Anticancer effect of modulating biological behaviors of cancer stem cells by blocking STAT3/CD133/survivin signaling pathway in colon cancer

Wanlu Li

Department of Medicine

The Graduate School

Yonsei University

Anticancer effect of modulating biological behaviors of cancer stem cells by blocking STAT3/CD133/survivin signaling pathway in colon cancer

Directed by Professor Mee-Yon Cho

A Dissertation Submitted to the Department of Medicine and the Graduate School of Yonsei University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Wanlu Li

January 2018

This certifies that the Doctoral Dissertation of

Wanlu Li is approved.

_____

Professor Mee-Yon Cho, M.D., Ph.D.: Thesis supervisor

_____

Professor Hyun-Soo Kim, M.D., Ph.D.: Committee member

_____

Professor Minseob Eom, M.D., Ph.D.: Committee member

_____

Professor Young Wan Kim, M.D., Ph.D.: Committee member

_____

Professor Ki-woo Kim, Ph.D.: Committee member

The Graduate School, Yonsei University

January 2018

ACKNOWLEDGEMENT

First of all, I would like to express my heartfelt gratitude to my supervisor Professor Mee-Yon Cho who gave me the opportunity to challenge the degree of Ph.D. during last several years. She has advised me with her invaluable insight, frequent encouragement, and endless support throughout my doctoral course. I would also like to express my sincere gratitude to the members of my dissertation committee, including Professor Hyun-Soo Kim, Professor Minseob Eom, Professor Young-wan Kim, and Professor Ki-woo Kim for their valuable advice and patience. I do gratefully thank our professors at the Department of Pathology including Professor Soon Hee Jung, Professor Kwang Hwa Park, Professor Hyeong Ju Kwon and Professor Jiwoon Choi for the unsparing share of their knowledge and their concerns in my living and work at the hospital. I am grateful to Professor Young-wan Kim (Division of Colorectal Surgery) for his assistance in the statistical analysis of my study.

Secondly, I would like to thank my colleague Taeyeong Kim, Mi-Ra Lee and Mirae Jang, all the laboratory members of Department of

Pathology, especially Mrs. Eun-sook Her, Mrs. Ji-eun Jung, Mr. Tae-Young Kang, Miss. Hye-mi Cho and Mrs. Sun-young Ji for their assistance and support in my research work. I am also indebted to my Ethiopian friend Dr. Taye Jemberu. Importantly, I am still gratefully thank you to Professor Jung Ja Lee during my stay in Korea.

Finally, I am forever indebted to and feel a deep sense of gratitude to my parents and my sister for their understanding, endless patience and encouragement when it was most required. Thank you to my Heavenly Father.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Anticancer effect of modulating biological behaviors of cancer stem cells by blocking STAT3/CD133/survivin signaling pathway in colon cancer

Wanlu Li

Department of Medicine

The Graduate School, Yonsei University

Directed by Professor Mee-Yon Cho

Background: Colon cancer is the third most prevalent cancer worldwide. The carcinogenesis of colon cancer is well known to be related to one or combination mechanisms, including chromosomal instability (CIN), CpG island methylator phenotype (GIMP), and microsatellite instability (MSI). Recently, new studies have shown that inflammation contributes to colon cancer carcinogenesis. Pro-inflammatory factors, like interleukin (IL)-6, have been reported to be related with colon cancer development by inducing the phosphorylation of signal transducer and activator of transcription 3

(STAT3). However, the clinical significance of increased p-STAT3 expression in colon cancer is controversial. Nowadays, increased experimental evidences show that cancer stem cells (CSCs) are responsible for the initiation and development of cancer. CD133, as a CSC marker, has been proposed to characterize colon cancer stem cells. A recent study revealed that the STAT3 signaling pathway plays a vital role in cell survival and tumorsphere formation in stem cell-like colon cancer cells. But the mechanism of how activated STAT3 regulates the colon cancer progression and its relationship with CSC marker expression have not been explained. In a previous study, higher chemoresistance of $CD133^+$ cells than $CD133^-$ cells through up-regulating survivin expression was demonstrated. However, the relationship between activated STAT3 and CD133 as well as survivin remains unclear yet. Additionally, the clinical significance of p-STAT3, CD133 and survivin expression and their relationships with clinicopathological parameters are not clear in colon cancer. In this thesis, I hypothesize that STAT3/CD133/survivin signaling pathway is participated in colonic carcinogenesis and activated STAT3 could induce cancer stem cell marker, CD133. To understand the role of

STAT3 / CD133 / survivin signaling pathway in colon cancer, I evaluated the changes of gene expression and biologic behavior of primary and metastatic colon cancer cell lines after modulating STAT3 or CD133 expression. In addition, I also evaluated p-STAT3, CD133 and survivin expression in different stages of colon cancers as well as pre-invasive adenoma and matched non-tumor mucosa to know the relationships between each protein expression and the clinicopathological parameters of colon cancer patients.

Methods: In this study, I comparatively investigated the effect of STAT3 on CD133 and survivin expression after the inhibition of STAT3 by STAT3-siRNA and small molecule STAT3 inhibitor (stattic) and the activation by IL-6 treatment in primary (Caco-2) and metastatic (LoVo and SNU407) colon cancer cells. I also analyzed the changes in the biological behaviors of cancer cells by colony formation assay, cell migration assay, cell availability assay and apoptosis assay after STAT3 or CD133 inhibition. In addition, we comparatively investigated the survivin expression on $CD133^{+}$ cells and siRNA-induced $CD133^{-}$ cells and anti-cancer effects of survivin inhibitor (YM155). Furtheremore, we analyzed the distribution of p-STAT3,

CD133 and survivin expression according to the clinicopathological parameters using the immunohistochemical (IHC) staining in resected colonic adenoma and adenocarcinoma tissues from 397 patients. Furthermore, we performed terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay to analysis apoptosis according to survivin and CD133 expression.

Results: In vitro, inhibition of STAT3 significantly reduced expression of CD133 and survivin compared with a control in both primary (Caco-2) and metastatic colon cancer cells (LoVo and SNU407). In addition, IL-6 induced an increase in the expression of p-STAT3, CD133 and survivin proteins. Inhibition of STAT3 also reduced the colony formation, migration ability and viability of colon cancer cells. In apoptosis assay, Caco-2 cells showed a higher rate of apoptosis than LoVo and SNU407 cells after stattic treatment (68.3, 22.7 and 27.9%, respectively). In addition, inhibition of CD133 by CD133-siRNA transfection also reduced survivin expression in colon cancer cells. With increasing dose of YM155 treatment, significantly increased level of apoptosis were observed in all groups. In cell proliferation assay, $CD133^{+}$ cells (mock and ctrl-siRNA groups) had slightly greater

chemoresistance to 5-fluorouracil (5-FU) compared with the cells in the CD133-siRNA group. In histopathological study, p-STAT3 expression was more common in the preinvasive (pTis) and early stages (pT1&T2) of colon cancer, which also inversely associated with depth of tumor invasion (p=0.001, HR=0.328, 95%CI: 0.170–0.632). In contrast, CD133 and survivin expressions were associated with advanced stage of colon cancer (p=0.000, HR=4.971, 95%CI: 0.551–9.687; and p=0.000, HR=3.617, 95%CI: 1.913–6.835, respectively). We also found a correlation between CD133 and survivin expression (p=0.018). Tumors with an immunoprofile of p-STAT3^{+}/CD133^{-}/survivin^{-} were significantly associated with less risk of tumor invasion (p = 0.003, HR = 0.042, 95%CI: 0.005–0.348) compared with p-STAT3^{+}/CD133^{+}/survivin^{+} tumors. However, the p-STAT3^{-}/CD133^{+}/survivin^{+} immunoprofile was significantly associated with the invasion of colon cancer (p = 0.002, HR = 6.480, 95%CI: 1.957–21.462).

Conclusions: IL-6/STAT3/CD133/survivin signaling pathway may participate in the progression of colon cancer. Therefore, STAT3 can be an effective target for early stages colon cancer by suppressing

cancer stem cell marker expression. And the small molecule survivin inhibitor YM155 may be an effective treatment modality for advanced colon cancer through increasing apoptosis.

Key words: Colon cancer, Cancer stem cells, STAT3, CD133, Survivin, YM155, Stattic

Anticancer effect of modulating biological behaviors of cancer stem cells by blocking STAT3/CD133/survivin signaling pathway in colon cancer

Wanlu Li

Department of Medicine

The Graduate School, Yonsei University

Directed by Professor Mee-Yon Cho

I. INTRODUCTION

Colon cancer is the third most prevalent cancer and the third leading cause of cancer-related death worldwide [1]. Three mechanisms of colon cancer carcinogenesis have already been established [2]. Colon cancer arises from one or combination mechanisms, including chromosomal instability, CpG island methylator phenotype, and microsatellite instability [2]. Recently, new studies have shown that inflammation contributes to colon cancer carcinogenesis [3]. Most colon cancer cases are non-hereditary, and are associated with environmental factors that

cause intestinal epithelial gene mutations and alterations to the gut microbiota. Huipeng *et al*. demonstrated differences in the colonic mucosal microbiota between healthy individuals and patients with colon cancer [4].

In support the role of chronic inflammation in colon cancers, some researchers reported that pro-inflammatory molecules and growth factors produced by the intestinal microbiota can stimulate the proliferation of crypt cells, and directly contribute to tumorigenesis [5,6]. Inflammation involves a variety of immune cells, inflammatory cells, chemokines, cytokines and pro-inflammatory mediators and their interactions that may lead to signaling, tumor cell proliferation, growth and invasion [3]. Specifically, the pro-inflammatory cytokine interleukin (IL)-6 has been reported associated with cancer development and metastasis through activation of signal transducer and activator of transcription 3 (STAT3) [7,8].

STAT3 responds to IL-6 in an inflammatory microenvironment which has been reported in colon cancer [9]. The increased level of phosphorylated STAT3 (p-STAT3) have been found in a lot malignancies, including liver [10], breast [11], head and neck [12], prostate [13],

as well as inflammatory bowel disease (IBD) [14] and colon cancers [15,16]. STAT3 becomes activated after phosphorylation of tyrosine 705 or serine 727. Clinical studies have revealed that elevated levels of STAT3 phosphorylation are correlated with tumor invasion, metastasis [15,17], and poor prognosis [18,19]. However, favorable prognoses resulting from STAT3 activation in malignancies have also been reported recently [20-22]. Therefore, the role of activated STAT3 in the prognosis of colon cancer is still controversial. On the other hand, blocking STAT3 has been reported to reduce cell viability, and suppresses cell migration and invasion in various cancers [23-25]. Kim *et al*. reported that STAT3 inhibition induced apoptosis through the generation of reactive oxygen species (ROS) in a colon cancer cell line [26]. However, the mechanism about how activated STAT3 regulate tumor initiation and progression are not clearly understood.

Within the tumor initiation and progression, cancer stem cells (CSCs) play an important role. CSCs are capable of self-renewal, and may be involved in tumorigenesis, differentiation, and resistance to chemo- and radiotherapy [27]. In colon cancer, CSCs can be identified by the expression of CSC markers such as CD133, CD44, and ALDH1,

which are reported to be associated with the Wnt, Notch, and JAK–STAT signaling pathways. In previous studies, we have reported that CD133 expression is significantly associated with survivin expression, both in vitro and in surgically resected colon cancer tissue [27,28]. Because CSCs play an important role in cell survival and drug resistance, we previously reported that targeting CSCs may be an important strategy for cancer therapy [28,29]. A recent study revealed that the STAT3 signaling pathway plays a vital role in cell survival and tumorsphere formation in $ALDH^+/CD133^+$ stem cell-like colon cancer cells [30]. However, the relationship between activated STAT3 and cancer stem cell markers remains unclear. Elucidation of the upstream mechanism of CSCs marker expression would facilitate the development of an improved therapy capable of targeting tumors before the onset of tumor heterogeneity.

In our previous study result, we found that CD133 knockdown by CD133-siRNA transfection could induce the decrease of survivin expression in colon cancer cells [27]. Survivin, a member of the inhibitors of apoptosis protein family, has diverse functions and plays a dual role in cell proliferation. One role is to regulate the cell cycle in

the G2/M phase as a subunit of the chromosomal passenger complex, which can be tagged in the nucleus [31]. The other is to inhibit apoptosis by preventing the activation of caspase-3 and caspase-7. Survivin has been identified in a mitochondrion-specific pool that can be detected in the cytoplasm [32]. Meta-analysis in colon cancer patients has shown that survivin expression is closely related to mortality but is unrelated to clinicopathological parameters [33,34]. Most studies have used immunohistochemistry to determine survivin expression in cytoplasm. However, a recent report showed that nuclear survivin expression is significantly higher than cytoplasmic expression in colorectal cancer [35]. The clinical significance of subcellular survivin expression remains controversial [36-38] although abnormal p53 has been associated with survivin expression [39], and the regulatory mechanism of survivin expression is not yet completely understood.

In the present study, we hypothesized that IL-6/p-STAT3/CD133/survivin signaling pathway play an important role in colon cancer, which can modulate biological behaviors of colon cancer cells. In addition, to determine the clinical implications of the expression of these molecules in colon cancer progressio, we examined

the association between p-STAT3/CD133/survivin immunoexpression and the pathological features of colon cancer using resected colonic adenoma and adenocarcinoma samples from the patients. We also comparatively analyzed apoptosis according to CD133 and survivin expression in surgically resected colon cancers.

II. MATERIALS AND METHODS

1. In vitro study

1.1. Cell lines and culture

The caco-2 cells were cultured in minimum essential medium with Earle's balanced salt solution (MEM/EBSS, Hyclone, Logan, Utah, USA), and the LoVo and SNU407 cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI-1640) (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL; Gibco) in a humidified atmosphere at 37℃ and 5% CO_2.

1.2. Inhibition of STAT3 and CD133 via siRNA transfection

The siRNAs (Qiagen, Hilden, Germany) targeting the STAT3 gene and CD133 gene sequence were mixed with 200 μL phosphate-buffered saline (PBS) and 4 μL G-fectin (Genolution, Seoul, Korea), and incubated for 10 min at room temperature. Approximately 8×10^4 cells were plated on six-well plates and treated with the transfection

mixture, as described above. The cells were harvested 24, 48, and 72 h post-transfection. Knockdown of STAT3 and CD133 expression was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting.

1.3. Small molecule inhibitors

The nonpeptidic small-molecule inhibitor stattic (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to inhibit the activation of STAT3 by blocking phosphorylation and dimerization events. The cells were treated with different concentrations of stattic for 24, 48, and 72 h. The effect of stattic was confirmed by qRT-PCR and western blotting.

