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이학석사 학위논문

S100A14 regulates stemness in chemoresistant colon cancer cells by regulation of STAT3 stability

항암제에 내성을 보이는 대장암 세포에서 S100A14의 기능에 대한 연구

2021년 2월

서울대학교 대학원

분자의학 및 바이오제약학과 분자의학 및 바이오제약전공

심 정 연

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2021년 1월

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분자의학 및 바이오제약학과 분자의학 및 바이오제약 전공

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2021년 1월

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ABSTRACT

S100A14 regulates stemness in chemoresistant colon cancer cells by regulation of STAT3 stability

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Colon cancer is the third most common cancer. Of the several therapies for colon cancer, chemotherapy has been used most frequently. 5-Flourouracil (5-Fu) and oxaliplatin are first-line chemotherapeutic agents used for treatment of colon cancer. Although these drugs are commonly used for therapy, several evidences have indicated that anti-cancer drugs induce chemoresistance, therefore reducing the effectiveness of treatment. Because of heterogeneity, cancer cells respond to treatments differently. Remarkably, the stem cell property of cancer cells has been reported to be highly correlated with chemoresistance. In this paper, when cancer cells become resistant to anticancer drugs, we

investigate the changes in cell properties and internal cell signaling pathways

Here, we generated drug resistant colon cancer cells after chronic exposure to 5-Fu and oxaliplatin. We found that resistant cells have highly upregulated stemness property. Through microarray and big data analysis, we found that S100A14 level was most decreased in drug resistant cells. Increased stemness property in resistant cells was reduced after S100A14 transfection. We also found that S100A14 level was decreased in colon cancer with high stem cell population. To investigate how S100A14 regulates stemness, we analyzed resistant cells using luciferase assay to find increased signaling pathway. STAT3 pathway was most upregulated in both cells.

HCT116 resistant cells have reduced S100A14 expression and increased STAT3 expression. To determine the relation between S100A14 and STAT3, we analyzed STAT3 level in S100A14 modulated cells. STAT3 protein level was downregulated in S100A14 overexpression cells, while it was increased in S100A14 knockdown cells. Next, we investigated that S100A14 regulated STAT3 at a mRNA level or protein level. There are no significant changes in mRNA levels between parental cell and resistant cells. On the other hand, STAT3 proteins were rapidly degraded when S100A14 level was high; therefore, we have demonstrated S100A14 regulates STAT3 stability. Finally, we found that STAT3 stability was regulated by interaction with S100A14. Through immunoprecipitation, we confirmed that STAT3 degradation was increased because S100A14 facilitated binding of more ubiquitin to STAT3.

These findings suggest novel function of S100A14 in regulation of stemness property in chemoresistant colon cancer. Moreover, S100A14 level can be used to estimate stem cell population in colon

cancer. Thus, S100A14 can be a potential biomarker for chemotherapy of colon cancer

Key words: Colon cancer, chemoresistance, Cancer stem cell, stemness, S100A14, STAT3

Student number: 2019-22493

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INTRODUCTION

Colon cancer is the third most common cancer and one of the major leading causes of death worldwide. During colon cancer development, occurrence of genetic mutation in epithelial cells of the gastrointestinal tract results in increased proliferation and self-renewal. The epithelial cells form polyps, which may contribute to tumor progression. It is well known that colon cancer can metastasize easily. Therapies for colon cancer include surgery, radiation therapy, and chemotherapy. Surgery is the first choice of treatment when colon cancer is in early stage. Depending on colon cancer progression, chemotherapy can be used with surgery as adjuvant therapy [1]. 5-Fluorouracil (5-FU) and oxaliplatin are the main drugs used for colon cancer treatment. 5-Fu is an uracil analog; it blocks the cell growth through thymidylate synthase inhibition [2]. Oxaliplatin can bind DNA, resulting in formation of platinum-DNA adducts that block DNA replication and induce apoptosis [3]. While these drugs are usually used for resolving colon cancer, response rates towards these drugs are low and they are associated with problems such as chemoresistance. Chemoresistance reduces the efficiency of drugs and results in bad prognosis [4, 5]. Therefore, there are many studies on overcoming drug resistance.

Cancer heterogeneity is the major reason of chemotherapy failure. Because of heterogeneity, cancer cells respond differently to drugs. Cancers consist of a subgroup of cells that is resistant to chemotherapy called cancer stem cells (CSCs). CSCs have self-renewal and differentiation properties. Chemotherapy can remove proliferating cells, but CSCs can avoid drugs and promote tumor relapse, resulting in bad prognosis [6]. To overcome chemoresistance and increase drug efficiency in cancer therapy, there are many studies on mechanism of CSCs. In metastasis patients,

there is a correlation between epithelial mesenchymal transition (EMT) markers and stem cell markers. It has been reported that activated Notch-1 upregulates EMT in gefitinib resistant lung cancer cell [7]. STAT3, upregulated in various types of cancer, also induces CSC properties. Activated JAK/STAT3 signaling increases tumorigenic characteristics, metastatic ability, transition to CSCs, and chemoresistance in cancers through enhancing EMT [8]. Therefore, CSCs should be investigated in detail for cancer therapy efficacy.

S100A14 is the one of the S100 family proteins. S100 family proteins consist of a calcium-binding region and they can regulate cellular response. The expression level of S100 proteins varies in different human cancers. For example, S100A11 is overexpressed in Non-small cell lung cancer, but its expression is decreased in small-cell lung cancer. S100A7 is expressed in early-stage oral squamous cell carcinomas. S100A1, S100A4, S100A6, S100A7, S100A8, S100A9, S100A11, and S100A14 have been reported to be overexpressed in breast cancer [9]. S100A14 has been discovered recently and has different expression patterns in many cancers. S100A14 is upregulated in lung and breast cancers, but is downregulated in colorectal cancer and oral squamous cell carcinoma [10]. Several studies have showed that increased S100A14 regulates the expression of metalloproteinases (MMPs) in gastrointestinal cancer, resulting in inhibition of cell metastasis [11]. However, no relation has been discovered between S100A14 and chemoresistance.

In this study, we investigated the alterations in tumor when it became resistant to anticancer drugs. We showed that CSC phenotype increased after chronic exposure to 5-Fu/oxaliplatin. We conducted microarray analysis to investigate the change in gene expression in resistant cells and found that S100A14 expression was decreased. Moreover, we showed that S100A14 regulated CSC characteristics in colon cancer cell. In addition, we found that STAT3

was commonly upregulated in resistant cells and its level was controlled by S100A14. Our data reveal the mechanism of increase in stemness properties in colon cancer cells and indicate that S100A14 expression is highly related to stemness. Based on this, we suggest that S100A14 can be a potential biomarker for prediction of response to chemotherapy.

METARIALS AND METHODS

Cell lines, culture conditions

Human colon cancer cell lines, HCT116, HT29 were provided by Prof. Sang kook Lee (Seoul national university). Resistant cell lines were generated by increasing dose of 5-Fluorouracil (Sigma Aldrich, F6627), and Oxaliplatin (Sigma Aldrich, O9512). All cell lines were cultured in RPMI1640 media (Welgene biopharmaceuticals) with 10% fetal bovine serum (FBS) and 1% antibiotics. All cells were incubated at 37℃ and 5% CO_2

MTT assay

1 x 10^3 cells of HCT116, HCT116/ R were seeded into 96 well plate. After 24 hours, fresh media with different concentration of drugs was added. After 2 days, media (plus drugs) were changed one more. One day later, 0,5mg/ml of MTT solution was added to each well and incubated at 37℃ for 4 hours. Each MTT solution was removed and DMSO was added to each well for dissolving the crystals and then plates were read at 570nm.

Anchorage independent and dependent assay

To do soft agar assay, 4% agar was diluted in RPMI solution to make 1% concentration. 1% agar was loaded in 24 well plates for bottom. After solidification, 1% agar was diluted with RPMI to make 0.4% concentration and 200 cells was added in this solution. These cell solutions were loaded on the bottom agar. Every 3-4 days, 0.5ml of media with drugs was changed into each soft agar plates. After 12-14 days, the cells were stained by 1% MTT solution in 4 hours

and then counted. In anchorage dependent assay, 200 cells were seeded in 12 well plates and grown for 10-12 days while changing the media. The colonies were fixed in methanol, stained with hematoxilin and counted.

Western blot and immunoprecipitation assay

Western blot was performed using protein lysates of colon cancer cells. RIPA buffer with protease inhibitor was used to prepare the lysates. BCA protein assay kit was used for measuring protein concentration. Each sample was loaded to separate proteins using 8-14% SDS-PAGE. After running gel, protein on the gels transferred to PVDF (ATTO). The primary antibodies were used to detect S100A14 (1:1000, Proteintech 10489-1-AP), STAT3 (1:1000, Santa Cruz 482), pSTAT3 (1:1000, Cell signaling Technology 9145), Acetyl STAT3 (1:1000, Cell signaling Technology 2523), Oct4 (1:1000, Abcam 19857), Nanog (1:1000, Cell signaling Technology 4903), Sox2 (1:1000, Abcam 97959), cMyc (1:1000, Santa Cruz 40), CD44 (1:1000, R&D BBA10), cleaved caspase 3(1:1000, Cell signaling Technology 9661), cleaved PARP (1:1000, Cell signaling Technology 9544) and β-actin(1:1000, Santa Cruz 47778). The horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-rabbit/mouse IgG (GeneTex 213110/ 13111) were used.

Immunoprecipitation analysis was performed using protein lysates of colon cancer cells. Before protein extraction, MG132 10uM was added in cultured cells. After 4 hours incubation, cells were lysed with IP buffer. 1mg of protein lysates were incubated with STAT3 (1:200, Santa Cruz 482) at 4℃ overnight. Agarose A beads were added to down STAT3 attached proteins. Equal amount of protein was loaded gel and transferred. The primary antibodies were used to detect Ubiquitin (1:1000, Santa Cruz 8017), STAT3, S100A14.

