 e168.

[30] Han TS, Voon DC, Oshima H, et al: Interleukin 1 Up-regulates MicroRNA 135b to Promote Inflammation-Associated Gastric Carcinogenesis in Mice. Gastroenterology. 2019;156(4):1140-1155.e4.

[31] Wroblewski LE, Peek RM Jr, Wilson KT: Helicobacter pylori and Gastric Cancer: Factors That Modulate Disease Risk. Clin Microbiol Rev. 2010;23(4):713-39.

[32] Szasz AM, Lanczky A, Nagy A, et al: Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. Oncotarget. 2016;7(31):49322-33.