The small-molecule inhibitor YM155 (Selleckchem, Houston, TX, USA) was used to inhibit the activation of survivin by inhibiting survivin promoter activity. The cells were treated with different concentrations of stattic for 24, 48, and 72 h. The effect of stattic was confirmed by qRT-PCR and western blotting.

1.4. Colony formation assay

The cells transfected with control-siRNA or STAT3-siRNA were cultured for 48 h. The culture medium was changed once every 4 days. Colonies were fixed with methanol for 5 min and visualized using 1% methylene blue on Day 12 of culture.

The cells were grown on six-well cell culture plates and treated with varying concentrations of stattic for 48 h. The culture medium was changed once every 4 days. Colonies were visualized by methylene blue staining on Day 12 of culture. All experiments were performed in triplicate wells for each condition.

1.5. Western blotting

Total proteins were extracted using PRO-PREP (Intron Biotechnology, Daejeon, South Korea). Equal amounts of protein from each sample were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to 0.45-mm-thick polyvinylidene fluoride membranes. After blocking with 5% skim milk for 1 h, the membranes were incubated with anti-STAT3 (Cell Signaling Technology, MA, USA), anti-p-STAT3 (S727) (Cell

Signaling Technology, MA, USA), anti-p-STAT3 (Y705) (Cell Signaling Technology, MA, USA), anti-CD133/1 (AC133, Miltenyi Biotec), anti-survivin (Abcam, Cambridge, MA, USA), anti-cleaved poly-ADP-ribose polymerase (PARP; Cell Signaling Technology, Danvers, MA, USA), and anti-cleaved caspase-3 (Cell Signaling Technology, MA, USA) at 4°C overnight. β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the loading control. After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), the immune complexes were visualized with Luminata Forte western HRP substrate (Millipore, Billerica, MA, USA). Images were obtained using a FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA, USA). All experiments were performed in triplicate wells for each condition.

1.6. Quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. A QuantiTect® Reverse Transcription Kit (QIAGEN) was used for complementary DNA (cDNA) synthesis from 1 μg total RNA. Quantitative RT-PCR

was performed as described elsewhere [40] in 384-well PCR plates containing Fast SYBR Green Master Mix (Applied Biosystems, California, USA), the cDNA template, and the primers to a final concentration of 10 pmole each. Each primer/cDNA set was set up in triplicate. The qRT-PCR mixture comprised a 7900HT Fast Real-Time PCR System (Applied Biosystems), and the reaction regimen was as follows: initiation by heating at 50℃ for 2 min; heating at 95℃ for 10 min; followed by 40 cycles of 95℃ (15 s) and 60℃ (60 s). The relative quantification of gene expression was carried out using the ΔCt method. All experiments were performed in triplicate wells for each condition, and were repeated three times.

1.7. Wound-healing assay

The cells were seeded on six-well plates with complete growth medium and cultured to 100% confluence. They were then treated with 0.1 μg/mL mitomycin C (Sigma-Aldrich) for 2 h before scratching to distinguish cell migration from proliferation [41]. The monolayer was scratched using a 200-μL sterile pipette tip, and washed once with sterile PBS to remove non-adherent cells. The cells were then treated

with varying concentrations of stattic (0, 10, and 20 μM). After 24 h, cell migration was examined by microscopy and analyzed objectively using Image J software. The wound-healing rate was calculated using the formula: wound healing rate (%) = (width at 0 h – width at 24 h) / (width at 0 h) × 100. All experiments were performed in triplicate wells for each condition and repeated three times.

1.8. Cell viability assay

The cells (5×10^5 cells/mL) were seeded into 96-well microplates and treated with varying concentrations of stattic. After incubating for 24 h, 10 μL WST-1 reagent (Roche, Indianapolis, IN, USA) was added to each well and the plates were incubated for an additional 1 h at 37℃ in 5% CO2. The absorbance of the samples was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm wavelength. All experiments were performed in triplicate wells for each condition and repeated three times.

Caco-2 cells were seeded at a concentration of 8×10^4 cells per well onto six-well plates. The cells were transfected with ctrl-siRNA or CD133 siRNA for 48 h and then treated with 10 μM 5-FU (Sigma-

Aldrich, St. Louis, MO, USA) and 10 nM YM155 for 72 h. Then, 10 μL WST-1 reagent was added to each well, and the plates were incubated for 1 h at 37 ℃ and 5% CO_2. The absorbance of the samples was measured at a 450 nm by using an enzyme-linked immunosorbent assay reader.

1.9. Apoptosis assay

The cells were treated with 20 nM stattic for 24 h. They were then stained with Annexin V-PE and 7-aminoactinomycin D (7-ADD) using a PE Annexin V apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA). The trypsinized cells were washed twice with cold PBS, then resuspended in 100 mL 1X Binding Buffer (1×10^5 cells). Annexin V-PE (5 μL) and 7-AAD (5 μL) were then added, and the mixture was incubated for 15 min at room temperature in the dark. After 15 min, 400 μL binding buffer was added and the mixture was analyzed by flow cytometry using a BD FACSAria III system (BD bioscience, San Jose, CD, USA). All experiments were performed in triplicate wells for each condition and repeated three times.

2. Histopathological study using clinical samples

2.1. Patient and tissue samples

We used formalin-fixed and paraffin-embedded tissues from patients registered at the Department of Pathology, Yonsei University, Wonju Severance Christian Hospital, South Korea. To evaluate p-STAT3, CD133 and survivin expression according to colonic adenoma and different stages of colon cancer, we randomly selected 210 resected adenoma and colon cancer patients from the archives Between January 2009 and December 2013. Additionally, to evaluate CD133 and survivin expression in advanced colon cancer, we used surgically resected stage II and III colorectal cancers from 187 patients with follow-up information for survival analysis between January 2000 and December 2006. Patients who had received preoperative chemotherapy or radiotherapy were excluded. Clinicopathological data comprising patient age, sex, tumor location, invasion depth, histological differentiation, and lymph node metastasis were collected from the pathology reports.

2.2. Ethics approval

This study was approved by the Institutional Ethics Commit¬tee of Yonsei University, Wonju College of Medicine (CR316116 and YWMR-14-4-102), and was carried out in compliance with the guidelines of the Declaration of Helsinki.

2.3. Immunohistochemical analysis

Immunohistochemical (IHC) staining of the paraffin-embedded tissue sections was performed as described elsewhere [28]. Sections were deparaffinized with EZPrep (Ventana Medical Systems, Tucson, AZ, USA), and an antigen-retrieval step was performed for 60 min. Endogenous peroxidase activity was blocked by exposing the sections to an ultraviolet inhibitor for 4 min. After washing several times with the reaction buffer at room temperature, an Ultra View Universal DAB Detection Kit (Ventana Medical Systems) was used for IHC staining. The slides were incubated with monoclonal antibodies against p-STAT3 (Y705) (Cell Signaling Technology, MA, USA) and survivin (Abcam, Cambridge, MA, USA) for 1 h, and with antibodies against CD133/1 (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) for

2 h at 37℃ in an autostainer (Benchmark XT, Ventana Medical Systems). The slides were then rinsed with the reaction buffer. Drops of HRP UNIV MULT, DAB, and DAB H2O2 (Ventana Medical Systems) were applied sequentially to each slide (8 min per reagent), and the slides were then rinsed again with the reaction buffer. The slides were subsequently treated with a drop of COPPER for 4 min. Finally, a blue color development agent was added and rinsed with reaction buffer, and the sections were counterstained with hematoxylin to staining the nuclei.

IHC staining for p-STAT3 was graded according to the percentage of epithelial cells that were negative (< 10%) or positive (≥ 10%) for the nuclear stain [7,42]. IHC staining for CD133 was according to the percentage of epithelial cells that were negative (< 10%) or positive (≥ 10%) for the epithelial cell membrane stain [28]. IHC staining for survivin was graded according to the percentage of epithelial cells that were negative (< 25%) or positive (≥ 25%) for the nuclear stain [28]. The expression levels of p-STAT3, CD133, and survivin were quantified

by manually counting the positive cells in the selected hot-spot fields. The IHC staining results were independently evaluated by two pathologists who were blinded to the clinical and pathological patient information. Discrepancies between the pathologist evaluations were resolved by consensus.

2.4. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay

All patient samples were stained using an In situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) in formalin-fixed and paraffin-embedded sections. The slides were deparaffinized and rehydrated. After washing, the slides were incubated for 30 minutes at RT with 0.1 M Tris-HCl (pH 7.5), 3% bovine serum albumin, and 20% normal bovine serum for blocking. Then, the slides were washed twice with phosphate-buffered saline (PBS). The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) reaction mixture (50 μL) was applied to each section for 1 hour at 37℃ in a humidified chamber. After three washes with PBS,

the slides were treated with 0.3% H2O2 in methanol for 10 minutes (RT). The blocking step was then repeated.

After washing with PBS, 50 mL Converter-POD was applied to each slide, and the slides were incubated for 30 minutes at 37℃ in a humidified chamber. After three washes with PBS, 100 μL DAB substrate solution was added, and the slides were incubated for 1–3 minutes at RT. The slides were then washed thoroughly with tap water and counterstained with hematoxylin before mounting. Under light microscopy, we calculated the apoptotic index (AI) by counting the number of apoptotic nuclei per 1,000 cells in a high-power field. Cells were considered positive if the entire nuclear area of the cell was stained brown. Cells in areas with necrosis and the margins of sections were excluded from the analysis.

2.5. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows (version 23.0; IBM Corporation, Armonk, NY, USA). Categorical variables were described by frequencies and percentages, and compared using a chi-square or Fisher's exact test as appropriate.

Continuous variables are described as the mean ± standard deviation, and were analyzed by Student's t-tests and analysis of variance (ANOVA). Factors associated with advanced pathologic T classification were assessed by logistic regression analysis. A p-value of < 0.05 was considered significant.

III. RESULTS

1. Modulating STAT3/CD133/survivin signaling pathway in colon cancer cell lines study

1.1. The role of STAT3 in primary and metastatic colon cancer cells

1.1.1. The effect of STAT3 inhibition by STAT3-siRNA and stattic on CD133 and survivin mRNA and proteins expressions

We used STAT3 small interfering RNA (STAT3-siRNA) and small molecule STAT3 inhibitor (stattic) to determine the effect of STAT3 inactivation on colon cancer cells. As shown in Figure 1, both STAT3-siRNA and stattic significantly reduced CD133 mRNA expression in all the cell lines, but protein expression was more obviously reduced in the primary colon cancer cells than in the metastatic cells. There was a significant reduction in CD133 mRNA expression 72 h after STAT3-siRNA transfection and 24 h after stattic treatment in primary (Caco-2) and metastatic (LoVo and SNU407) colon cancer cells. However, the

decrease in survivin mRNA expression was most prevalent in the Caco-2 cells after stattic treatment compared with the control. After treatment with stattic, the level of STAT3 mRNA was not reduced because stattic acts by inhibiting the phosphorylation and dimerization of STAT3. Survivin expression was also reduced at the mRNA and protein levels. However, siRNA transfection apparently disrupted the results owing to the cell-damaging effect of the transfection procedure.

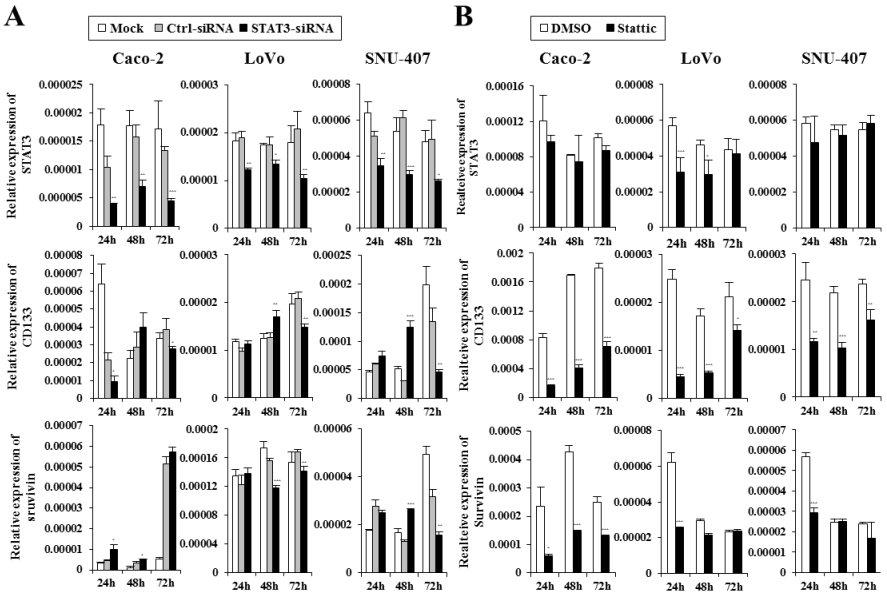

Figure 1. Changes in STAT3, CD133 and survivin mRNA expression after inhibiting STAT3 in primary and metastatic colon cancer cells

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed that the expression of CD133 and survivin decreased after STAT3-siRNA transfection (A) and stattic treatment (B). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

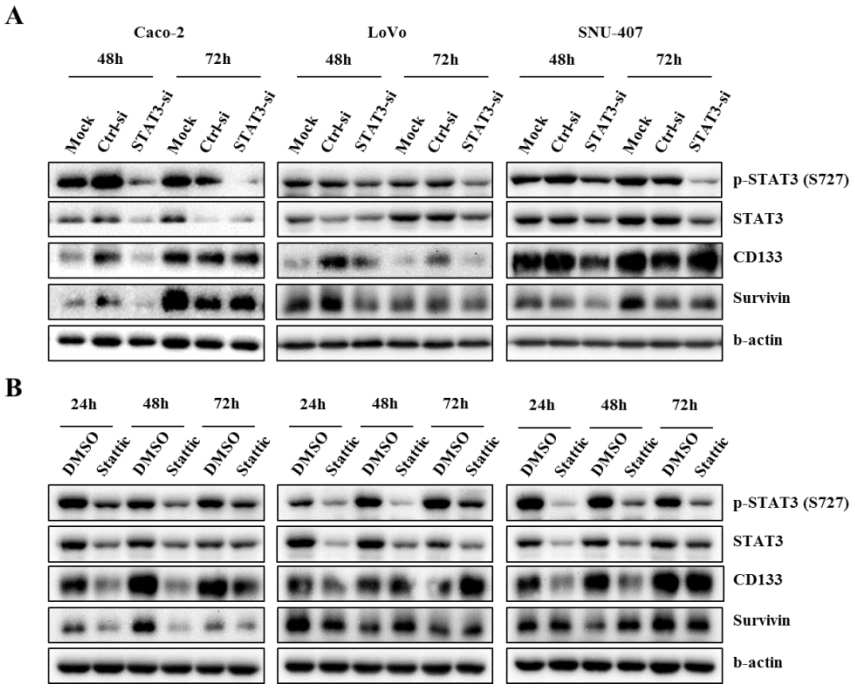

Figure 2. Changes in p-STAT3/STAT3, CD133 and survivin protein expression after inhibiting STAT3 in primary and metastatic colon cancer cells

Western blotting analysis following treatment with STAT3-siRNA (A) and stattic (B) revealed a more obvious decrease in p-STAT3 serine727 (S727) and CD133 in primary colon cancer cells than in metastatic cancer cells.