Real-time PCR

Total RNA isolation was synthesized to cDNA and used for the quantification of S100A14, Oct4, Nanog, Sox2, cMyc level in cells. SYBR green with high ROX qPCR premix (Enzynomics) was used. And all DNA mount was calculated by using Real-time PCR system (Applied Biosystem, Prism 7300).

Sphere formation assay

Serum-free medium for sphere culture was composed of DMEM/F-12 media supplemented with 1% B27 supplements (Gibco), 20ng/ml human FGF, 20ng/ml human EGF, 1% antibiotics. 1000-2000 cells of HCT116, HT29 were cultured at low attachment 96 well plate. (Corning). The spheres were counted to confirm their sphere forming ability.

In vivo serial dilution

Small amounts of cell need to be prepared. 10^2, 10^3, 10^4 of HCT116/ HT29 cells in PBS were mixed with 25% Matrigel (BD 354234). 5 weeks FvB mice were injected these cells in subcutaneous. After about 2 month later, tumor incidence was checked.

Flow cytometric cell Sorting

All sorting was performed on BD FACs Aria Ⅱ. For the staining of stemness population, cells and PDXs were stained with FITC-conjugated anti human CD44 (BD Pharmingen) and PE-conjugated anti human CD133 (Miltenyl Biotec). Double positive (about 10% high populations) and double negative (about 10% low populations) cells

were collected.

Immunofluorescence

Sorted cells were attached to saline coated slide glass by cytocentrifuge (cytopro 7620) and then fixed in 4% PFA for 5 minutes in room temperature (RT). Fixed cells were permeabilized in 0.1% Triton X-100. After 1 hour blocking, S100A14 (1:200, Proteintech 10489-1-AP) was stained overnight. Secondary staining was performed at RT for 2 hours with Donkey anti-rabbit Alexa 594 (1:1000, Invitrogen A21207) and the coverslips were mounted with a mounting solution (Dako, Agilent, Santa Clara, CA, USA) containing DAPI. Fluorescence was observed under a fluorescence microscope.

Luciferase reporter assay

To find upregulated pathway in resistant cells, Cignal 45-Pathway Reporter Array (QIAGEN, CCA-901L) was used. The Cignal 45-Pathway Reporter Array can measured the activity of 45 signaling pathways. HCT116 parental cell and resistant cells were seeded into plate using JetPRIME transfection reagent (Polyplus-Transfection SA). After 48 hours incubation, cells were harvested with lysis-Juice (PJK GmbH, Kleinblittersdorf, Germany). Luciferase activity was monitored with transcription factor responsive firefly luciferase construct (for signal activity) and constitutively expression renilla luciferase construct (for control) using a microplate luminometer (Berthold Technologies GmbH & Co. KG, Germany).

RESULTS

Generation of 5-Flourouracil/ Oxaliplatin resistant cells

To investigate the cause of chemoresistance in colon cancer therapy, we used HCT116 and HT29 human colon cancer cell lines, and generated resistant cells. We used 5-Fu and oxaliplatin to induce drug resistance, which was acquired after 6 months of chronic treatment (Figure 1A). The resistance of cells was confirmed via MTT assay, anchorage dependent assay, anchorage independent assay, and determined PARP caspase cleavage through western blotting. After 5-Fu treatment, about 50% parental HCT116/ HT29 cells survived in 10 ㎛M 5-Fu whereas HCT116 5-FuR/HT29 5-FuR cells survived in more than 50 ㎛M 5-Fu. After oxaliplatin treatment, approximately 50% parental HCT116/HT29 cells survived in 5 ㎛M, whereas HCT116 OxaR/HT29 OxaR cells survived in more than 10 ㎛M oxaliplatin (Figure 1B). We also found that HCT116/HT29 resistant cells developed more colonies compared to their parental cells when treated with drugs (Figure 1B and C). Finally, we confirmed each cell's apoptotic response after exposure to the drugs. Western blot showed that resistant cells were less sensitive to the drugs (Figure 1D). These data indicated that HCT116/HT29 resistant cells have drug resistance characteristics.

Resistant colon cancer cells increase stemness phenotype compared with parental cell

Chemoresistant cancer cells have stemness property [6]. We confirmed the stemness phenotype of the generated resistant cells. First, we found the stemness marker well known in colon cancer and determined its expression level. The mRNA expression of Oct4,

Nanog, Sox2, and cMyc, the colon cancer stemness markers, was increased (Figure 2A). In addition, we determined that the protein level was increased in resistant cells and found that the level of the stemness markers was increased in 5-Fu/oxaliplatin resistant cells (Figure 2B). Next, we cultured the cells in serum-free, non-adherent conditions. We observed that our cells developed spheres in those conditions. Drug resistant cells generated more spheres compared to parental HCT116/HT29 cells (Figure 2C). Finally, we subcutaneously injected a small number of cells in mice to monitor tumor incidence. Two months later, we found increased incidence of resistant cells (Figure 2D). These data indicate that 5-Fu/oxaliplatin resistant cells have cancer stem cell properties.

S100A14 expression was lowered after drug treatment in colon cancer cells

In previous study, we found that S100A14 gene has the lowest expression in HCT116 5-FuR cells through microarray [12]. We checked whether S100A14 expression was decreased in our resistant cells. S100A14 mRNA and protein levels were remarkably lowered in resistant cells (Figure 3A and B). As shown in Figure 2, resistant cells comprise stemness and chemoresistance. Colon cancer patients have anticancer drug resistance and show increased stemness properties during cancer progression. We analyzed GSE39582 dataset, which contains data on gene expression of colon cancer patients in different cancer progression stages. We found that stemness genes (cMyc, Oct4) had reverse correlation with S100A14 and as the cancer progresses, their correlation became more negative (Figure 3C). Through these results, we found S100A14 expression is decreased in resistant cells, indicating that S100A14 regulates stemness and tumor progression.

S100A14 level in cells affects stemness properties

To investigate whether S100A14 regulates stemness property in colon cancer cells, we generated S100A14 knockdown parental HCT116 cells and S100A14 overexpressing HCT116 resistant cells. We investigated their colony forming ability using anchorage dependent and independent assays. Colonies were elevated when S100A14 level was low (Figure 4A and B). To determine whether S100A14 induces stemness properties in cells, we analyzed the gene expression level and protein level of stemness markers. Both gene and protein levels were decreased in S100A14 overexpressed cells (Figure 4C and D). Moreover, when S100A14 level was decreased in the cells, their sphere-forming ability was elevated (Figure 4E and F). In addition, tumor incidence was increased when S100A14 was decreased (Figure 4G). These results indicated that S100A14 alleviates stemness characteristics in colon cancer cells.