1.1.2. pSTAT3 and STAT3 expression by western blot after CD133-siRNA treatment

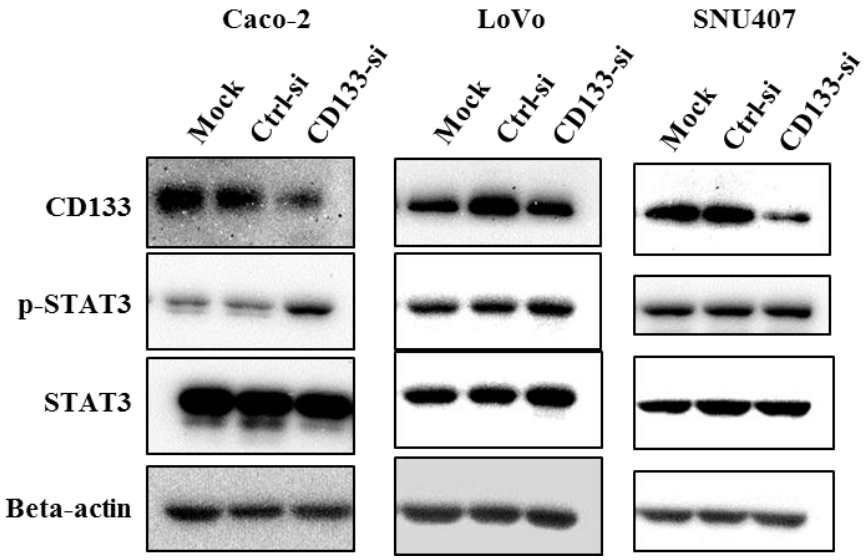

Figure 3. CD133, p-STAT3/STAT3 protein expression after CD133-siRNA transfection

p-STAT3 and STAT3 expression showed no significant change after CD133-siRNA transfection in primary (Caco-2) and metastatic (LoVo and SNU407) colon cancer cell lines.

1.1.3. Effect of IL-6 induced STAT3 activation on CD133 and survivin protein expression

Expression of the phosphorylated STAT3 (p-STAT3) tyrosine705 (Y705) phenotype was obvious at 6, 12, 24, and 36 h after treatment of the primary and metastatic colon cancer cell lines (Caco-2, LoVo, and SNU407) with IL-6 (20 ng/mL) (Figure 4). In all cell lines, CD133 and survivin expression levels reached their maxima at 6 h, and then declined slightly.

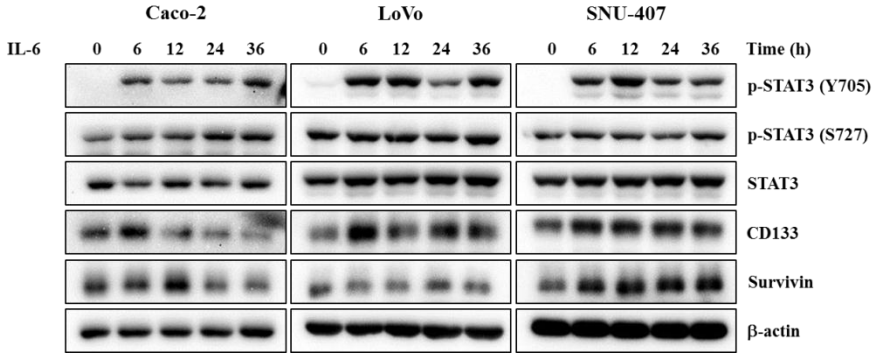

Figure 4. Changes in p-STAT3/STAT3, CD133 and survivin protein expression after IL-6 treatment in primary and metastatic colon cancer cells

p-STAT3 (Y705) expression was elevated at 6, 12, 24, and 36 h after IL-6 treatment in all cell lines. The expression of CD133 and p-STAT3 also increased in all cell lines, but survivin expression only increased in the Caco-2 and SNU407 cell lines.

1.1.4. The effect of STAT3 inhibition by STAT3-siRNA and stattic on biological behaviors of cancer cells

We examined the effect of STAT3 on the stemness of colon cancer cells using a colony formation assay. Following STAT3-siRNA transfection and treatment with stattic, colony-forming ability was markedly reduced in both the primary and metastatic colon cancer cells compared with the control (Figure 5).

We next evaluated cell migration using a wound-healing assay. To exclude the effect of cell proliferation, all wound-healing assays were performed following mitomycin C treatment, as described in the literature [41]. As shown in Figure 6, the migration ability of all the colon cancer cells was significantly attenuated in a dose-dependent manner by treatment with stattic at concentrations of 10–20 μM.

A WST-1 assay revealed that stattic lowered cell viability in a dose-dependent manner in both primary and metastatic colon cancer cells, but had a more immediate effect on the primary colon cancer cells (Figure 7A). The half maximal inhibitory concentration (IC_{50}) values were 15, 20, and 20 μM for the Caco-2, LoVo, and SNU407 cell lines,

respectively. Flow cytometry analysis with 7AAD–Annexin V staining revealed a marked increase in apoptosis after treatment with stattic in the primary colon cancer cells compared with the metastatic cancer cells (Figure 7B). Western blotting confirmed the increase in apoptosis by detecting the increase in the expression levels of cleaved caspase-3 and cleaved PARP compared with the control (Figure 7C). The expression of cleaved caspase-3 and cleaved PARP was more persistent in the primary colon cancer cells than in the metastatic cancer cells. This phenomenon seems to be consistent with changes in survivin expression at the mRNA and protein levels following treatment with stattic (Figure 1B and 2B). We think that the increase in apoptosis following treatment with stattic may be related to the reduction in survivin expression caused by reduced CD133 expression.

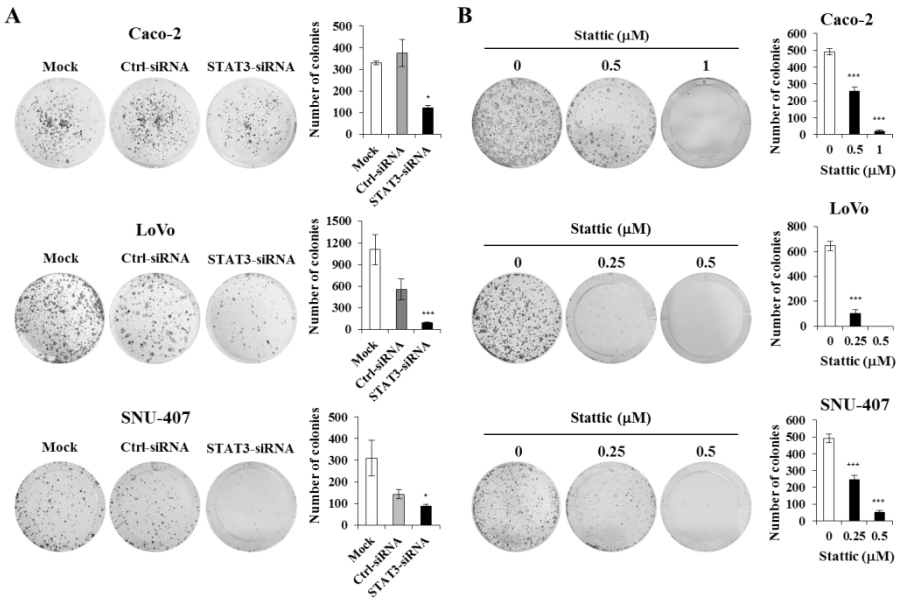

Figure 5. The effect of STAT3 on colony formation in primary and metastatic colon cancer cells

Following STAT3-siRNA transfection, a colony formation assay revealed a significant reduction in the number of colonies compared with the control-siRNA- and STAT3-siRNA-transfected groups in all cell lines (Caco-2, $p = 0.0296$; LoVo, $p = 0.0007$; SNU407, $p = 0.0131$) (A). Treatment with stattic inhibited the ability to form colonies in primary and metastatic colon cancer cells in a dose-dependent manner. (***$p < 0.0001$) (B).

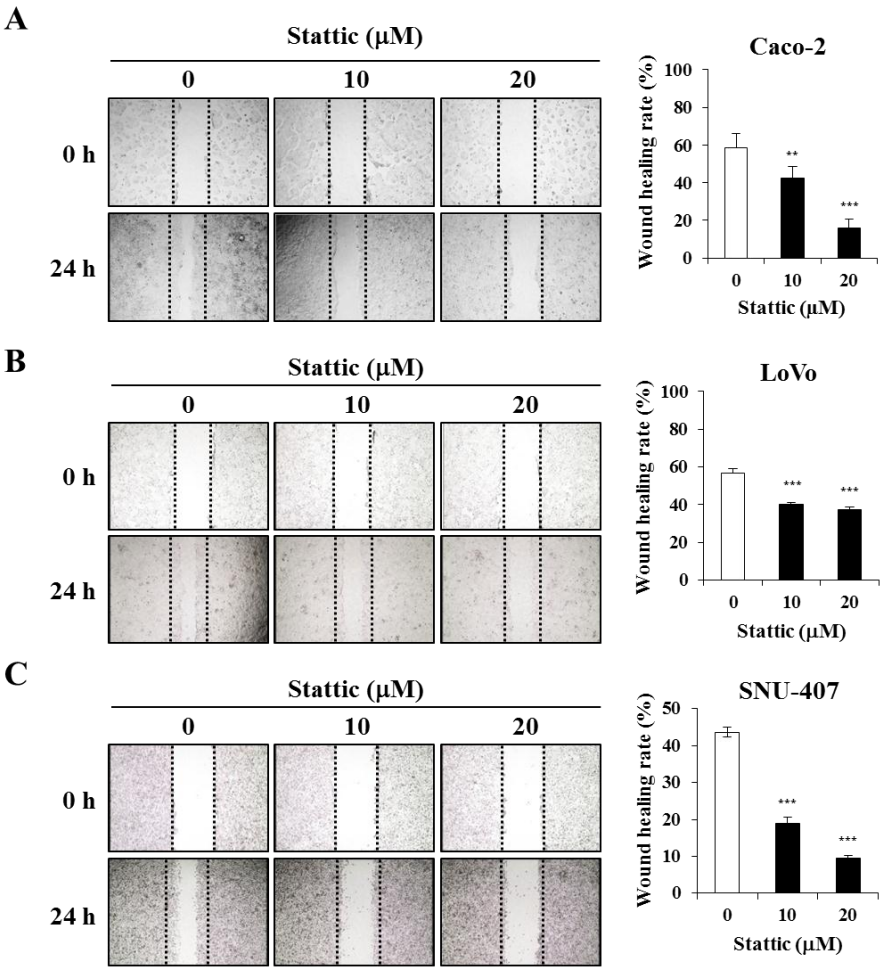

Figure 6. The effect of STAT3 on cell migration in primary and metastatic colon cancer cells

Wound-healing assays revealed a significant difference in cell migration between the control and stattic treatment groups (A, Caco-2; B, LoVo; C, SNU407). The ability to migrate was attenuated by

treatment with stattic in the primary and metastatic colon cancer cells. (**$p < 0.01$, ***$p < 0.001$).

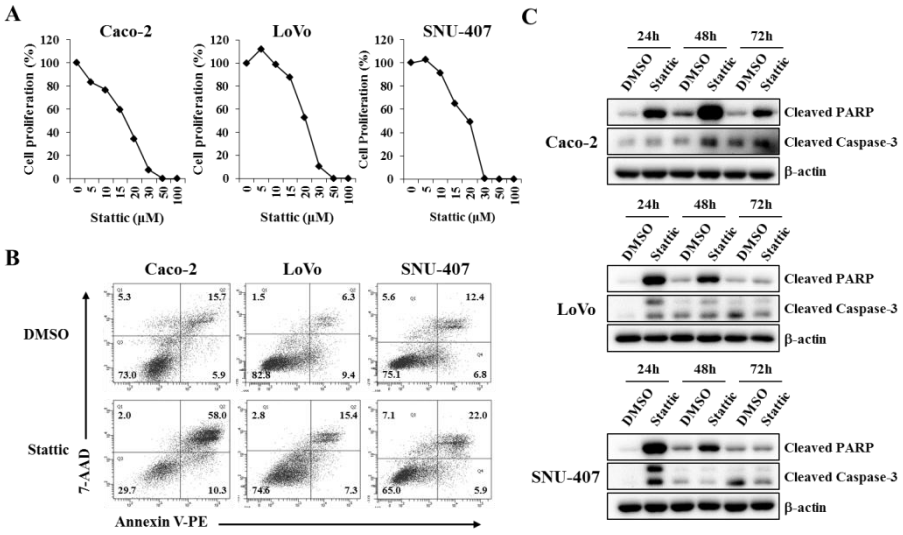

Figure 7. The effect of STAT3 on cell proliferation and apoptosis in primary and metastatic colon cancer cells

A WST-1 assay revealed that the proliferation of cancer cells was inhibited by treatment with stattic in a dose-dependent manner in primary and metastatic colon cancer cells (A). Following treatment with stattic, cytometric flow analysis revealed increased levels of Annexin-V- and 7-AAD-positive cells compared with the control (B). The apoptosis rates in the Caco-2, LoVo, and SNU407 cell lines increased in stattic treatment groups (68.3, 22.7, and 27.9%, respectively) compared with control groups (21.6, 15.7, and 19.2%, respectively. The expression levels of cleaved PARP and cleaved

caspase-3 increased following treatment with stattic compared with the control (C). The increase in apoptosis and the expression levels of cleaved caspase-3 and cleaved PARP were more pronounced in the primary colon cancer cells than in the metastatic cancer cells.

1.2. The role of CD133 in colon cancer cells

1.2.1. The effect of CD133 inhibition by CD133-siRNA on survivin protein expression

Western blot analysis of the cells in the colonies on day 12 of the colony formation assay showed that survivin expression levels in the ctrl-siRNA group were higher than those in the CD133-siRNA group, whereas the mock group had the lowest level of survivin expression among the three groups (Figure 8). Survivin expression seemed to be related to not only CD133 expression but also to the transfection procedure because compared with the mock group, the ctrl-siRNA and CD133-siRNA groups had a higher level of survivin expression.