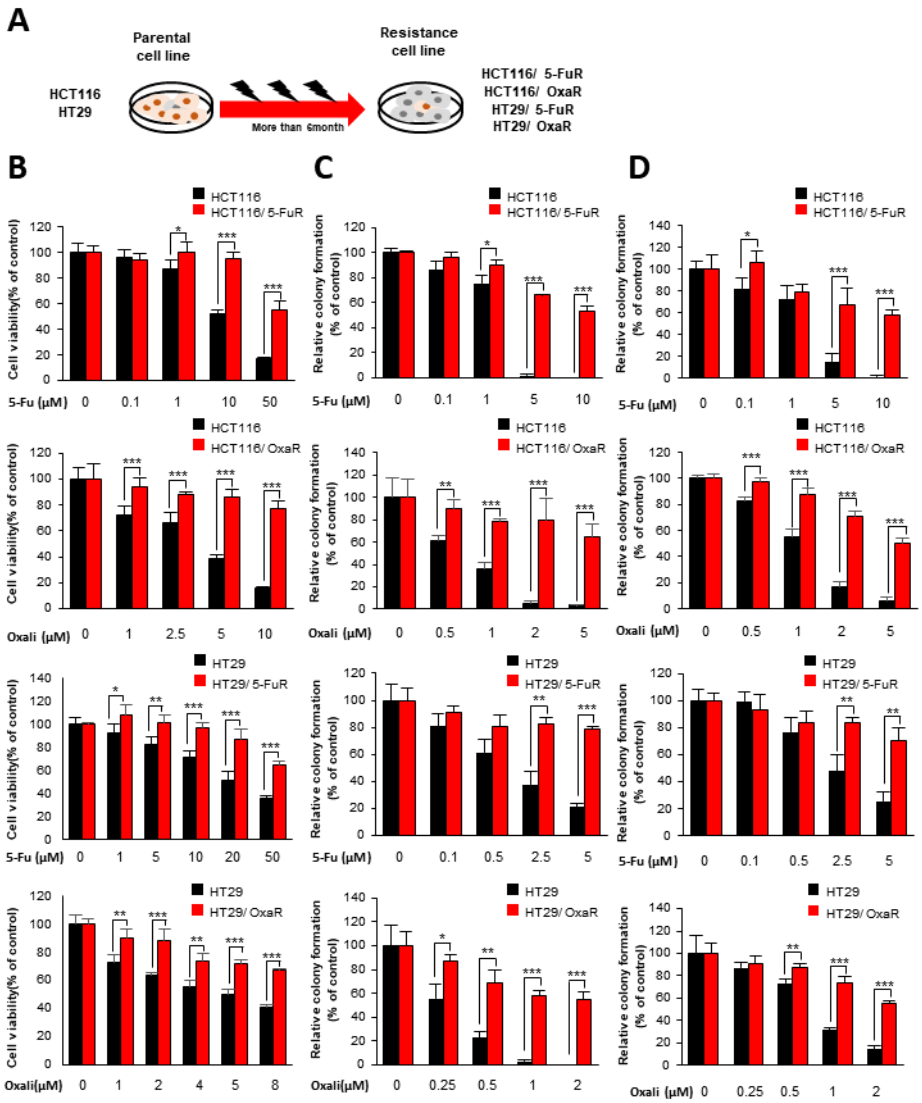
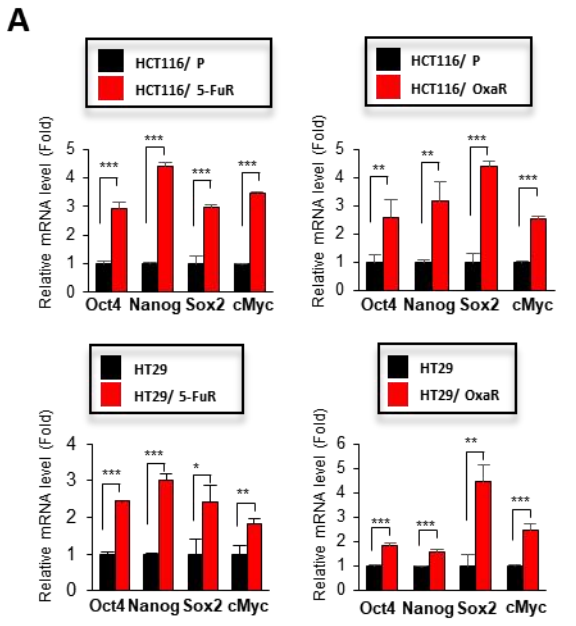
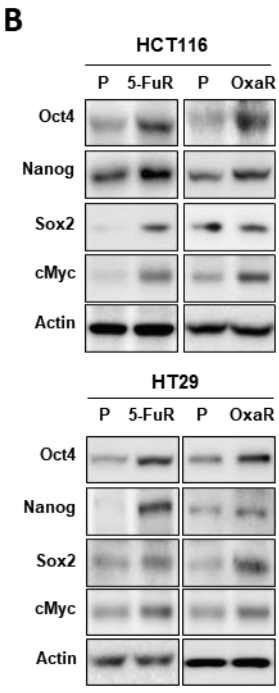
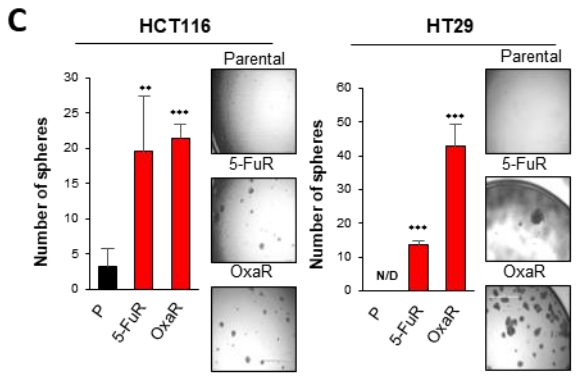

A
Parental cell line
Resistance cell line
HCT116
HT29
More than 6month
HCT116/ 5-FuR
HCT116/ OxaR
HT29/ 5-FuR
HT29/ OxaR
B
C
D
HCT116
HCT116/ 5-FuR
HCT116/ OxaR
HT29
HT29/ 5-FuR
HT29/ OxaR
Cell viability(% of control)
Relative colony formation (% of control)
5-Fu (μM)
Oxali (μM)
E
HCT116 P
HCT116/ 5-FuR
HT29
HT29/ 5-FuR
HCT116/ OxaR
HT29/ OxaR
5-Fu(μM)
Oxa(μM)
cl-Cas3
cl-PARP
Actin

Figure 1. Generation of 5-Flourouracil/ Oxaliplatin resistant cell.

A. Schematic diagram showing the generation of resistant cells. B-E. Acquisition of resistance to the chemotherapy in drug treated cells compared with the corresponding parental cells, as determined by a MTT assay (B) and anchorage-dependent (C) independent assay (D) and PARP/ Caspase3 cleavage (E). *P<0.05, ** P <0.01 and *** P <0.001







D

HCT116

Number of Incidence	Number of cells	P	5-FuR
	10^4	5/5	5/5
	10^3	1/5	5/5
	10^2	0/5	3/5

Number of Incidence	Number of cells	P	OxaR
	10^4	6/6	6/6
	10^3	3/6	6/6
	10^2	0/6	4/6

HT29

Number of Incidence	Number of cells	P	5-FuR
	10^4	4/5	5/5
	10^3	3/5	5/5
	10^2	1/5	4/5

Number of Incidence	Number of cells	P	OxaR
	10^4	4/5	4/5
	10^3	3/5	4/5
	10^2	1/5	3/5

Figure 2. Drug resistant cells show increasing stemness property. A, B. Alteration of stemness markers expression in resistant cells were evaluated in gene expression level by real-time PCR (A), and in protein level by western blot (B). **C.** Increased stemness property was monitored by in vitro sphere formation. **D.** In vivo tumor incidence test was performed to confirm increased stemness in resistant cells compared with parental cells. $*P<0.05$, $** P<0.01$ and $*** P<0.001$

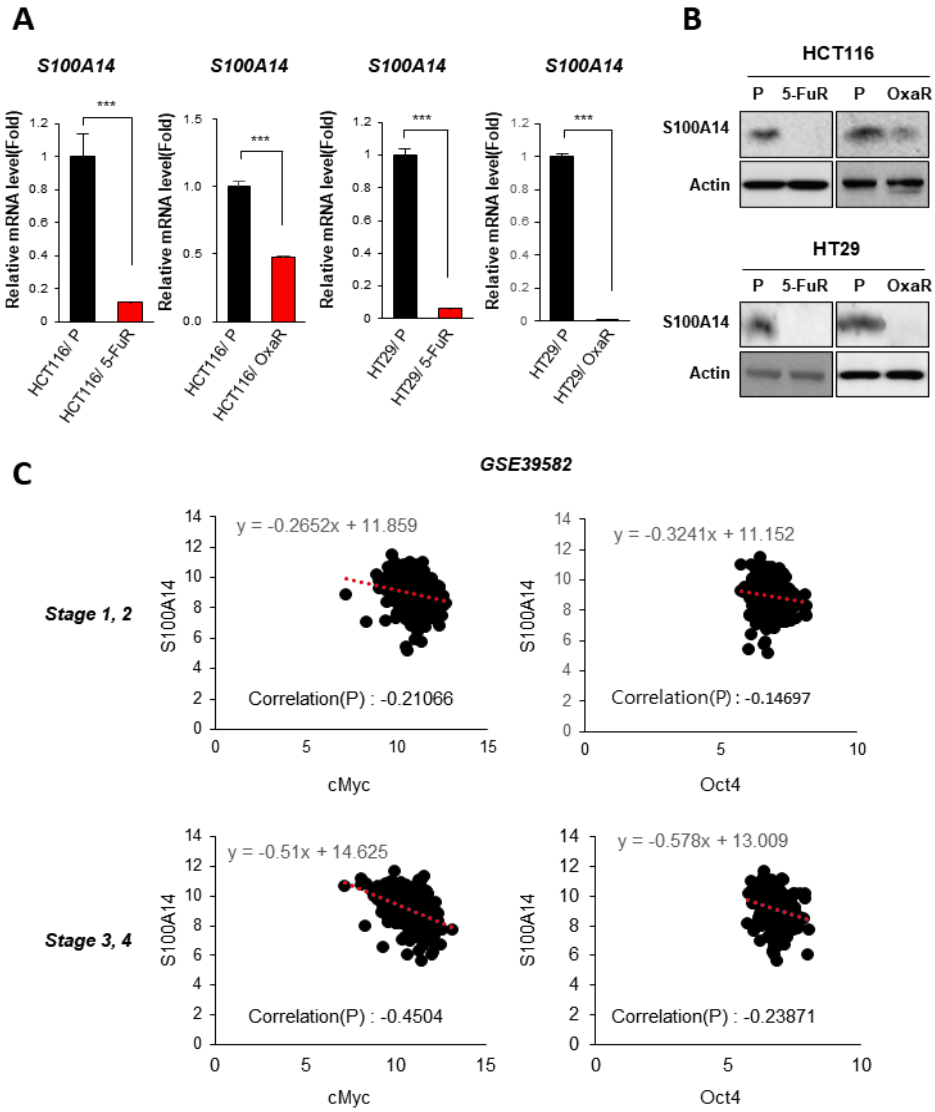


Figure 3. Chemoresistant colon cancer cells have lowered S100A14 expression.