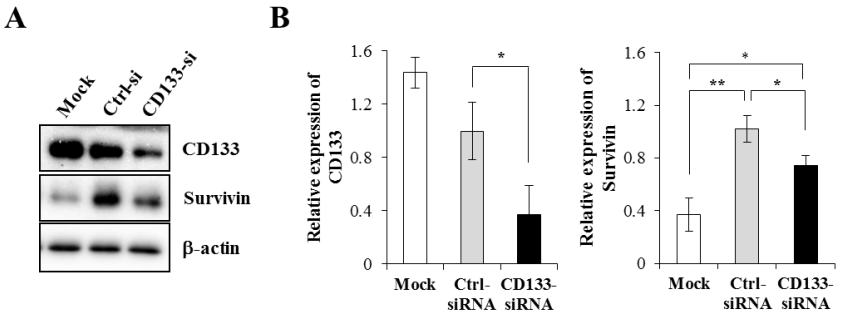

Figure 8. Changes in CD133 and survivin protein expression after CD133-siRNA transfection in primary colon cancer cell line

Western blot analysis showed that survivin expression levels in cells of the ctrl-siRNA group were significantly higher than those of the CD133-siRNA group (p=0.0191), but survivin expression levels in cells of the mock group were significantly lower than those of the ctrl-siRNA group (p=0.0022) even though the colonies in both groups were composed of $CD133^+$ cells. CD133-siRNA group showed significantly higher survivin expression than mock group (P=0.0115) (*$p < 0.05$, **$p < 0.01$). (Adopted[43])

1.2.2. CD133 and p-STAT3/STAT3 expression by western blot after YM155 treatment

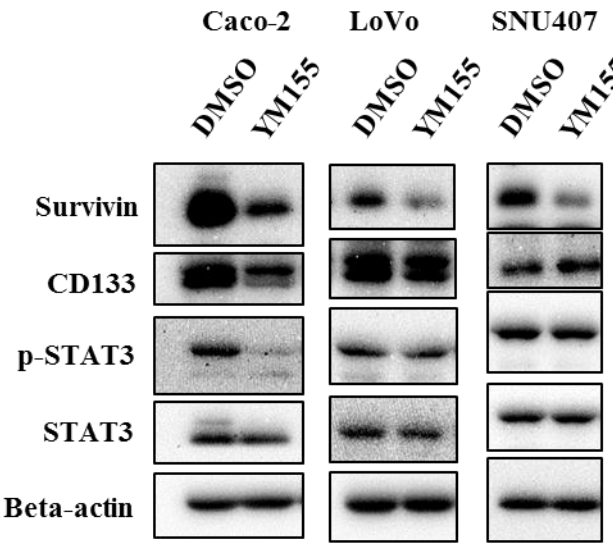

Figure 9. Survivin, CD133 and p-STAT3/STAT3 protein expression after YM155 treatment in primary and metastatic colon cancer cells

CD133 expression showed no significant change after YM155 treatment for 24h in primary (Caco-2) and metastatic (LoVo and SNU407) colon cancer cell lines. p-STAT3 showed no decrease expression only in metastatic (LoVo and SNU407) cell lines but not primary (Caco-2) cell lines.

1.2.3. The effect of CD133 inhibition by CD133-siRNA on biological behaviors of cancer cells

To elucidate the effect of CD133 expression on the tumorigenicity of Caco-2 colon cancer cells, we analyzed $CD133^+$ cells (a mock group and a ctrl-siRNA group) and siRNA-induced $CD133^-$ cells (CD133-siRNA group) with the colony formation assay. As seen in Figure 11, the mock group had the highest tumorigenicity of the three groups, and no significant difference was found in the number of colonies in the ctrl-siRNA and CD133-siRNA groups at 12 days after inoculation. Flow cytometry analysis of the colonies on day 12 of the colony formation assay showed that the CD133-siRNA group was composed of 66.2% $CD133^+$ cells in contrast to 95.6% and 93.8% in the mock and ctrl-siRNA groups, respectively (Figure 10B). Notably, this assay demonstrated that a significant number of $CD133^-$ cells formed colonies in the CD133-siRNA group.

Flow cytometric analysis with Annexin V staining showed that YM155 effectively induced apoptosis in a dose-dependent manner in the mock, ctrl-siRNA and CD133-siRNA groups (Figure 11A). However, no significant difference in anticancer effect of YM155 was

found among the groups. Compared with untreated colon cancer cells, YM155-treated (10 nM) colon cancer cells showed increased levels of cleaved PARP and cleaved caspase-3 expression at the protein level (Figure 11B).

However, the apoptosis analysis showed that the mock group with the lowest survivin expression level had the lowest rate of apoptosis (Figure 11 and 8).

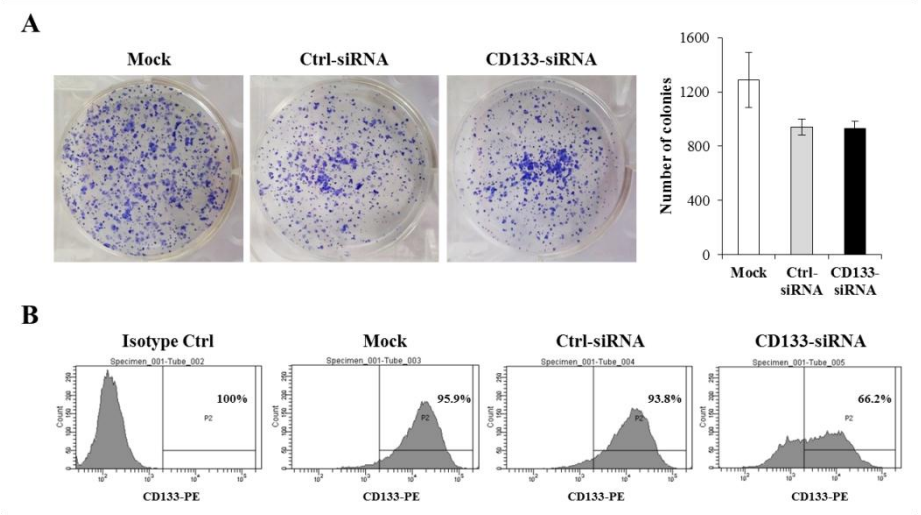

Figure 10. The effect of CD133 on colony formation in primary colon cancer cell

(A) Representative photographs of the colony formation assay demonstrate that the highest tumorigenicity was observed in the mock group. No significant difference in tumorigenicity was found between the ctrl-siRNA and CD133-siRNA groups. (B) Flow cytometry analysis using cells from the colony formation assay showed that most of the cells in the mock and ctrl-siRNA groups were $CD133^{+}$, but the colonies in the CD133-siRNA group were composed of $CD133^{+}$ cells (66.2%) as well as $CD133^{-}$ cells (33.8%). PE, phycoerythrin. (Adopted[43])

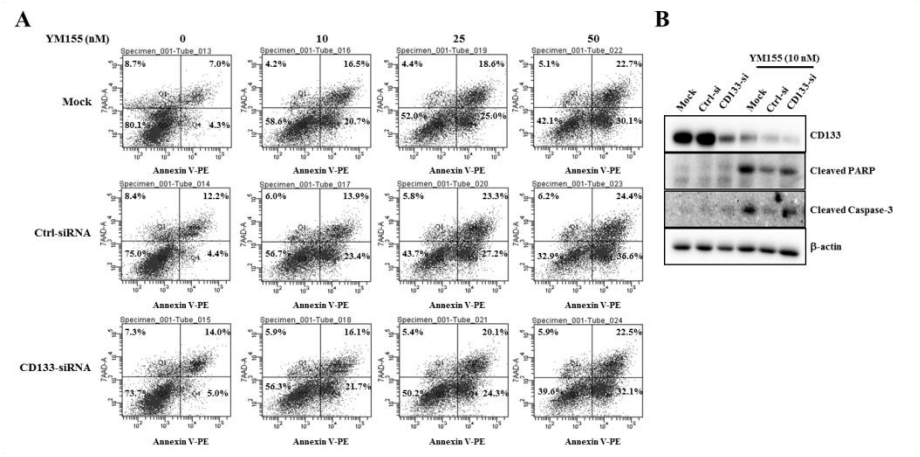

Figure 11. The effect of CD133 and survivin on cell apoptosis in primary colon cancer cells

Flow cytometric (A) and western blot (B) analyses after treatment with the survivin inhibitor YM155 showed that although the apoptosis rate increased significantly in a dose-dependent manner, no significant differences in anticancer effect were found among the groups. PARP, poly-ADP-ribose polymerase. (Adopted[43])

1.2.4. Anticancer effect of survivin inhibitor YM155 on colon cancer cells

WST-1 assay after survivin inhibitor treatment showed that YM155 and a combination of YM155 and 5-FU were more effective than 5-FU alone in all three groups (Figure 12). These results indicated that YM155 is an effective treatment modality for colon cancer through increasing apoptosis; however, the relationship between the effectiveness and CD133 expression is unclear.

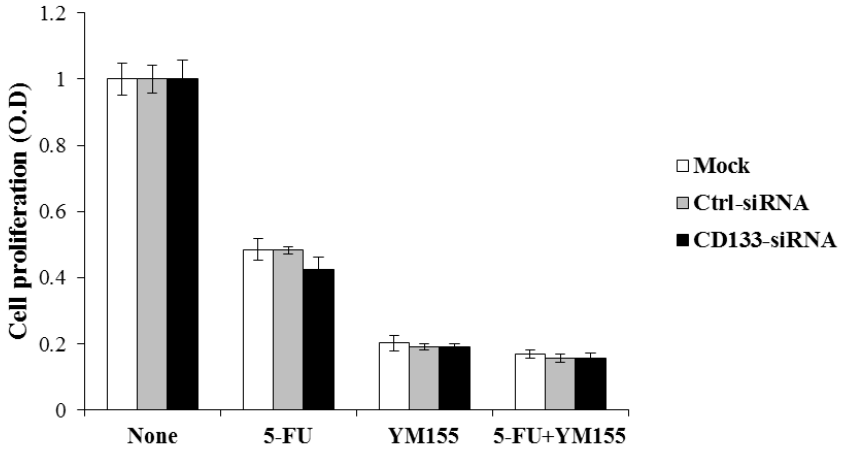

Figure 12. Anticancer effect of survivin inhibitor YM155 by WST-1 assay

The results of the cell proliferation assay with WST-1 kit showed that, compared with the cells in the CD133-siRNA group, the $CD133^{+}$ cells (mock and ctrl-siRNA groups) had slightly greater chemoresistance to 5-fluorouracil (5-FU). However, in all groups, treatment with YM155 and a combination of YM155 and 5-FU had greater anticancer effect than treatment with 5-FU alone. (Adopted[43])

2. Histological study using clinical samples

2.1. Association of p-STAT3, CD133 and survivin expression with clinico-pathological parameters

2.1.1. The result of IHC stain for p-STAT3 expression in non-tumoral mucosa, adenoma, and adenocarcinoma of colon

We investigated the possible association between the expression of p-STAT3, CD133, and survivin and the pathological features of colonic adenoma and adenocarcinoma by examining the immunohistochemical (IHC) expression of these proteins in 42 resected colonic adenomas, 62 carcinomas in situ (pTis = high-grade dysplasia and intramucosal adenocarcinoma), and 106 adenocarcinomas. The clinicopathological features and the distribution of p-STAT3, CD133, and survivin expression in the patients included in this study are presented in Table 1. The mean age of the 210 patients was 65.6 ± 10.7 years (range 36–95 years).

P-STAT3 (Y705) expression was observed in the nuclei of the cells on the surfaces of the tumors (Figure 13). Interestingly, p-STAT3 expression was more pronounced in pTis and pT1&T2 colon cancer than in pT3&T4 cancer (Table 2). As shown in Figure 13, several p-STAT3-positive cells were found in the non-tumor mucosa around many adenocarcinomas (15 out of 40 in pT1&T2; 22 out of 66 in pT3&T4); however, these cells were rarely found in the non-tumor mucosa around preinvasive tumors (1 out of 42 in the adenomas; 2 out of 62 in the pTis).

Table 1. Patient demographics for the current study (n=210)

Feature	Classification	n (%)
Sex	Male	127
	Female	83 (39.5)
Age	≥50	194
	<50	16 (7.62)
Location	Right (Sigmoid, Descending)	98 (46.7)
	Left (Transverse, Ascending,	112
Invasion depth (pT)	Adenoma	42 (20)
	pTis	62 (29.5)
	T1 & T2	40 (19.0)
	T3 & T4	66 (31.4)
Regional lymph	N0	69 (32.9)
	N1 & N2	42 (20.0)
	No lymph node dissection	99 (47.1)
Lymphatic invasion	Not identified	154
	Identified	56 (26.6)
Venous invasion	Not identified	200
	Identified	10 (4.8)
Perineural invasion	Not identified	189 (90)
	Identified	21 (10)

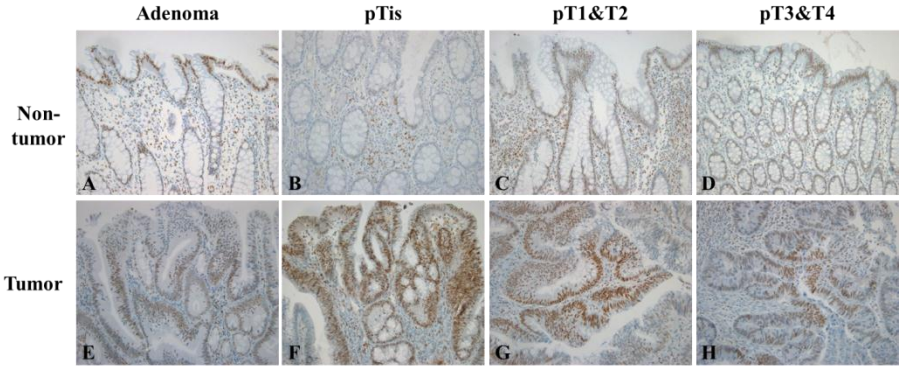

Figure 13. The immunohistochemical expression of p-STAT3 in the non-tumor, adenoma, and adenocarcinoma of the colon

Nuclear p-STAT3 expression was observed in the non-tumoral mucosa around the tumor (A-D) and in the surface area, but not in the invasive border of the tumor (E-H).

2.1.2. p-STAT3, CD133 and Survivin expression according to the clinicopathological parameters

We also observed CD133 and survivin expression according to p-STAT3 and clinicopathological parameters. The CD133 expression showed luminal membranous patterns mostly in the adenocarcinomas, rarely in the adenomas, and very rarely in the non-tumor mucosa. Nuclear survivin expression was found mostly in advanced colon cancer (42% of pT3&T4), but also in adenomas (14.3%), pTis (21%), and pT1&T2 (22.7%).

The expression levels of p-STAT3, CD133, and survivin were all significant independent factors associated with tumor invasion (p = 0.001, 0.000, and 0.000, respectively; Table 2). Additionally, there were significance between p-STAT3 expression and pN, lymphatic invasion, venous invasion and perineural invasion (Table 2).

Table 2. Distribution of p-STAT3, CD133 and survivin expression according to the clinicopathological parameters

Parameter		p-STAT3			CD133			Survivin		
		-	+	p-value*	-	+	p-value*	-	+	p-value*
Age**		66.0±10.3	65.1±11.3	0.54 (0.366)	62.9±11.9	67.4±9.4	0.004 (0.019)	65.4±9.6	65.8±11.5	0.775 (0.550)
Sex	Male	66(58.9%)	61(62.2%)	0.143	50(59.5%)	77(61.1%)	0.931	50(54.9%)	77(64.7%)	0.197
	Female	46(41.1%)	37(37.8%)		34(40.5%)	49(38.9%)		41(45.1%)	42(35.3%)	
Location	Right	44(39.3%)	54(55.1%)	0.173	37(44.0%)	61(48.4%)	0.631	37(40.7%)	61(51.3%)	0.166
	Left	68(60.7%)	44(44.9%)		47(56.0%)	65(51.6%)		54(59.3%)	58(48.7%)	
Invasion depth (pT)	Adenoma	24(21.4%)	18(18.4%)	0.001	37(44.0%)	5(4.0%)	<0.001	25(27.5%)	17(14.3%)	<0.001
	pTis	23(20.5%)	39(39.8%)		23(27.4%)	39(31.0%)		37(40.7%)	25(21.0%)	
	pT1 & T2	13(11.6%)	27(27.6%)		7(8.3%)	33(26.2%)		13(14.3%)	27(22.7%)	
	pT3 & T4	52(46.4%)	14(14.3%)		17(20.2%)	49(38.9%)		16(17.6%)	50(42.0%)	
pN†	N0	35(31.3%)	34(34.7%)	0.012	11(45.8%)	58(66.7%)	0.104	17(54.8%)	52(65.0%)	0.440
	N1 & N2	32(28.6%)	10(10.2%)		13(54.2%)	29(33.3%)		14(45.2%)	28(35.0%)	
Lymphatic invasion	Not identified	69(61.6%)	85(86.7%)	0.023	70(83.3%)	84(66.7%)	0.012	74(81.3%)	80(67.2%)	0.033
	Identified	43(38.4%)	13(13.3%)		14(16.7%)	42(33.3%)		17(18.7%)	39(32.8%)	
Venous invasion††	Not identified	102(91.1%)	98(100.0%)	0.006	82(97.6%)	118(93.7%)	0.322	87(95.6%)	113(95.0%)	1.000
	Identified	10(8.0%)	0(0%)		2(2.4%)	8(6.3%)		4(4.4%)	6(5.0%)	
Perineural invasion	Not identified	93(83.0%)	96(98.0%)	0.005	76(90.5%)	113(89.7%)	1.000	84(92.3%)	105(88.2%)	0.458
	Identified	19(17.0%)	2(2.0%)		8(9.5%)	13(10.3%)		7(7.7%)	14(11.8%)	

*Chi-square test (or Fisher's Exact test)

**As age is a continuous variable, so the Independent-Samples T-test and the Wilcoxon rank sum test were performed.

†Patients of adenoma or pTis without lymph node dissection were excluded.

†† The p-values of venous invasion were performed by Fisher's Extract test.

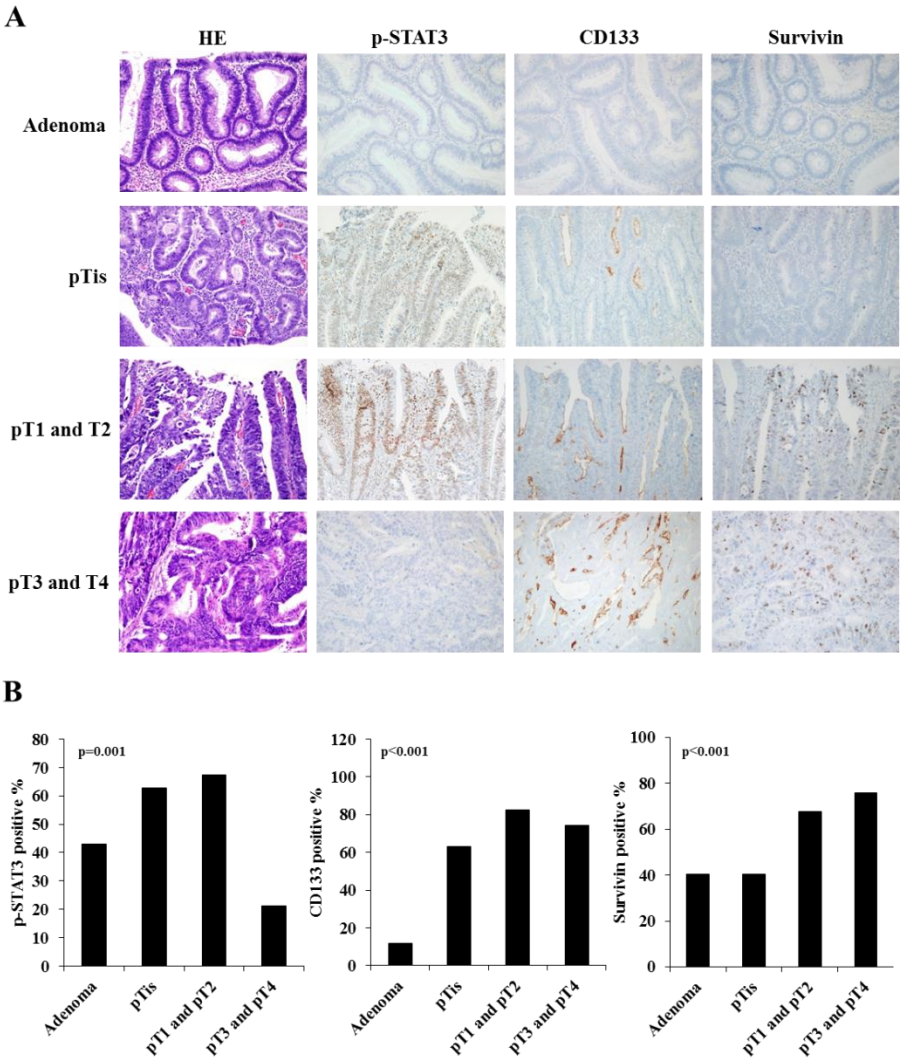

Figure 14. Immunohistochemical expression of p-STAT3, CD133 and survivin and their distribution according to tumor invasion depth in adenoma and adenocarcinoma of colon

The photomicrographs revealed the features of immunohistochemical (IHC) staining from representative cases according to tumor invasion. p-STAT3 was more common in pTis and T1&T2 colon cancer than in the adenomas or T3&T4. In contrast, CD133 expression was found mainly in T1&T2 and T3&T4, and was rare in adenomas (B). The immunoprofile of p-STAT3/CD133/survivin varied significantly associated with tumor invasion (A). The most common immunoprofiles were: p-STAT3^{+}/CD133^{-}/survivin^{-} for adenoma; p-STAT3^{+}/CD133^{+}/survivin^{+} for pTis and pT1&T2; and p-STAT3^{-}/CD133^{+}/survivin^{+} for pT3&T4.

2.1.3. Association between IHC results of p-STAT3/CD133/survivin and advanced T-stage based on logistic regression analysis

For the logistic regression analysis, the tumors were divided into two groups according to the depth of invasion: preinvasive (adenoma, pTis) and invasive (pT1-T4). The expression levels of p-STAT3, CD133, and survivin were all significant independent factors associated with tumor invasion (p = 0.001, 0.000, and 0.000, respectively; Table 3). As shown in Table 3, p-STAT3 expression was inversely related to tumor invasion (hazard ratio (HR) = 0.328, 95% confidence interval (95%CI): 0.17–0.632), whereas CD133 and survivin expression exhibited significant direct association. We also determined whether the expression patterns of the three molecules in the same tumor (the immunoprofile) were related to tumor invasion. The immunoprofile according to the invasion depth of the tumors is presented in Figure 14. Tumors with an immunoprofile of p-STAT3^{+}/CD133^{-}/survivin^{-} were significantly associated with less risk of tumor invasion compared with p-STAT3^{+}/CD133^{+}/survivin^{+} tumors. However, the p-STAT3^{-}/CD133^{+}/survivin^{+} immunoprofile was

significantly associated with the invasion of colon cancer (p = 0.002, HR = 6.480, 95%CI: 1.957–21.462).

Table 3. Aassociation of p-STAT3/CD133/survivin expression with tumor invasion* by logistic regression analysis

		Hazard ratio (95%CI)	P
p-STAT3		**0.328** (0.170-0.632)	**0.001**
CD133		**4.971** (2.551-9.687)	**0.000**
survivin		**3.617** (1.913-6.835)	**0.000**
p-STAT3/CD133/survivin			
n (%)			
+/+/+	43 (20.5)	1	0.000
+/+/-	22 (10.5)	0.498 (0.176-1.416)	0.191
+/-/+	15 (7.1)	0.480 (0.145-1.590)	0.230
+/-/-	18 (8.6)	**0.042** (0.005-0.348)	**0.003**
-/+/+	40 (19.0)	**6.480** (1.957-21.462)	**0.002**
-/+/-	21 (10.0)	0.960 (0.334-2.758)	0.940
-/-/+	21 (10.0)	0.655 (0.229-1.869)	0.428
-/-/-	30 (14.3)	**0.219** (0.077-0.620)	**0.004**

* Tumor invasion: adenoma & pTis *vs* T1-4

2.2. Relationships between survivin and CD133 expression and clinico-pathological parameters

2.2.1. Immunohistochemical expression of CD133 and survivin expression

The demography of cases examined in this study presented in Table 3. Representative cases with high and low survivin expression are shown in Figure 15A–D with matched CD133 expression. CD133 showed a luminal membranous expression pattern (Figure 15A), whereas survivin showed distinct nuclear expression with weak and vague cytoplasmic expression in CRC cells (Figure 15B). We analyzed only the nuclear survivin expression in this study, because the cytoplasmic expression was too diffuse and weak to be scored.

Table 4. Patients demographics for the current study (n=187)

Feature	Classification	n (%)
Sex	Male	117
	Female	70
Age	>50	156
	<50	31
Tumor	Well differentiated	10
	Moderately	159
	Poorly	18
Tumor stage	II	72
	III	115
Location	Cecum, appendix	44
	Transverse,	64
	Rectum	79
Regional lymph	N0	73 (39)
	N1	70
	N2	44

(Adopted[28])

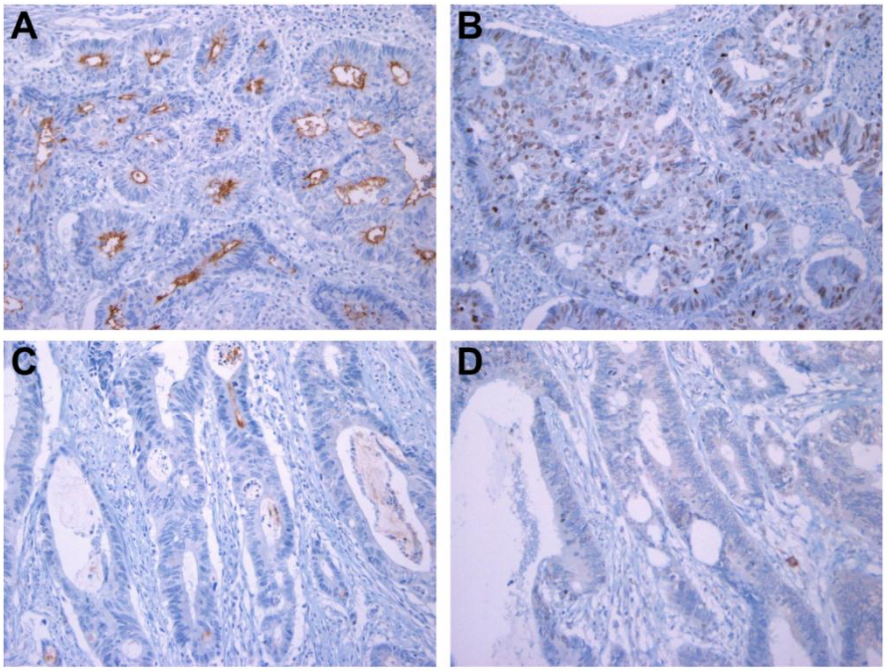

Figure 15. The immunohistochemical expression of CD133 and survivin in advanced colon cancer

Photomicrographs showing representative cases of high CD133 (A) and survivin (B) expression and low CD133 (C) and survivin (D) expression in colon cancer tumor cells. CD133 expression localized in the luminal surface of the cytoplasm, and survivin expression localized in the nucleus (×400). (Adopted [28])

2.2.2. Survivin expression according to the pathologic parameters

Survivin expression showed a significant correlation with CD133 expression ($p = 0.0178$) when analyzed by the Mantel-Haenszel test. However, survivin showed no significant relationship with invasion depth ($p = 0.2674$), histologic differentiation ($p = 0.5314$), or lymph node metastasis ($p = 0.7128$).

Table 5. Survivin expression according to the pathologic parameters

Parameters	Survivin expression (n)				Total	p
	1+	2+	3+	4+		
Histologic differentiation						
Well	2	4	4	0	10	0.5314
Moderate	32	52	59	16	159	
Poor	5	3	7	3	18	
Invasion depth						
T2	1	3	3	1	8	0.2674
T3	36	52	60	16	164	
T4	2	4	7	2	15	
Lymphnode metastasis						
N0	17	20	26	10	73	0.8149
N1	13	25	27	5	70	
N2	9	14	17	4	44	
Adjuvant therapy						
Yes	32	54	65	18	169	0.2973
No	7	5	5	1	18	
CD133 IHC expression						
0	18	14	18	3	53	**0.0178**
1+	11	12	15	6	44	
2+	3	16	12	5	36	
3+	7	17	25	5	54	

IHC, immunohistochemistry.

(Adopted[28])

2.2.3. Association of apoptosis with CD133 and survivin expression

Apoptosis detected by TUNEL analysis showed rare positive cells in normal mucosa (Figure 16A), in contrast to occasionally scattered positive cells in colon cancer cells (Figure 16B). The AI was significantly higher in $CD133^+$ tumors (5.1654 ± 4.9607) than in $CD133^-$ tumors (4.2308 ± 3.8123; p = 0.0336). Further, compared with $survivin^-$ tumors, $survivin^+$ tumors had a higher AI (5.1164 ± 4.8941 versus 4.1026 ± 3.6906; p = 0.0443; Figure 17).