A, B. Expression of S100A14 was evaluated by mRNA level (A) and protein level (B) in resistant cells compared with the corresponding parental cells. C. The correlation between stemness gene expression and S100A14 expression in colon cancer patients was analyzed using publicly available datasets deposited in the Gene Expression Omnibus database (GSE39582). *** P <0.001

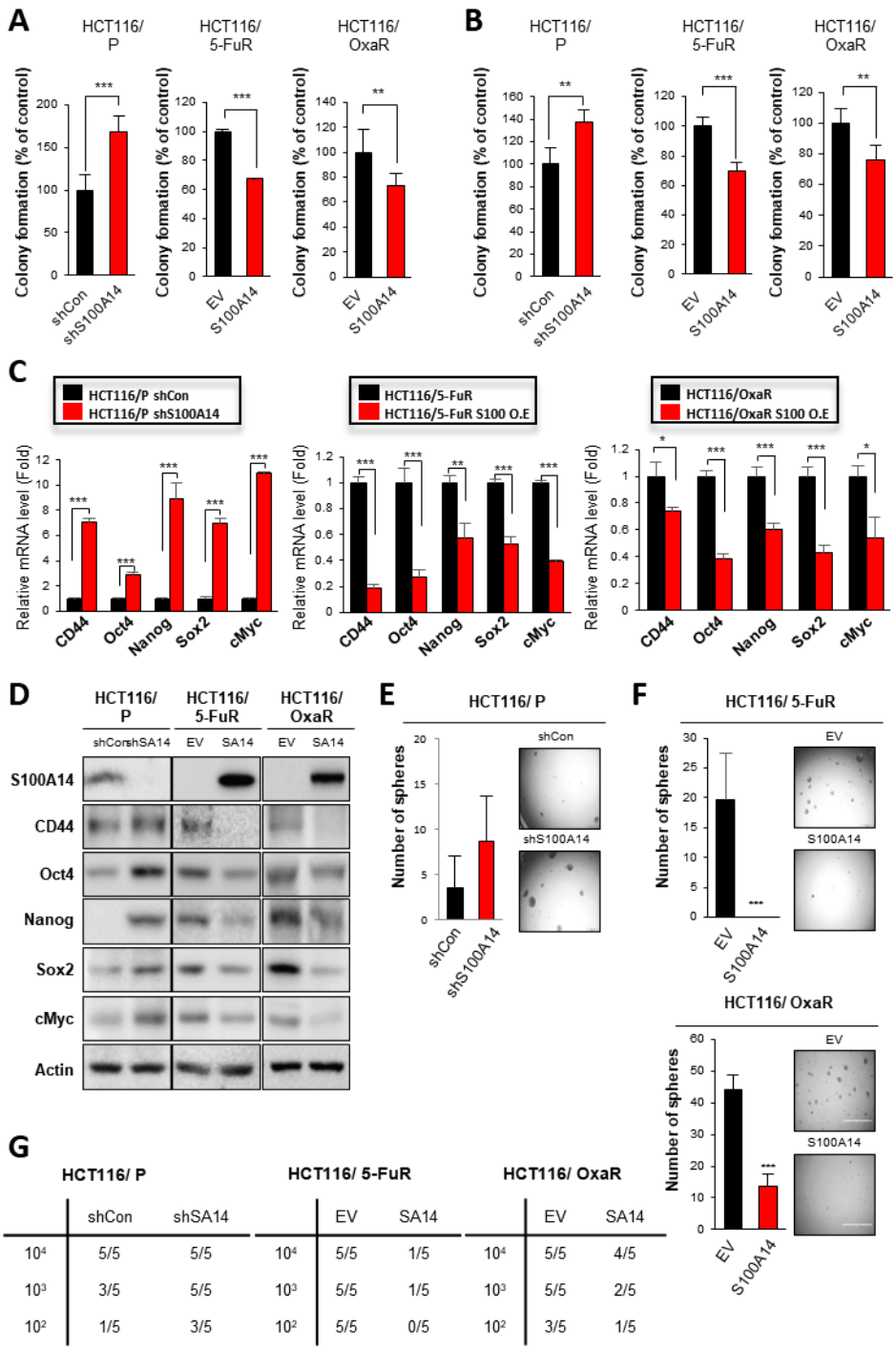


Figure 4. S100A14 is related with stemness property.

A, B. S100A14 effect on colony formation was determined by

anchorage dependent (A), independent assay (B). **C, D.** Representative stemness marker change in colon cancer cells depending on S100A14 was evaluated by real time PCR (C) and western blot (D). **E-G.** Decreased stemness property in S100A14 overexpressed cells was monitored by in vitro sphere formation (E, F) and in vivo tumor incidence (G).

Stemness population in colon cancer expressed S100A14 at decreased levels

Because of cancer heterogeneity, there are different populations in same tumor such as CSC population. CD44 and CD133 are surface proteins that are well known to be highly expressed in colon cancer [13]; therefore, they can be considered stemness markers. HCT116/HT29 cells were stained with CD44 and CD133 for determining stemness population. We sorted the cells into CD44+CD133 high population and low population (each cell gated 10% of positive and negative regions). Later, we monitored the RNA and protein levels of S100A14 in sorted population. Double positive region indicated stemness population among cells. S100A14 expression level was lowered in double positive region. Immunofluorescence revealed that S100A14 protein level was also decreased in stemness high region (Figure 5). We attempted to confirm whether stemness population in human cancer model had also decreased S100A14 level. Two colon cancer patient-derived xenografts (PDX) were sorted in same condition. Double positive cells in colon cancer PDX also had decreased S100A14 level (Figure 6). These findings indicate that colon cancer CSC population has decreased S100A14 expression and they have reverse correlation.

S100A14 downregulates STAT3 and stemness axis

S100A14 has negative correlation with stemness markers and it can regulate stem cell property. Next, we wanted to know how S100A14 regulates stemness. To find which pathway changed in resistant cells, we performed luciferase assay in HCT116 resistant cells. We used cignal 45-pathway (QIAGEN) to measure the activity of signaling pathways. Among all the pathways, STAT3 pathway was most upregulated in both resistant cells (Figure 7A and B). We confirmed that STAT3 expression was indeed increased in resistant

cells. Notably, resistant cells showed upregulation of not only STAT3 active form but also STAT3 total form (Figure 7C). Upregulation of STAT3 has been discovered in various cancer cells and increased STAT3 activation is related to tumor growth, chemoresistance, and stemness property. To investigate whether increased STAT3 expression affected the stemness property of resistant cells, we analyzed the stemness markers after STAT3 inhibition. STAT3 inhibitor stattic reduced STAT3 and stemness markers (Figure 8A). siRNA was used to knockdown STAT3. Inhibition of STAT3 decreased stemness markers (Figure 8B). To confirm whether STAT3 regulates stemness phenotype, resistant cells were pre-treated with stattic. After that, STAT3 inhibited resistant cells were seeded with serum free sphere media. In vitro sphere forming ability was decreased compared to normal resistant cells (Figure 8C). We also treated same procedures to confirm stemness phenotype in vivo. Tumor incidence also reduced after inhibition of STAT3 (Figure 8D).

We found that S100A14 was decreased and STAT3 was increased in resistant cells, which resulted in upregulated stemness properties. To find which relation between S100A14 and STAT3, we confirmed STAT3 level in S100A14 variant cells. S100A14 overexpression reduced STAT3 level; therefore, we speculated that S100A14 regulated STAT3 (Figure 9A). To confirm this hypothesis, we transfected S100A14 and STAT3 Y705D, which is the constitutively active form of STAT3, in HCT116 5-FuR cell. In resistant cells, transfected S100A14 decreased the expression of both STAT3 and stemness markers. STAT3 transfection increased the expression of stemness markers, but those markers were decreased when STAT3 and S100A14 were co-transfected (Figure 9B). This result indicates that S100A14 regulates stemness property through regulation of STAT3.

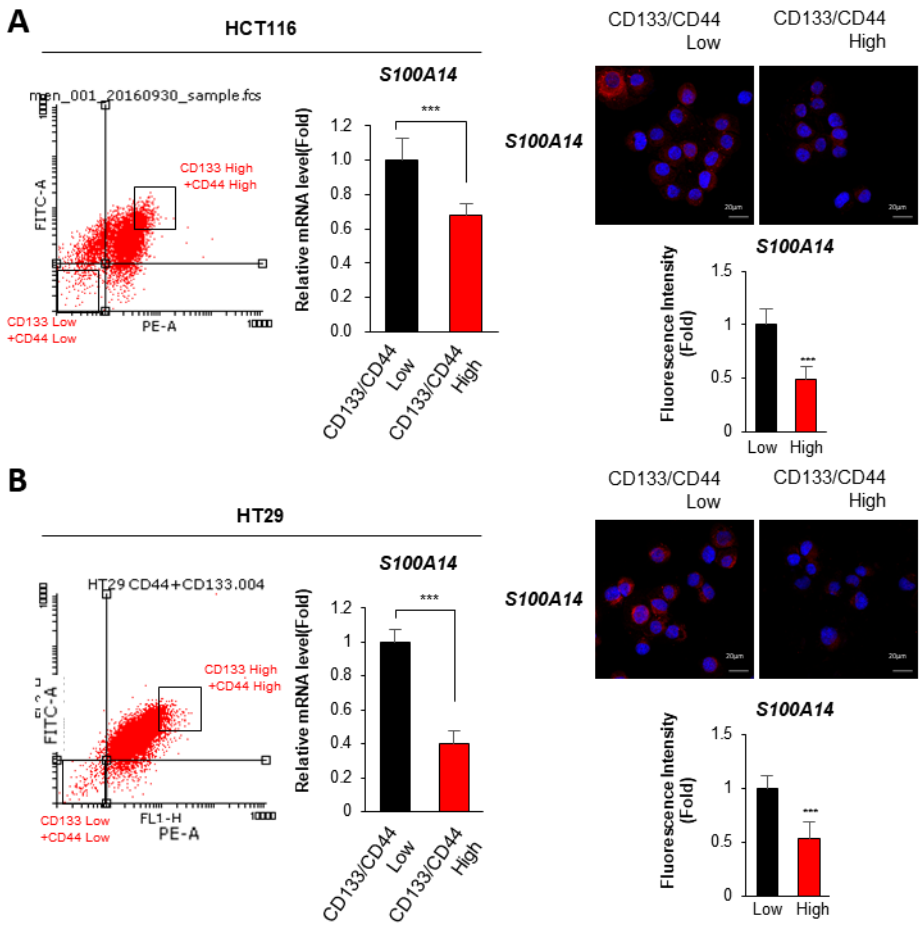


Figure 5. Stemness populations in Colon Cancer cells expressed lowered S100A14.