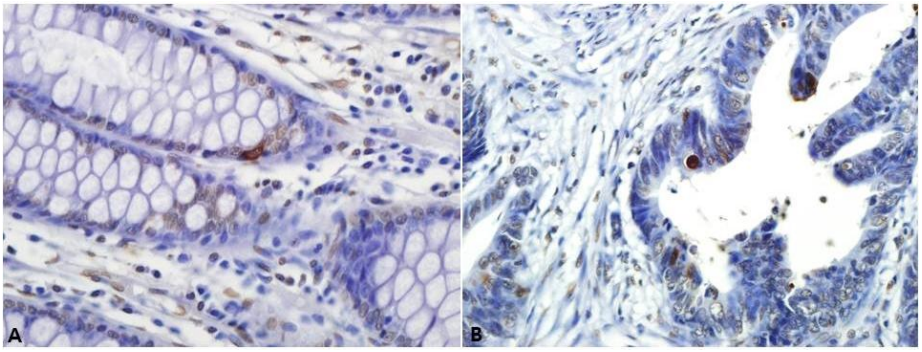

Figure 16. Representative microphotograph of TUNEL staining of section from normal mucosa and colon cancer

Rare apoptosis in normal mucosa (A), but scattered positively stained apoptotic cells in colon cancer (B) were observed by TUNEL assay (×400). (Adopted[28])

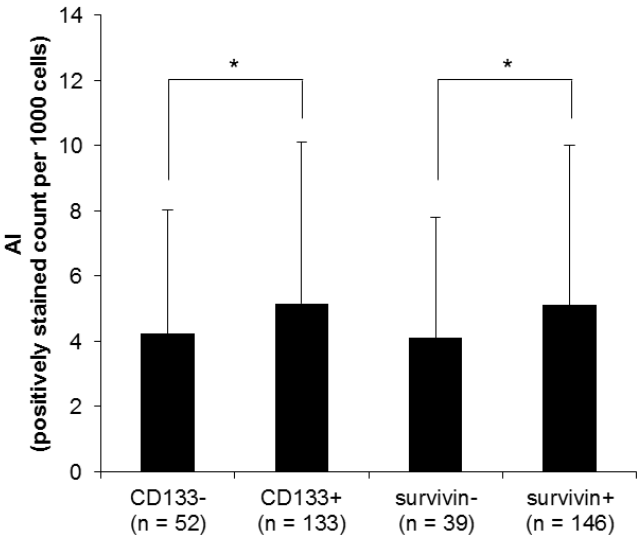

Figure 17. Difference in apoptosis according to survivin and CD133 expression

Compared with that in $CD133^-$ and $survivin^-$ tumors, the mean apoptotic index (AI) values obtained by TUNEL assay was significantly higher in $CD133^+$ and $survivin^+$ tumors (*$p < 0.05$). (Adopted[28])

2.2.4. Survivin expression was not related with patient's overall survival

The mean overall survival (OS) duration according to survivin expression was 113.149 ± 9.072 months for score 1+, 47.008 ± 1.657 months for score 2+, 41.781 ± 1.351 months for score 3+, and 7.411 ± 0.077 months for score 4+. However, survivin expression had no prognostic significance in the survival analysis (p = 0.4804; Figure 18).

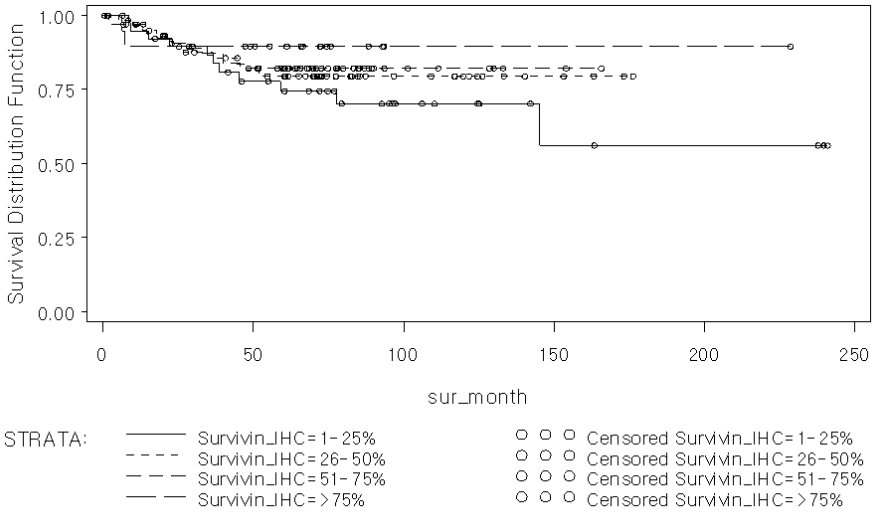

Figure 18. Survival analysis according to survivin expression

IV. DISSCUSSION

Colon cancer is formed by an abnormal growth of epithelial cells which contains mutated genes. Recently, the role of inflammation in carcinogenesis of colon cancer has been studied. At the same time, relationship between colorectal cancer and immune reaction has been researched for several years. The recent study have reported differences between the gut microbiota of healthy people and that of colorectal cancer sufferers [4]. An abnormal gut microbiota can activate innate immune responses that play a key role in gut inflammation. It is well known that inflammation plays a role in the development of colorectal cancer, but the mechanism by which it acts remains unclear. Accumulating evidence suggests that inflammatory cells and their products in the tumor microenvironment can enhance the progression of cancer [44,45]. Inflammatory bowel disease (IBD) including ulcerative colitis (UC) and Crohn's disease (CD) is characterized by recurrent chronic inflammation, which significantly increases the risk of colorectal cancer [46]. An in vivo study revealed that the microbiota-induced activation of IL-6 signaling induces colon cancer [47].

IL-6 and its intracellular signaling mediator, STAT3 play an important role in chronic inflammation and inflammation-induced colorectal cancer [48]. Several studies have shown that the IL-6/STAT3 signaling pathway is involved in the development of colon cancer [49-51]. Ren *et al*. showed that the IL-6-activated Jak–STAT3 pathway promotes the development of colon cancer [52]. Kim *et al*. showed that bone morphogenetic protein-2 (BMP-2)-induced STAT3 activation promotes colon cancer stemness via epithelial–mesenchymal transition (EMT) [53]. This has also been demonstrated by Bak *et al*. [54]. These results provide strong support for our hypothesis that IL-6-induced p-STAT3 positively regulates colon cancer stem cell marker CD133 expression. A recent study showed that $ALDH^+/CD133^+$ cells express higher levels of IL-6 and STAT3 phosphorylation than $ALDH^-/CD133^-$ cells [24]. Kryczek *et al*. showed that IL-22 can also promote stemness in human colorectal cancer via STAT3 activation-mediated epigenetic regulation of stem cell genes [55]. As expected, in the present study we found that IL-6 induced increased p-STAT3 expression accompanied by upregulated CD133 expression. Furthermore, the inhibition of STAT3 caused the downregulation of CD133 expression, which was

accompanied by changes to the biological behaviors of the cancer cells. Interestingly, CD133 protein expression decreased more in the primary colon cancer than in the metastatic colon cancer after STAT3 inhibition. We have previously reported that promoter methylation is a regulatory mechanism of CD133 expression in colorectal cancer [40]. Therefore, we attempted to determine whether STAT3 inhibition can induce a change in CD133 promoter methylation. Unfortunately, we could not detect any change in CD133 promoter methylation following STAT3 inhibition (data not shown), probably because STAT3 was temporarily inhibited in the present experiment. To clarify the regulatory mechanism of the IL-6/STAT3/CD133 pathway, it will be necessary to conduct additional experiments that are sufficiently long-lasting to register change. In the present study, stattic induced dramatic increase of apoptosis in all the cell lines. Our results are consistent with those obtained by Li *et al*. and Zhao *et al*., who used other STAT3 inhibitors such as FLLL32 and LY5 [23,30]. We think phosphorylated STAT3 contributes to the biological behaviors of colon cancer cells by inducing survivin-regulated apoptosis. In our previous research, we demonstrated that the knockdown of CD33 by CD133-siRNA reduced

survivin expression, and treatment with the survivin inhibitor (YM155) markedly induced apoptosis [43]. Considering the consequences of the changes to biological properties during STAT3 suppression in the present study, we speculate that STAT3 regulates proliferation, invasiveness, and apoptosis by inducing the cancer stem cell marker CD133 in colon cancer. Moreover, one of the noteworthy findings of this study was that following treatment with stattic, apoptosis was more obvious in primary colon cancer than in metastatic colon cancer.

In order to obtain a more purified cell population of $CD133^-$ cells, we used a CD133 Microbead kit (Miltenyi Biotec) to separate $CD133^+$ cells from $CD133^-$ cells in the CD133-siRNA group. However, the results of the population analysis by flow cytometry showed that the $CD133^-$ colonies still contained 38.4% $CD133^+$ cells even after sorting (Date was not shown). We found a similar population pattern in the cells that underwent ctrl-siRNA or CD133-siRNA transfection without sorting. To avoid the bias induced by the damaging force of sorting, we used the data from the mock, ctrl-siRNA, and CD133-siRNA groups to analyze the biological characteristics of the tumor cells.

We found a number of $CD133^-$ cells (33.8%) in CD133-siRNA group by flow cytometry analysis of the cells from these colonies. However, there is no significant difference in the colony number between Ctrl-siRNA and CD133-siRNA groups (Figure 10). This result suggested that there may be other molecules that can affect colony formation ability of colon cancer cells. Notably, we found that STAT3 inhibition significantly reduced colony formation both in primary and metastatic colon cancer cells. These findings suggested STAT3 played an important role in maintaining the stemness of colon cancer cells.

Given the results of our previous study, which concluded that survivin overexpression induces chemoresistance in $CD133^+$ colon cancer cells [56], we hypothesized that a survivin inhibitor would have a specific effect on $CD133^+$colon cancer. In the current study, we analyzed colony formation and apoptosis assays for survivin expression after CD133 knockdown in vitro to determine the biological effect of CD133 expression in relation to survivin expression. However, we found no significant differences in tumorigenicity and apoptosis related to CD133 or survivin expression. Furthermore, the mock group

had the lowest level of survivin expression and the highest number of colonies on day 12 of the colony formation assay. No significant difference was found between the number of colonies in the ctrl-siRNA and CD133-siRNA groups, although survivin expression levels in cells in the ctrl-siRNA group were slightly higher than the CD133-siRNA group. These results may indicate that survivin expression is also related with a cell-damaging force such as a transfection procedure. The results may also support the hypothesis that survivin expression is related to abnormal p53 expression [39], which can be induced by cell damage.

The strategies explored for survivin-targeting therapies include dominant-negative survivin [57], gene silencing [58], and an aptamer-based drug delivery system [59]. YM155, a small-molecule inhibitor of survivin, targets the core promoter of survivin, which leads to tumor regression via the mitotic arrest of cells in the G2/M phase of the cell cycle. Antitumor activities of YM155 have been reported in a variety of human cancer cell lines, including colon cancer cells and the Caco-2 cell line [60,61]. However, the mode of action is not clear yet. In addition, survivin has a physiologic role in regulating the proliferation and

survival in normal cells such as polymorphonuclear cells [62], vascular endothelial cells [63], T cells [64], hematopoietic progenitor cells [65], and erythroid cells [66]. Therefore, it must be considered that survivin is expressed not only in tumor cells but also in normal colon mucosa [67]. Unavoidable limitations of survivin-targeted therapies, including the toxicity of agents, tumor specificity, and treatment sustainability, are the leading causes of treatment failure.

YM155 acts as an inhibitor of survivin but not only on surviving. Wagner *et al.* and Na *et al.* reported that YM155 could also decreases p-STAT3 expression in multiple myeloma [68] and pancreatic cancer cells [69], respectively. Therefore, it was not enough to observe the change of p-STAT3 with YM155 to prove the role of survivin on p-STAT3 expression. In addition, p-STAT3/ survivin signaling pathway has been largely confirmed in breast cancer [70,71], lung cancer [72], ovarian cancer [73] and colorectal cancer[74].

We evaluated the apoptosis rate after treatment with YM155 to determine whether the anticancer effects of the inhibitor are related to CD133 expression. The apoptosis rate increased with the concentration of YM155. Increase of cleaved PARP and cleaved caspase-3

expression were also found in cells treated with YM155 regardless of in vitro CD133 expression. The results of our in vitro study using Caco-2 colon cancer cells showed that treatment with YM155 or a combination of YM155 and 5-FU was far more effective than treatment with 5-FU alone. However, we had difficulty demonstrating differences in the therapeutic effects on tumor cells according to CD133 expression because of the limitation of the experimental procedure and the complex mechanisms of survivin expression. An array-based expression analysis using a human HT-12 expression bead chip also showed that survivin expression in $CD133^+$ cells was 1.2-fold higher than that in $CD133^-$ cells transfected with CD133-siRNA (data not shown); however, we could not completely separate $CD133^+$ cells and $CD133^-$ cells as in our previous study [27]. Therefore, the lack of significant difference in therapeutic effect between the ctrl-siRNA and CD133-siRNA groups is thought to be related to the small difference in survivin expression between these groups. *In vitro* study, we described that $CD133^+$ colon cancer cells showed chemoresistance to 5-FU through high survivin expression. In addition, we demonstrated that the survivin inhibitor YM155 was more effective than 5-FU in

inducing cell death of colon cancer cells. Inhibitors of apoptosis have been used as new targeted therapies in colon cancer [75].