A. HCT116 cell was sorted in CD133+CD44 High/ Low population. Using sorted cell, S100A14 level were evaluated by real time PCR and immunofluorescence. B. HT29 cell did same methods. *** P <0.001

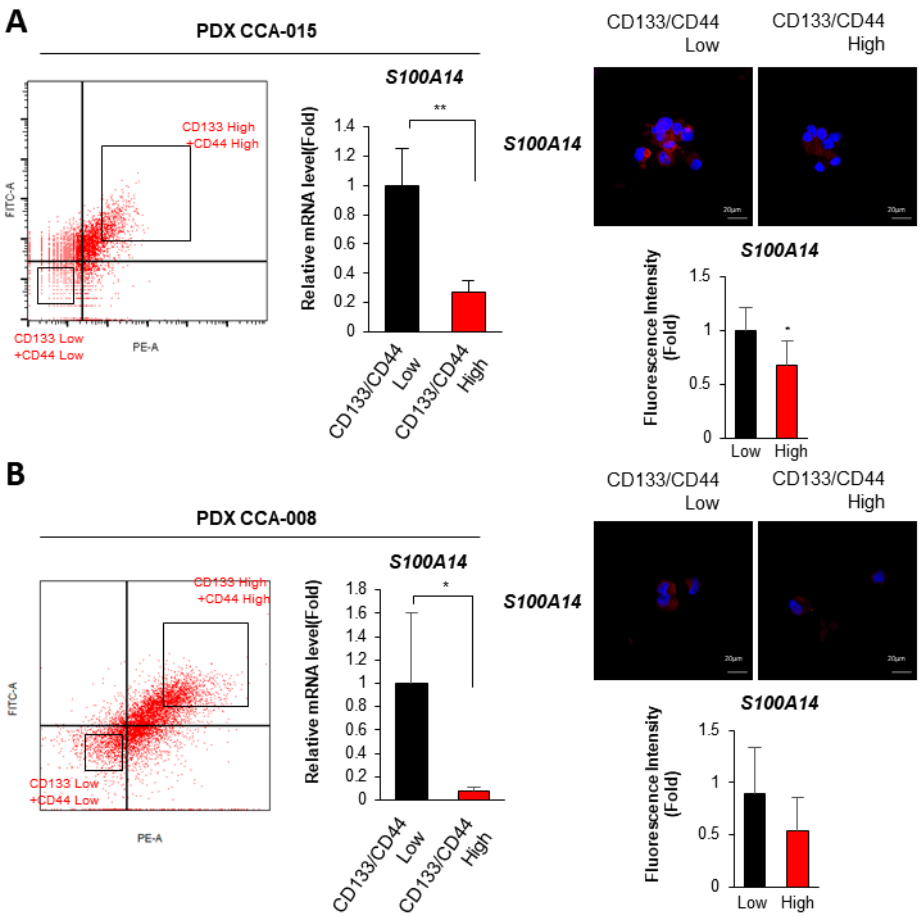


Figure 6. Human colon PDX with high stemness showed decreased level of S100A14.

A. Colon PDX CCA-015 was resected in mouse subcutaneous. After tumor dissociation, cell was sorted in CD133+CD44 High/ Low population. Using sorted cell, S100A14 level were evaluated by real time PCR and immunofluorescence. B. Colon PDX CCA-008 did same methods. *P<0.05, ** P <0.01

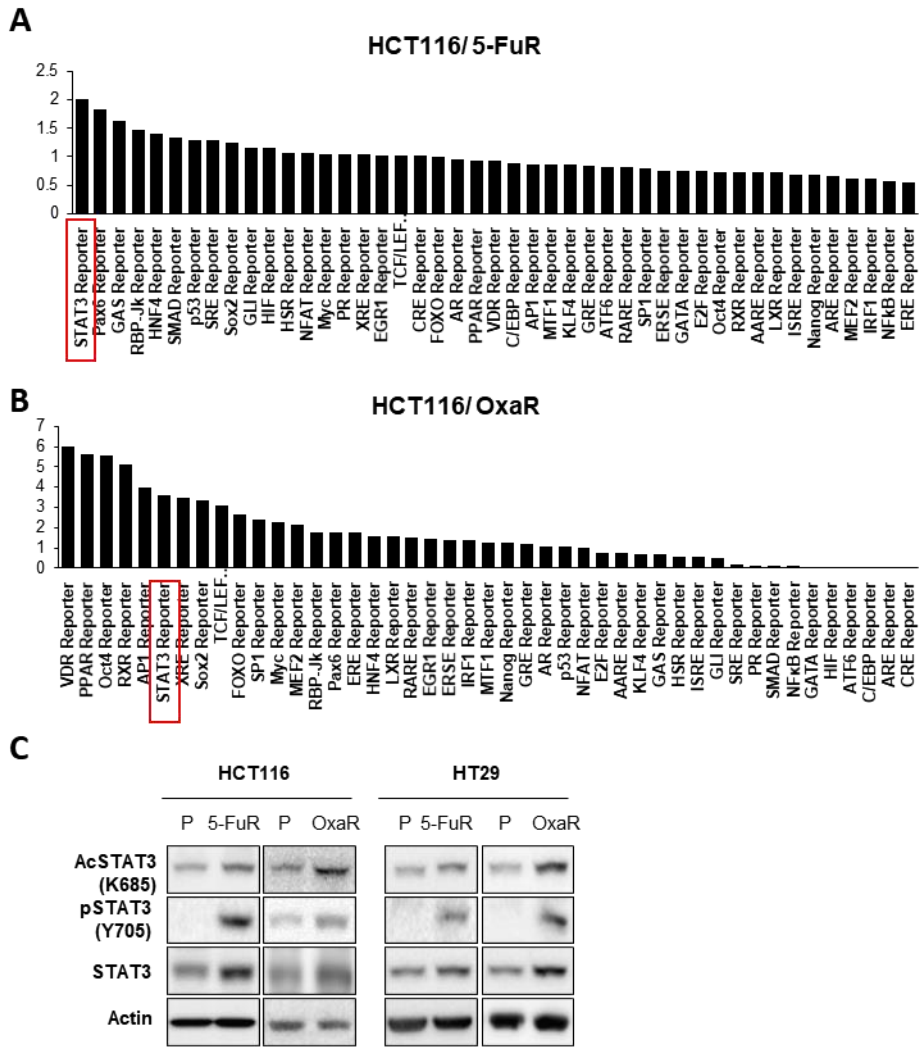


Figure 7. STAT3 is increased in HCT116/ R cell.

A, B. Luciferase assay between resistant cell and parental cell. 45 signaling pathway was presented. HCT116 5-FuR (A), HCT116 OxaR(B) C. HCT116/ HT29 parental cell and resistant cell were measured by western blot for monitoring STAT3 changes.

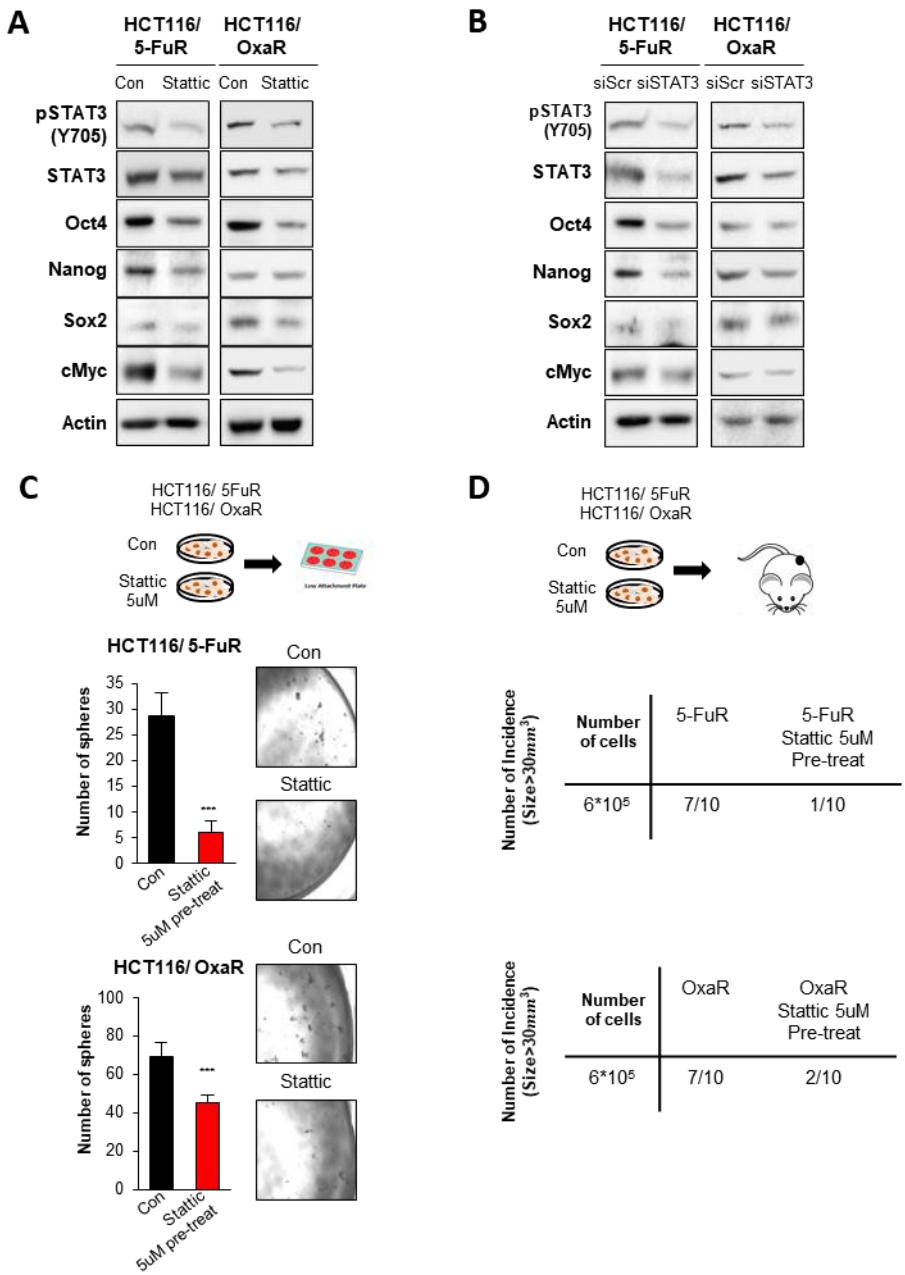


Figure 8. STAT3 regulates stemness property.

A, B. Stemness markers were evaluated when STAT3 was Knocked down by STAT3 inhibitor stattic (A) and siSTAT3 (B). C. Self renewal and proliferation capacity of stemness population were

confirmed after STAT3 inhibition. **D.** In vivo tumor incidence inhibition was monitored after STAT3 inhibition. *** P <0.001

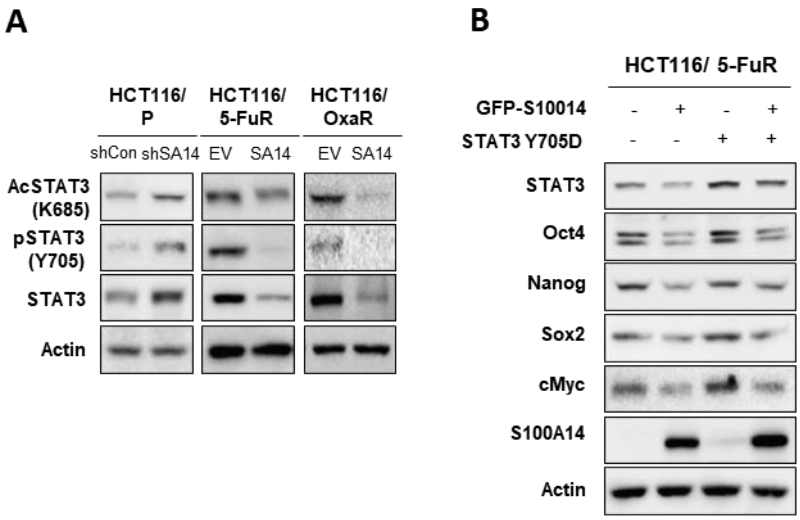


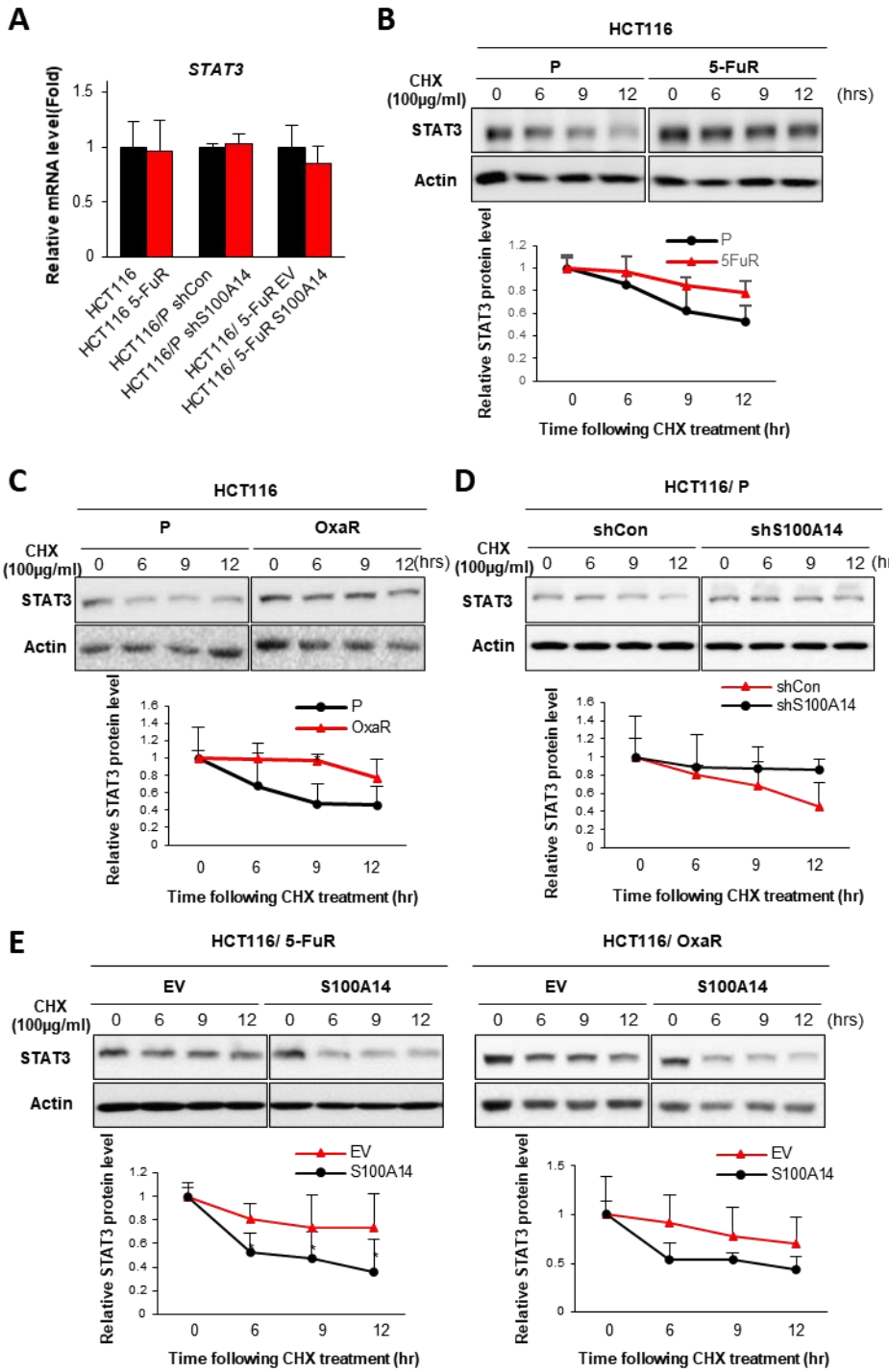
Figure 9. S100A14 decreases stemness property through STAT3 regulation

A. STAT3 and STAT3 active forms were evaluated between parental cell and S100A14 Knockdown cell, resistant cell and S100A14 overexpression cells. B. HCT116 5-FuR cell was transfected (1) EV, (2) GFP-S100A14, (3) STAT3 constitutive active form (4) both vector and confirmed stemness markers dynamics.

S100A14 regulates STAT3 stability through increasing ubiquitination

Proteins can be regulated by changing their expression and by controlling their stability. First, we confirmed STAT3 mRNA level (Figure 10A). There was no significant relation between STAT3 mRNA expression and S100A14. Next, we confirmed the protein stability. Protein synthesis was stopped through cyclohexamide treatment. Without protein synthesis, we found that STAT3 had been slowly degraded in resistant cells (Figure 10B and C). We speculated that decreased S100A14 level had an effect on protein degradation. Therefore, we analyzed STAT3 stability in S100A14 modulated cells. Increased S100A14 reduced STAT3 stability (Figure 10D and E).

Protein stability is regulated by the ubiquitin-proteasome system. In cytoplasm, degraded protein is marked by ubiquitin, which is followed by proteasomal degradation. To investigate how S100A14 regulates STAT3 stability, we transfected ubiquitin and monitored ubiquitin bound STAT3. We performed immunoprecipitation using STAT3 antibody to confirm STAT3 ubiquitination. In resistant cells, the level of polyubiquitin-bound STAT3 was decreased compared to that in parental cells, suggesting that STAT3 escaped protein degradation in resistant cells. However, ubiquitination was increased when S100A14 was co-transfected (Figure 11). These results indicate that S100A14 reduces STAT3 stability via increasing STAT3 ubiquitination.


A
STAT3
Relative mRNA level(Fold)
1.5
1
0.5
0
HCT116
HCT116 5-FuR
HCT116/P shCon
HCT116/P shS100A14
HCT116/ 5-FuR EV
HCT116/ 5-FuR S100A14
B
HCT116
P
5-FuR
CHX (100μg/ml)
0 6 9 12 0 6 9 12 (hrs)
STAT3
Actin
Relative STAT3 protein level
P
5FuR
Time following CHX treatment (hr)
C
HCT116
P
OxaR
CHX (100μg/ml)
0 6 9 12 0 6 9 12(hrs)
STAT3
Actin
Relative STAT3 protein level
P
OxaR
Time following CHX treatment (hr)
D
HCT116/ P
shCon
shS100A14
CHX (100μg/ml)
0 6 9 12 0 6 9 12 (hrs)
STAT3
Actin
Relative STAT3 protein level
shCon
shS100A14
Time following CHX treatment (hr)
E
HCT116/ 5-FuR
EV
S100A14
CHX (100μg/ml)
0 6 9 12 0 6 9 12
STAT3
Actin
Relative STAT3 protein level
EV
S100A14
Time following CHX treatment (hr)
HCT116/ OxaR
EV
S100A14
0 6 9 12 0 6 9 12 (hrs)
Relative STAT3 protein level
EV
S100A14
Time following CHX treatment (hr)

,

Figure 10. S100A14 regulates STAT3 stability.

A. mRNA level of STAT3 was checked HCT116 parental, resistant, S100A14 variant cell lines. B–E. Cyclohexamide (CHX) was treated and cells were prepped time dependent. STAT3 level was evaluated by western blot. The band intensity was calculated by image J.