To determine whether the results of our *in vitro* studies can applicable to clinical samples or not, we examined the study to found the relevance of p-STAT3, CD133 and survivin expression to the clinicopathological parameters. We found a correlation between CD133 expression and nuclear survivin expression in advanced stage of colon cancer. It agrees with that of our previous in vitro study, which indicates that both the mRNA and protein expression levels of survivin in $CD133^+$ cells are remarkably higher than those in small interfering RNA (siRNA)-induced CD133- cells. These findings are also concordant with those of the transcriptome analysis reported by Kim *et al* [37]. Elevated levels of p-STAT3 have been reported to be correlated with tumor invasion, metastasis [15,17], and poor prognosis [18]. However, Monnien *et al*. reported that p-STAT3 was significantly associated with improved overall survival in advanced rectal cancer [20]. The authors of another study reported that there was no significant difference between p-STAT3 expression and tumor stage [19]. Invasive colonic adenocarcinomas were enrolled in both studies. However, in

the present study, we included preinvasive tumors (tubular adenomas with low-grade dysplasia, high-grade dysplasia, and intramucosal adenocarcinoma (pTis)) as well as invasive adenocarcinomas (pT1&T2, pT3&T4) to investigate the distribution of p-STAT3 according to tumor progression. Interestingly, we found that p-STAT3 expression was more common in pTis (39.8%) and pT1&T2 (27.6%) colon cancer than in adenomas (18.4%) and advanced adenocarcinomas (pT3&T4, 14.3%). Logistic regression analysis revealed that p-STAT3 expression was inversely associated with tumor invasion (HR = 0.328, 95%CI: 0.170–0.632), whereas CD133 and survivin expression levels were significantly associated with the advanced stages of colon cancer. We also determined whether the immunoprofile of p-STAT3/CD133/survivin expression in the same tumor was associated with tumor progression. The immunoprofile of p-STAT3^{-}/CD133^{+}/survivin^{+} was significantly associated with advanced colon cancer (HR = 6.480, 95%CI: 1.957–21.462). However, the most common immunoprofile of adenoma was p-STAT3^{+}/CD133^{-}/survivin^{-} (28.6%), which was inversely associated with tumor invasion (p = 0.003, HR = 0.042, 95%CI: 0.005–0.348) compared with all the

positive tumors. Based on the results of our in vitro study, which suggest that p-STAT3 regulates CD33 expression, we believe that the results of the study using clinical samples support the signaling pathway in the order: STAT3/CD133/survivin. In the present study, we demonstrated that inhibition of STAT3 induced apoptosis and reduced CD133 expression more successfully in primary colon cancer cells than in metastatic colon cancer cells. These findings suggest that targeting STAT3 for colon cancer treatment is more effective in the early stages than in the advanced stages of colon cancer. Therefore, detection of p-STAT3 expression in primary colon cancer may be used to guide $CD133^{+}$ colon cancer treatment.

We also found that AI in tumor tissue was higher than that in the surrounding normal mucosa. In addition, $CD133^{+}$ and $survivin^{+}$ tumors showed higher mean of AI than $CD133^{-}$ and $survivin^{-}$ tumors. However, AI was not significantly related with either survivin or CD133 expression in this study. The balance between proliferation and apoptosis maintains the homeostasis of the colonic epithelium, but uncontrolled cell proliferation and apoptosis contribute to tumor development [76]. Alcaide *et al*. reported that AI in colorectal cancer is

higher than that in adenomas and normal tissue [77]. Bedi *et al*. reported that apoptosis is inhibited during CRC development, owing to abnormal expression of the BCL2 gene [78]. Zhao *et al*. reported that a low dose of bcl-2 inhibitor could up-regulate survivin expression in hepatocellular carcinoma [79]. They indicate that apoptosis is regulated by not only survivin but also bcl-2. In addition, the effects of apoptotic proteins on $CD133^+$ colon cancer cells have been rarely reported. In a study by Kemper *et al*., activated caspase-9 was found to induce a high level of apoptosis in $CD133^+$ colon cancer stem cells [80]. Sam *et al.* demonstrated that inhibiting survivin and caspase 3 trigger apoptosis in colon cancer stem-like cells [81].

According to the pathogenesis of colorectal cancer, looking for an initial cancer-causing promoter can be a more effective strategy for targeting treatment. Despite advances in cancer treatment, such as immunotherapy, tumor heterogeneity is one of the main causes of chemotherapy failure. After cancer stem cells are induced, it is easy to develop tumor heterogeneity owing to the asymmetric and autonomous proliferation of cancer cells. Therefore, suppressing the progression of

early cancer is thought to be the most effective approach to the treatment of colon cancer.

The experimental subjects in current study were human primary and metastatic human colon cancer cells lines, but didn't include *in vivo* study. Firstly, most animal models of colon cancer that are inflammatory bowel disease associated colon cancer. However, most of the cases in the study were sporadic colon cancer. Tumor microenvironment with inflammation in the animal model can interfere p-STAT3 related experimental results. Additionally, instead of animal model with we used clinical samples. The results of IHC stain could clearly and accurately explain the distribution of p-STAT3, CD133 and survivin in different stages of colon cancer as well as in the mucosa of normal colon.

There is a limitation for our current study, we think that the effect of gene blocking with siRNA might be limiting in the evaluation of functional effects. Further study of STAT3 expression regulation mechanism and its impact on the expression of stem cell marker CD133 are needed.

V. CONCLUSION

In conclusion, we suggested that IL-6/STAT3/CD133/survivin signaling pathway played an important role in transformation and progression of colon cancer. IL-6/p-STAT3 involved in the progression of colon cancer through up-regulation of CD133 that in turn induced survivin expression. CD133 could regulate tumor progression and chemoresistane by survivin expression.

To the best of our knowledge, the present study is the first demonstration that p-STAT3 expression causes cancer progression in preinvasive tumors by inducing a cancer stem cell marker, CD133, and apoptosis. We demonstrated p-STAT3 expression involved in the progression of early colon cancer. In contrast, CD133 and survivin expressions were associated with advanced stage of colon cancer. The level of nuclear survivin expression was significantly correlated with CD133 expression, although it was not significantly related to pathological parameters or patient prognosis. Apoptosis rate was higher in $CD133^{+}$ tumors than negative one.

Therefore, STAT3 can be an effective target for early stages colon cancer by suppressing cancer stem cell marker expression. And the small molecule survivin inhibitor YM155 may be an effective treatment modality for advanced colon cancer through increasing apoptosis.

VI. REFERENCE

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66:7-30.
2. Tariq K, Ghias K. Colorectal cancer carcinogenesis: a review of mechanisms. Cancer Biol Med 2016;13:120-35.
3. Janakiram NB, Rao CV. The role of inflammation in colon cancer. Adv Exp Med Biol 2014;816:25-52.
4. Huipeng W, Lifeng G, Chuang G, Jiaying Z, Yuankun C. The differences in colonic mucosal microbiota between normal individual and colon cancer patients by polymerase chain reaction-denaturing gradient gel electrophoresis. J Clin Gastroenterol 2014;48:138-44.
5. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883-99.
6. Zackular JP, Baxter NT, Iverson KD, Sadler WD, Petrosino JF, Chen GY, et al. The gut microbiome modulates colon tumorigenesis. MBio 2013;4:e00692-13.
7. Li Y, de Haar C, Chen M, Deuring J, Gerrits MM, Smits R, et al. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. Gut 2010;59:227-35.
8. Atreya R, Neurath MF. Involvement of IL-6 in the pathogenesis of inflammatory bowel disease and colon cancer. Clin Rev Allergy Immunol 2005;28:187-96.
9. Ahmad R, Kumar B, Chen Z, Chen X, Muller D, Lele SM, et al. Loss of claudin-3 expression induces IL6/gp130/Stat3 signaling to promote colon cancer malignancy by hyperactivating Wnt/beta-catenin signaling. Oncogene 2017;36:6592-604.
10. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. Cell Res 2011;21:159-68.
11. Banerjee K, Resat H. Constitutive activation of STAT3 in breast cancer cells: A review. Int J Cancer 2016;138:2570-8.
12. Mali SB. Review of STAT3 (Signal Transducers and Activators of Transcription) in head and neck cancer. Oral Oncol 2015;51:565-9.

13. Pencik J, Schlederer M, Gruber W, Unger C, Walker SM, Chalaris A, et al. STAT3 regulated ARF expression suppresses prostate cancer metastasis. Nat Commun 2015;6:7736.
14. Nguyen PM, Putoczki TL, Ernst M. STAT3-Activating Cytokines: A Therapeutic Opportunity for Inflammatory Bowel Disease? J Interferon Cytokine Res 2015;35:340-50.
15. Ma XT, Wang S, Ye YJ, Du RY, Cui ZR, Somsouk M. Constitutive activation of Stat3 signaling pathway in human colorectal carcinoma. World J Gastroenterol 2004;10:1569-73.
16. Ji K, Zhang M, Chu Q, Gan Y, Ren H, Zhang L, et al. The Role of p-STAT3 as a Prognostic and Clinicopathological Marker in Colorectal Cancer: A Systematic Review and Meta-Analysis. PLoS One 2016;11:e0160125.
17. Kusaba T, Nakayama T, Yamazumi K, Yakata Y, Yoshizaki A, Nagayasu T, et al. Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma; correlation with clinicopathological factors. J Clin Pathol 2005;58:833-8.
18. Kusaba T, Nakayama T, Yamazumi K, Yakata Y, Yoshizaki A, Inoue K, et al. Activation of STAT3 is a marker of poor prognosis in human colorectal cancer. Oncol Rep 2006;15:1445-51.
19. Morikawa T, Baba Y, Yamauchi M, Kuchiba A, Nosho K, Shima K, et al. STAT3 expression, molecular features, inflammation patterns, and prognosis in a database of 724 colorectal cancers. Clin Cancer Res 2011;17:1452-62.
20. Monnien F, Zaki H, Borg C, Mougin C, Bosset JF, Mercier M, et al. Prognostic value of phosphorylated STAT3 in advanced rectal cancer: a study from 104 French patients included in the EORTC 22921 trial. J Clin Pathol 2010;63:873-8.
21. Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activators of transcription 3 (Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clin Cancer Res 2003;9:594-600.
22. Woo S, Lee BL, Yoon J, Cho SJ, Baik TK, Chang MS, et al. Constitutive activation of signal transducers and activators of transcription 3 correlates with better prognosis, cell

proliferation and hypoxia-inducible factor-1alpha in human gastric cancer. Pathobiology 2011;78:295-301.

23. Zhao C, Wang W, Yu W, Jou D, Wang Y, Ma H, et al. A novel small molecule STAT3 inhibitor, LY5, inhibits cell viability, colony formation, and migration of colon and liver cancer cells. Oncotarget 2016;7:12917-26.
24. Lin L, Liu A, Peng Z, Lin HJ, Li PK, Li C, et al. STAT3 is necessary for proliferation and survival in colon cancer-initiating cells. Cancer Res 2011;71:7226-37.
25. Fan Y, Zhang YL, Wu Y, Zhang W, Wang YH, Cheng ZM, et al. Inhibition of signal transducer and activator of transcription 3 expression by RNA interference suppresses invasion through inducing anoikis in human colon cancer cells. World J Gastroenterol 2008;14:428-34.
26. Kim DH, Park KW, Chae IG, Kundu J, Kim EH, Kundu JK, et al. Carnosic acid inhibits STAT3 signaling and induces apoptosis through generation of ROS in human colon cancer HCT116 cells. Mol Carcinog 2016;55:1096-110.
27. Lee MR, Ji SY, Mia-Jan K, Cho MY. Chemoresistance of CD133(+) colon cancer may be related with increased survivin expression. Biochem Biophys Res Commun 2015;463:229-34.
28. Li W, Lee MR, Choi E, Cho MY. Clinicopathologic Significance of Survivin Expression in Relation to CD133 Expression in Surgically Resected Stage II or III Colorectal Cancer. J Pathol Transl Med 2017;51:17-23.
29. Kozovska Z, Gabrisova V, Kucerova L. Colon cancer: cancer stem cells markers, drug resistance and treatment. Biomed Pharmacother 2014;68:911-6.
30. Lin L, Fuchs J, Li C, Olson V, Bekaii-Saab T, Lin J. STAT3 signaling pathway is necessary for cell survival and tumorsphere forming capacity in ALDH(+)/CD133(+) stem cell-like human colon cancer cells. Biochem Biophys Res Commun 2011;416:246-51.
31. Knutsen A, Adell G, Sun XF. Survivin expression is an independent prognostic factor in rectal cancer patients with and without preoperative radiotherapy. Int J Radiat Oncol Biol Phys 2004;60:149-55.

32. Wheatley SP, McNeish IA. Survivin: a protein with dual roles in mitosis and apoptosis. Int Rev Cytol 2005;247:35-88.
33. Li Y, Ma X, Wu X, Liu X, Liu L. Prognostic significance of survivin in breast cancer: meta-analysis. Breast J 2014;20:514-24.
34. Krieg A, Werner TA, Verde PE, Stoecklein NH, Knoefel WT. Prognostic and clinicopathological significance of survivin in colorectal cancer: a meta-analysis. PLoS One 2013;8:e65338.
35. Shintani M, Sangawa A, Yamao N, Kamoshida S. Immunohistochemical expression of nuclear and cytoplasmic survivin in gastrointestinal carcinoma. Int J Clin Exp Pathol 2013;6:2919-27.
36. Qi G, Tuncel H, Aoki E, Tanaka S, Oka S, Kaneko I, et al. Intracellular localization of survivin determines biological behavior in colorectal cancer. Oncol Rep 2009;22:557-62.
37. Kim ST, Sohn I, Do IG, Jang J, Kim SH, Jung IH, et al. Transcriptome analysis of CD133-positive stem cells and prognostic value of survivin in colorectal cancer. Cancer Genomics Proteomics 2014;11:259-66.
38. Jung W, Hong KD, Jung WY, Lee E, Shin BK, Kim HK, et al. SIRT1 Expression Is Associated with Good Prognosis in Colorectal Cancer. Korean J Pathol 2013;47:332-9.
39. Seok JY, Kim SH, Lee YH, Kwon J, Kim TS. Vimentin and survivin expression rates as prognostic factors in medulloblastoma. Korean Journal of Pathology 2007;41:87-94.
40. Mia-Jan K, Jung SY, Kim IY, Oh SS, Choi E, Chang SJ, et al. CD133 expression is not an independent prognostic factor in stage II and III colorectal cancer but may predict the better outcome in patients with adjuvant therapy. BMC Cancer 2013;13:166.
41. Tomasz M. Mitomycin C: small, fast and deadly (but very selective). Chem Biol 1995;2:575-9.
42. Yakata Y, Nakayama T, Yoshizaki A, Kusaba T, Inoue K, Sekine I. Expression of p-STAT3 in human gastric carcinoma: significant correlation in tumour invasion and prognosis. Int J Oncol 2007;30:437-42.
43. Li WL, Lee MR, Cho MY. The small molecule survivin inhibitor YM155 may be an effective treatment modality for

colon cancer through increasing apoptosis. Biochem Biophys Res Commun 2016;471:309-14.
44. Jenkins BJ. Multifaceted Role of IRAK-M in the Promotion of Colon Carcinogenesis via Barrier Dysfunction and STAT3 Oncoprotein Stabilization in Tumors. Cancer Cell 2016;29:615-7.
45. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell 2005;7:211-7.
46. Terzic J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. Gastroenterology 2010;138:2101-14.e5.
47. Hu B, Elinav E, Huber S, Strowig T, Hao L, Hafemann A, et al. Microbiota-induced activation of epithelial IL-6 signaling links inflammasome-driven inflammation with transmissible cancer. Proc Natl Acad Sci U S A 2013;110:9862-7.
48. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103-13.
49. De Simone V, Franze E, Ronchetti G, Colantoni A, Fantini MC, Di Fusco D, et al. Th17-type cytokines, IL-6 and TNF-alpha synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth. Oncogene 2015;34:3493-503.
50. Rose-John S, Mitsuyama K, Matsumoto S, Thaiss WM, Scheller J. Interleukin-6 trans-signaling and colonic cancer associated with inflammatory bowel disease. Curr Pharm Des 2009;15:2095-103.
51. Atreya R, Neurath MF. Signaling molecules: the pathogenic role of the IL-6/STAT-3 trans signaling pathway in intestinal inflammation and in colonic cancer. Curr Drug Targets 2008;9:369-74.
52. Ren W, Shen S, Sun Z, Shu P, Shen X, Bu C, et al. Jak-STAT3 pathway triggers DICER1 for proteasomal degradation by ubiquitin ligase complex of CUL4A(DCAF1) to promote colon cancer development. Cancer Lett 2016;375:209-20.
53. Kim BR, Oh SC, Lee DH, Kim JL, Lee SY, Kang MH, et al. BMP-2 induces motility and invasiveness by promoting colon

cancer stemness through STAT3 activation. Tumour Biol 2015;36:9475-86.
54. Bak Y, Kwon T, Bak IS, Hong J, Yu DY, Yoon DY. IL-32theta inhibits stemness and epithelial-mesenchymal transition of cancer stem cells via the STAT3 pathway in colon cancer. Oncotarget 2016;7:7307-17.
55. Kryczek I, Lin Y, Nagarsheth N, Peng D, Zhao L, Zhao E, et al. IL-22(+)CD4(+) T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase DOT1L. Immunity 2014;40:772-84.
56. Mobahat M, Narendran A, Riabowol K. Survivin as a preferential target for cancer therapy. Int J Mol Sci 2014;15:2494-516.
57. Xu GC, Zhang P, Leng F, Pan L, Li ZY, Yu DD, et al. Inhibition of lymphatic metastases by a survivin dominant-negative mutant. Oncol Res 2012;20:579-87.
58. Kumar K, Maiti B, Kondaiah P, Bhattacharya S. Efficacious gene silencing in serum and significant apoptotic activity induction by survivin downregulation mediated by new cationic gemini tocopheryl lipids. Mol Pharm 2015;12:351-61.
59. Sa LT, Simmons S, Missailidis S, da Silva MI, Santos-Oliveira R. Aptamer-based nanoparticles for cancer targeting. J Drug Target 2013;21:427-34.
60. Nakahara T, Kita A, Yamanaka K, Mori M, Amino N, Takeuchi M, et al. Broad spectrum and potent antitumor activities of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models. Cancer Sci 2011;102:614-21.
61. Iwai M, Minematsu T, Li Q, Iwatsubo T, Usui T. Utility of P-glycoprotein and organic cation transporter 1 double-transfected LLC-PK1 cells for studying the interaction of YM155 monobromide, novel small-molecule survivin suppressant, with P-glycoprotein. Drug Metab Dispos 2011;39:2314-20.
62. Altznauer F, Martinelli S, Yousefi S, Thurig C, Schmid I, Conway EM, et al. Inflammation-associated cell cycle-independent block of apoptosis by survivin in terminally differentiated neutrophils. J Exp Med 2004;199:1343-54.

63. Mesri M, Morales-Ruiz M, Ackermann EJ, Bennett CF, Pober JS, Sessa WC, et al. Suppression of vascular endothelial growth factor-mediated endothelial cell protection by survivin targeting. Am J Pathol 2001;158:1757-65.
64. Kim SJ, Hong M, Do IG, Lee SH, Ryu KJ, Yoo HY, et al. Serum survivin and vascular endothelial growth factor in extranodal NK/T-cell lymphoma, nasal type: implications for a potential new prognostic indicator. Haematologica 2015;100:e106-9.
65. Fukuda S, Mantel CR, Pelus LM. Survivin regulates hematopoietic progenitor cell proliferation through p21WAF1/Cip1-dependent and -independent pathways. Blood 2004;103:120-7.
66. Leung CG, Xu Y, Mularski B, Liu H, Gurbuxani S, Crispino JD. Requirements for survivin in terminal differentiation of erythroid cells and maintenance of hematopoietic stem and progenitor cells. J Exp Med 2007;204:1603-11.
67. Hernandez JM, Farma JM, Coppola D, Hakam A, Fulp WJ, Chen DT, et al. Expression of the antiapoptotic protein survivin in colon cancer. Clin Colorectal Cancer 2011;10:188-93.
68. Wagner V, Hose D, Seckinger A, Weiz L, Meissner T, Reme T, et al. Preclinical efficacy of sepantronium bromide (YM155) in multiple myeloma is conferred by down regulation of Mcl-1. Oncotarget 2014;5:10237-50.
69. Na YS, Yang SJ, Kim SM, Jung KA, Moon JH, Shin JS, et al. YM155 induces EGFR suppression in pancreatic cancer cells. PLoS One 2012;7:e38625.
70. Wang X, Qiu W, Zhang G, Xu S, Gao Q, Yang Z. MicroRNA-204 targets JAK2 in breast cancer and induces cell apoptosis through the STAT3/BCl-2/survivin pathway. Int J Clin Exp Pathol 2015;8:5017-25.
71. Kim JS, Kim HA, Seong MK, Seol H, Oh JS, Kim EK, et al. STAT3-survivin signaling mediates a poor response to radiotherapy in HER2-positive breast cancers. Oncotarget 2016;7:7055-65.
72. Hu W, Jin P, Liu W. Periostin Contributes to Cisplatin Resistance in Human Non-Small Cell Lung Cancer A549 Cells

via Activation of Stat3 and Akt and Upregulation of Survivin. Cell Physiol Biochem 2016;38:1199-208.

73. Huang K, Li LA, Meng YG, You YQ, Fu XY, Song L. Arctigenin promotes apoptosis in ovarian cancer cells via the iNOS/NO/STAT3/survivin signalling. Basic Clin Pharmacol Toxicol 2014;115:507-11.
74. Wen K, Fu Z, Wu X, Feng J, Chen W, Qian J. Oct-4 is required for an antiapoptotic behavior of chemoresistant colorectal cancer cells enriched for cancer stem cells: effects associated with STAT3/Survivin. Cancer Lett 2013;333:56-65.
75. Miura K, Fujibuchi W, Ishida K, Naitoh T, Ogawa H, Ando T, et al. Inhibitor of apoptosis protein family as diagnostic markers and therapeutic targets of colorectal cancer. Surg Today 2011;41:175-82.
76. De Oliveira Lima F, De Oliveira Costa H, Barrezueta LF, Fujiyama Oshima CT, Silva JA, Jr., Gomes TS, et al. Immunoexpression of inhibitors of apoptosis proteins and their antagonist SMAC/DIABLO in colorectal carcinoma: correlation with apoptotic index, cellular proliferation and prognosis. Oncol Rep 2009;22:295-303.
77. Alcaide J, Funez R, Rueda A, Perez-Ruiz E, Pereda T, Rodrigo I, et al. The role and prognostic value of apoptosis in colorectal carcinoma. BMC Clin Pathol 2013;13:24.
78. Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, et al. Inhibition of apoptosis during development of colorectal cancer. Cancer Res 1995;55:1811-6.
79. Zhao X, Ogunwobi OO, Liu C. Survivin inhibition is critical for Bcl-2 inhibitor-induced apoptosis in hepatocellular carcinoma cells. PLoS One 2011;6:e21980.
80. Kemper K, Rodermond H, Colak S, Grandela C, Medema JP. Targeting colorectal cancer stem cells with inducible caspase-9. Apoptosis 2012;17:528-37.
81. Sam S, Sam MR, Esmaeillou M, Safaralizadeh R. Effective Targeting Survivin, Caspase-3 and MicroRNA-16-1 Expression by Methyl-3-pentyl-6-methoxyprodigiosene Triggers Apoptosis in Colorectal Cancer Stem-Like Cells. Pathol Oncol Res 2016.

VII. ABSTRACT IN KOREAN

대장암에서 STAT3/CD133/survivin signaling pathway 차단을 통한 암줄기세포 표적치료의 항암효과

이완루 (Wanlu Li)

연세대학교 대학원 의학과

<지도교수: 조미연>

연구 배경 : 대장암은 전 세계적으로 세 번째로 많이 발생하는 암이며 발암기전으로 염색체 불안정성 (CIN), CpG 섬 메틸화 및 미소위성체 불안정성을 포함하는 메커니즘이 잘 알려져 있다. 최근 연구에서 interleukin (IL)-6 와 같은 전 염증성 인자가 Signal

Transductor and Activator of Transcription (STAT3)의 활성화를 유도하는 것이 대장암 발생에 관여 한다고 보고되었다. 또한 암의 시작과 발달 유도한다고 생각하는 암 줄기 세포 (CSCs) 개념이 도입된 이후 CD133 은 대장 암에서 암 줄기 세포의 마커로써 잘 알려져 있다. 최근 한 연구에서 암줄기세포 표지자를 발현하는 암에서 세포의 증식에 STAT3 신호전달이 중요한 역할을 한다고 보고되었으나 대장암의 진행에서 STAT3/CD133 의 임상적 의의는 아직 충분히 설명되지 못하였다.

또한 이전 연구에서, $CD133^{+}$ 대장암 세포가 $CD133^{-}$ 세포보다 높은 항암제 저항성을 보이는데 이는 survivin 발현의 변화와 연관이 있다고 보고하였다.

그러므로 본 연구에서는 대장암 진행에 STAT3/CD133/survivin

signaling pathway 관여한다는 가설을 세우고 이를 검증하기위하여 아래와 같은 연구 목적을 설정하였다. **연구 목적 :** 1) STAT3 활성의 유도 및 억제가 CD133 와 survivin 의 발현 및 암세포의 생물학적 변화를 초래하는지 2) CD133 억제가 survivin 발현 및 생물학적 변화를 초래하는지 3) 환자에서 절제된 샘종과 대장암의 진행단계에서 p-STAT3, CD133, survivin 의 발현양상에 차이가 있는지를 비교 분석하였다.

연구 방법 : 원발 (Caco-2) 및 전이 (Lovo and SNU407) 대장 암 세포주를 이용하여 STAT3-siRNA 와 STAT3 억제제 (stattic)로 STAT3 를 억제하거나 IL-6 처리로 STAT3 활성화를 유도한 후 CD133 및 survivin 발현과 생물학적 특성의 변화를 비교 하였다. 또한 STAT3 나 CD133 을 억제한 후 암세포의 콜로니 형성, 이동 능력 및 생존력, 세포사멸율분석을 통해 암 세포의 생물학적 특징

변화를 확인했다. 또한 CD133RNA 를 이용하여 CD133 발현을 억제한 후 survivin 발현의 변화와 $CD133^+$ 세포 및 siRNA-induced $CD133^-$ 세포에서 survivin 억제제 (YM155) 의 항암 효과를 조사하였다. 397 명의 대장 선종 및 선암 환자의 조직에 p-STAT3, CD133 및 survivin 에 대한 면역 조직 화학 염색 을 시행하여, p-STAT3, CD133 및 survivin 발현의 분포와 환자들의 임상 병리학적 변수와의 연관성을 분석 하였다. 또한 terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay 를 이용하여 survivin 과 CD133 발현에 따른 세포 자멸빈도를 비교 분석 하였다.

연구 결과 : STAT3 의 억제는 원발암 (Caco-2) 및 전이성 대장암 세포 (LoVo 및 SNU407) 모두에서 CD133 및 survivin 의 발현을 대조군에 비해 유의하게 감소시켰으며 대장암 세포의 콜로니

형성, 이동 능력 및 생존력을 감소시켰다. 세포 자멸 분석에서 원발대장암 (Caco-2) 세포는 stattic 처리 후 전이암 (LoVo 및 SNU407) 세포보다 더 높은 세포 사멸율을 보였다 (각각 68.3, 22.7 및 27.9 %). 반면에 IL-6 처리는 p-STAT3, CD133 및 survivin 단백질 발현을 증가시켰다. CD133-siRNA transfection 에 의한 CD133 의 억제는 대장암 세포에서 survivin 의 발현을 감소시켰다. survivin 억제제인 YM155 는 용량이 증가함에 따라 세포 사멸 수준을 유의하게 증가 시켰다. 그러나 CD133 발현의 변화는 초래하지 않았다. 세포 증식 분석에서 $CD133^+$ 암세포 (mock 및 ctrl-siRNA 그룹)는 siRNA 에 의해 유도된 $CD133^-$ 세포와 비교하였을때 5-FU (5-FU)에 대해 항암제 저항성이 약간 증가되었으며 YM155 치료가 더 효과적임을 확인하였다. 환자 검체를 이용한 면역조직화학염색에서 p-STAT3 발현은

재자리암(pTis)과 조기 대장암 (pT1 & T2)에서 더 흔하게 나타난 반면 (p = 0.001, HR = 0.328, 95 % CI : 0.170-0.632), CD133 과 survivin 의 발현은 진행대장암 (T3& T4)에서 더 흔하였다 (p = 0.000, HR = 4.971, 95 % CI : 0.551-9.687, p = 0.000, HR = 3.617, 95 % CI : 1.913-6.835). 침윤이 없는 종양 (샘종과 재자리암종)과 침윤이 있는 종양 (T1-4)으로 구분하여 비교하였을 때 p-STAT3^{+}/CD133^{-}/survivin^{-}의 immunoprofile 을 가진 종양은 침윤 위험이 유의하게 낮았으나 (p = 0.003, HR = 0.042, 95%CI: 0.005–0.348), p-STAT3^{-}/CD133^{+}/survivin^{+} immunoprofile 을 가진 종양은 침윤 위험이 유의 하게 높았다 (p = 0.002, HR = 6.480, 95 % CI : 1.957-21.462).

결론 : IL-6 / STAT3 / CD133 / survivin 신호 전달 경로에서 pSTAT3 는 전암 또는 조기 대장암에서 암줄기세포 표지자 CD133

발현을 유도하여 다장암의 진행에 관여하여 CD133 은 주로 진행암에서 survivin 발현을 증가하여 세포사멸을 억제함으로써 대장암의 진행에 관여하는 것으로 생각된다. 따라서 STAT3 는 대장 암의 초기 단계에서 암 줄기 세포 마커 발현을 억제함으로써 진행을 억제하는데 유용한 표적치료 대상이며, survivin 억제제인 YM155 는 진행성 대장암의 유용한 표적치료제로 생각된다.

VIII. PUBLICATION LIST

1. **Li W**, Lee MR, Cho MY. The small molecule survivin inhibitor YM155 may be an effective treatment modality for colon cancer through increasing apoptosis. Biochem Biophys Res Commun 2016;471:309-14.

2. **Li W**, Lee MR, Choi E, Cho MY. Clinicopathologic Significance of Survivin Expression in Relation to CD133 Expression in Surgically Resected Stage II or III Colorectal Cancer. J Pathol Transl Med 2017;51:17-23.