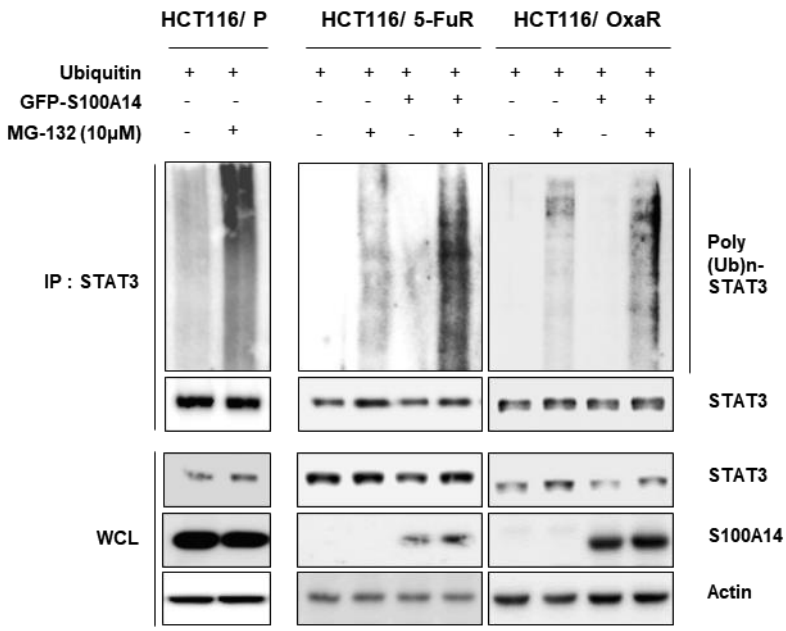


Figure 11. S100A14 increases STAT3 degradation through inducing ubiquitination via interaction

Immunoprecipitation was performed with HCT116 Parental cell and resistant cells. Ubiquitin was transfected into cell with or without S100A14. MG-132 was added 4 hours before prepping to stop protein degradation.

DISCUSSION

Chemotherapy drugs induce resistance in colon cancer cells. This study demonstrates that CSC property is induced when cells get resistance after chronic drug treatment. Moreover, we find that S100A14, a protein with lowest expression in resistant cells, can downregulates stemness characteristic. Chemotherapy has been used for cancer treatment since a long time. However, it is hard to completely disseminate cancer with chemotherapy alone and a large number of patients suffer from side effects. Moreover, there are many negative results such as recurrence and metastasis because patients become resistant to therapy. Because of chemoresistance to drugs, patients cannot see the effect of commonly used anticancer therapy. Patients also become resistant to 5-Fu/oxaliplatin, which are commonly used drugs in colon cancer therapy [13]. Therefore, in order to increase treatment efficiency, research to overcome cancer resistance is still active. In our previous study, we developed 5-Fu resistant HCT116 cells and elucidated the mechanism of chemoresistance. Epithelial-mesenchymal transition (EMT) property was increased in colon cancer cells, contributing to emergence of acquired resistance to 5-Fu [14]. In addition to this research, we used more cell lines and drugs to determine the mechanism of resistance. There are various studies about relation between resistant cells and stemness. Our resistant cells remarkably show increased stemness properties than corresponding parental cells. In cancer, increased CSC population is related with drug resistance [15]. Therefore, we found that upregulated CSC properties result in drug resistant phenotype in colon cancer.

CSCs are the major source of drug resistance. Tumors have subpopulation that retains self-renewal and differentiation capacity and has high tumorigenicity. Stem cell population can live after

therapy and cause tumor relapse. In this case, reorganized tumor has resistance to therapy; therefore, it is more dangerous than before [16]. In lung cancer, high resistance against therapies is associated with CSCs. Patient-derived NSCLC tumor spheres showed high self-renewal and growth potential, and showed resistance against chemotherapeutic agents. The spheres had increased expression of stemness markers such as NANOG, CD44, and ITGA6. Those markers have been correlated with bad prognosis [17]. Many researchers are trying to determine how to regulate the CSC population for treatment. For this reason, there are many studies on CSC regulation. Upregulated Wnt signaling is crucial for colon CSC activation; therefore, many researchers have attempted to inhibit this pathway [18]. In a previous study, we performed microarray with HCT116 and HCT116 5-Fu resistant cells [12]. Among various genes whose expression was altered, we found that S100A14 gene had the lowest expression in HCT116 5-Fu resistant cell line. S100A14 has been reported to be expressed at a low level in colon cancer; however, the function of S100A14 has not been addressed. We generated S100A14 knockdown and overexpression cell lines to determine the role of S100A14 in CSC population. High level of S100A14 expression in tumor reduced CSC phenotype. In addition, we found that S100A14 level was downregulated in colon cancer's stemness subgroups. Because S100A14 expression depends on CSC population in tumors, it is possible that S100A14 can be used as a biomarker for prediction of response to chemotherapy.

STAT3 is a transcription factor that regulates cell proliferation, differentiation, and immune response. STAT3 is upregulated in many types of cancers and aberrant STAT3 activation triggers tumor progression [19]. The FDA-approved drug atovaquone, a novel, clinically available inhibitor of STAT3, has efficacy in AML patients [20]. We showed that STAT3 pathway was increased in resistant colon cancer cells through the luciferase assay. STAT3 regulates

CSC markers such as OCT4, NANOG, SOX2, and cMYC as their transcription factor. Because S100A14 was decreased and STAT3 was increased in resistant cells, we tried to determine the relationship between them. We found that S100A14 modulated STAT3 stability through regulation of STAT3 degradation. Protein stability can be regulated via ubiquitination, a post-translational modification (PTM). Ubiquitin is attached to a target protein and then proteasome recognizes the ubiquitin-conjugated protein. Through this process, the protein is destined to be degraded [21, 22]. We confirmed that resistant cells reduced ubiquitination and following transfection of S100A14 in resistant cells, STAT3 ubiquitination was increased. In addition, we found that S100A14 regulated STAT3 ubiquitination through binding between these proteins.

In conclusion, we demonstrated that increased CSC properties in chemoresistance are related to decreased S100A14 expression. CSC population is the major problem for drug treatment. STAT3 is upregulated in many cancer stem cell populations. S10014 regulates STAT3 stability through modulating STAT3 ubiquitination. Because S100A14 regulates CSC characteristics and is associated with CSC population in tumor, we can suggest S100A14 as a biomarker for prediction of therapeutic efficacy. Recently, combination cancer therapies have been used to improve therapeutic response. The combination of chemotherapy and immune checkpoint inhibitors is associated with significantly prolonged survival compared with only chemotherapy in NSCLC [24]. PD-L1 is the major target of immune checkpoint inhibitors. Previous studies have reported that 5-Fu causes upregulation of PD-L1 in cancer cells [25]. STAT3, major upregulated protein in our study, is the transcription factor of PD-L1 [26]. We found that PD-L1 was increased in colon resistant cells (not described in this research). Therefore, by defining the relation between S100A14 and PD-L1 via further studies, we may find a new alternative for cancer treatment.

REFERENCES

[1] Marina de rosa, et al. Genetics, diagnosis and management of colorectal cancer (Review). Oncol Rep. 2015 Sep; 34(3): 1087-1096.

[2] Daniel B. Longley, D. Paul Harkin & Patrick G. Johnston, 5-Fluorouracil: mechanisms of action and clinical strategies. Nature Reviews Cancer volume 3, pages330-338(2003)

[3] T. Alcindor, MD* and N. Beauger. Oxaliplatin: a review in the era of molecularly targeted therapy. Curr Oncol. 2011 Feb; 18(1): 18-25.

[4] Nikolaos A. Dallas, et al. Chemoresistant Colorectal Cancer Cells, the Cancer Stem Cell Phenotype and Increased Sensitivity to Insulin-like Growth Factor Receptor-1 Inhibition. Cancer Res. 2009 Mar 1; 69(5): 1951-1957.

[5] Tao Hu, Zhen Li, Chun-Ying Gao, and Chi Hin Cho. Mechanisms of drug resistance in colon cancer and its therapeutic strategies. World J Gastroenterol. 2016 Aug 14; 22(30): 6876-6889.

[6] Lan Thi Hanh Phi, et al. Cancer Stem Cells (CSCs) in Drug Resistance and their Therapeutic Implications in Cancer Treatment. Stem Cells Int. 2018; 2018: 5416923.

[7] Mian Xie, et al. Activation of Notch-1 enhances epithelial-mesenchymal transition in gefitinib-acquired resistant lung cancer cells. J Cell Biochem. 2012 May;113(5):1501-13.

[8] Wook Jin. Role of JAK/STAT3 Signaling in the Regulation of Metastasis, the Transition of Cancer Stem Cells, and Chemoresistance of Cancer by Epithelial-Mesenchymal Transition. Cells. 2020 Jan; 9(1): 217.

[9] Anne R. Bresnick, David J. Weber & Danna B. Zimmer. S100

proteins in cancer. Nature Reviews Cancer 15, 96-109(2015)

[10] Suyog Basnet, Sunita Sharma, Daniela Elena Costea, and Dipak Sapkota. Expression profile and functional role of S100A14 in human cancer. Oncotarget. 2019 Apr 26; 10(31): 2996-3012.

[11] Min Zhu, et al. Calcium-binding protein S100A14 induces differentiation and suppresses metastasis in gastric cancer. Cell Death Dis. 2017 Jul 20;8(7):e2938

[12] Ji-Young Ahn. 5-Fu resistance and acquisiton of EMT phenotype in colon cancer cells are mediated by down regulation of S100A14. Seoul national university, college of pharmacy master corse gradutation research

[13] Kevin Van der Jeught, Han-Chen Xu, Yu-Jing Li, Xiong-Bin Lu, and Guang Ji. Drug resistance and new therapies in colorectal cancer. World J Gastroenterol. 2018 Sep 14; 24(34): 3834-3848.

[14] Ji-Young Ahn, Ji-Sun Lee, Hye-Young Min, Ho-Young Lee. Acquired resistance to 5-fluorouracil via HSP90/Src-mediated increase in thymidylate synthase expression in colon cancer. Oncotarget. 2015 Oct 20;6(32):32622-33.

[15] Plabon Kumar Das, Farhadul Islam, and Alfred K. Lam. The Roles of Cancer Stem Cells and Therapy Resistance in Colorectal Carcinoma. Cells 2020, 9, 1392

[16] Marta Prieto-Vila, Ryou-u Takahashi, Wataru Usuba, Isaku Kohama, and Takahiro Ochiya. Drug Resistance Driven by Cancer Stem Cells and Their Niche. Int J Mol Sci. 2017 Dec; 18(12): 2574

[17] Alejandro Herreros-Pomares, et al. Lung tumorspheres reveal cancer stem cell-like properties and a score with prognostic impact in resected non-small-cell lung cancer. Cell Death and Disease (2019) 10:660

[18] E. Melo Felipe de Sousa, Louis Vermeulen, Dick Richel and Jan

Paul Medema. Targeting Wnt Signaling in Colon Cancer Stem Cells. Clin Cancer Res. 2011 Feb 15;17(4):647-53

[19] Haeri Lee1, Ae Jin Jeong1 & Sang-Kyu Ye. Highlighted STAT3 as a potential drug target for cancer therapy. BMB Reports 2019; 52(7): 415-423

[20] Michael Xiang, et al. Gene expression-based discovery of atovaquone as a STAT3 inhibitor and anticancer agent. Blood (2016) 128 (14): 1845-1853.

[21] Guoqiang Xu and Samie R. Jaffrey. The new landscape of protein ubiquitination. Nat Biotechnol. 2011 Dec; 29(12): 1098-1100

[22] Jeffrey H. Stack, Michael Whitney, Steven M. Rodems & Brian A. Pollok. A ubiquitin-based tagging system for controlled modulation of protein stability. Nature Biotechnology volume 18, pages1298-1302(2000)

[24] Alfredo Addeo1, Giuseppe Luigi Banna, Giulio Metro and Massimo Di Maio. Chemotherapy in Combination With Immune Checkpoint Inhibitors for the First-Line Treatment of Patients With Advanced Non-small Cell Lung Cancer: A Systematic Review and Literature-Based Meta-Analysis. Front. Oncol. 9:264

[25] Lauren Van Der Kraak, et al. 5-Fluorouracil upregulates cell surface B7-H1 (PD-L1) expression in gastrointestinal cancers. J Immunother Cancer. 2016; 4: 65.

[26] Xiangfeng Shen, et al. Recent Findings in the Regulation of Programmed Death Ligand 1 Expression. Front Immunol. 2019; 10: 1337

국문초록

대장암은 전 세계적으로 세 번째로 흔한 암종으로 주요 사망 원인 중 하나이다. 대장암 치료에 사용되는 다양한 방법 중에서, 항암제를 이용하는 화학요법은 가장 많이 사용되어 진다. 5-플로오로 우라실 (5-Fluorouracil)과 옥살리플라틴 (oxaliplatin)은 대장암 치료에 있어 주로 사용되는 화학요법제이다. 이와 같은 약물은 치료에 있어 흔히 사용되지만, 다양한 연구들은 항암제가 오히려 약에 대한 저항성을 유도해 치료 효율을 낮춘다는 것을 보여주고 있다. 암세포 내부의 이질성 (heterogeneity)으로 인해, 암세포는 치료에 있어 다양한 반응성을 가지게 된다. 특히 암 줄기세포는 항암제 저항성과 높은 상관관계를 가진다는 것으로 보고되고 있다. 본 논문에서는 암세포가 항암제 저항성을 가지게 되었을 때, 세포의 성질 변화와 그리고 이를 유도하는 내부 기작을 밝히고자 하였다.

우선 대장암 치료에 많이 쓰이는 항암제인 5-플로오로우라실 (5-Fluorouracil)과 옥살리플라틴 (oxaliplatin)을 세포에 장기간 노출시켜 약물에 저항성을 가지는 세포를 구축하였다. 이렇게 만들어진 항암제 내성 세포는 증가된 줄기세포능 (Stemness)을 가진다는 것을 발견하였다. 그리고 구축된 세포 간의 마이크로어레이 실험을 통해 저항성을 가진 세포에서 변화하는 유전자를 분석하였고, 이를 통해 S100A14가 저항성 세포에서 가장 낮아져 있다는 것을 발견하였다. 저항성 세포에서 증가되어진 줄기세포능은 S100A14가 transfection될 경우 낮아졌으며, 대장암에서 줄기세포능을 많이 가지는 부분일수록 낮은 S100A14 발현을 보인다는 것을 확인하였다. S100A14가 어떻게 줄기세포능을 조절하는지 알아보기 위해서, 본 실험에서는 luciferase assay를 실행하여 항암제 저항성 세포에서 증가되어진 기작을 찾고자 하였다. 다양한 신호전달 경로 중에서 STAT3와 관련된 신호전달 기작이 대장암 항암제 저항성 세포에서 큰 폭으로 증가 되어있는 것을 확인하였다.

화학 항암제에 저항성을 가진 HCT116 세포는 낮은 S100A14

발현 보이며, 저항성이 없는 기존의 HCT116 세포에 비해 높은 수준의 STAT3를 가진다. S100A14와 STAT3 사이의 관계를 알아보기 위해, S100A14를 없애거나, 과발현시켜 양을 조절하였을 경우 STAT3가 달라지는지를 확인해 보았다. 저항성 세포에서 STAT3 단백질은 S100A14를 transfection할 경우 줄어들었으며, 반대로 S100A14를 없앤 세포에서는 STAT3 단백질이 증가하였다. 우리는 이러한 과정에서 S100A14가 STAT3를 mRNA 발현이 아닌 단백질 수준에서 조절한다는 것을 찾아냈고, S100A14가 STAT3 분해를 조절한다는 것을 알아냈다. S100A14가 어떻게 STAT3 분해를 조절하는지 알아보기 위해 면역침강실험을 진행하였고, 이를 통해 S100A14가 STAT3에 결합하여 STAT3의 유비퀴틴화 (ubiquitination)를 조절하여 STAT3 분해를 유도한다는 것을 밝혔다.

이 연구를 통해, 화학 항암제에 저항성을 보이는 대장암 세포에서 줄기세포능 조절에 있어서 S100A14의 새로운 기능을 규명하였다. S100A14은 STAT3의 분해를 조절하는데, 저항성 세포에서는 S100A14의 발현이 낮아져 STAT3가 증가하게 되고, 이로 인해 줄기세포능을 유도하는 여러 요소들이 증가하게 된다는 것을 본 논문을 통해 밝힌 것이다. 더 나아가, S100A14가 항암제 치료의 효과를 예측할 수 있는 바이오 마커로 사용될 수 있다는 점을 본 논문을 통해 제시하였다.

주요어: 대장암, 항암제 내성, 암 줄기세포, 줄기세포능, S100A14, STAT3

학 번: 2019-22493

ACKNOWLEDGEMENT

막 실험실에 들어온 지가 엊그제 같은데 시간이 2년이나 흘러 벌써 석사를 마무리하게 되었습니다. 논문 작성을 다 마치고 이렇게 마무리 글을 적고 있으니 석사 생활을 돌아보게 되며 도움을 받은 많은 분들이 떠오릅니다.

우선, 아무것도 모르던 학부생 한 명을 받아 주시고 이렇게 졸업 논문을 쓰고 석사 졸업을 하기까지 많은 가르침을 주신 이호영 교수님, 감사드립니다. 학생들보다 더 많이, 밤낮으로 연구에 매진하시고 끊임없이 공부하시는 모습에서 많은 것을 배웠습니다. 한 분야에서 전문가로서 안주하지 않고 계속해서 공부하며 발전을 멈추지 않으시는 교수님의 모습은 제가 연구자로 나아가는데 있어 어떤 태도를 가져야 하는지를 일깨워 주셨습니다. 교수님께서 해주신 지도 덕분에 석사 생활을 잘 마무리할 수 있었습니다. 정말 감사드립니다. 그리고 항상 친절하신 민혜영 박사님, 제가 무언가를 여쭤볼 때마다 늘 여쭤본 것 이상으로 더 좋은 대답과 말씀을 해 주셔서 감사합니다. 그리고 항상 좋은 조언과 위로로 저에게 많은 도움을 주신 혜진 언니, 늘 친절하고 상냥하신, 고양이 구조도 같이 한 혜정 언니, 처음 들어왔을 때 쫌 많이 물어봐서 가끔 귀찮았을텐데도 잘 알려주신 첫 사수 재범 오빠, 고민상담 할 때마다 여러가지로 좋은 조언해주시고 처음 실험실 생활 시작할 때부터 편하게 대해 주셔서 감사한 현지 언니, 대화할 때마다 유머러스한, 어쩌다보니 듀티를 자주 같이하는 승엽 오빠, 박사 졸업이지만 같은 해에 졸업하게 된, 베트남으로 돌아가면 보기 힘들어져 아쉬울 Huong 언니, 실험이나 일상적인 고민을 털어놓을 때 많이 공감해주시고 위로해주시는, 같이 프로젝트 하는데 있어 부족한 저에게 많은 도움과 실험적 조언을 아끼지 않는 고마운 명경언니, mbti가 비슷하게 나온, 재밌는 프로그램을 추천해 주셔서 취향에 맞는 드라마를 발견하게 해주는 호진 언니, 가끔 내가 들어도 이상한 영어를 찰떡같이 알아들어주어서 즐겁게 대화할 수 있는 친구 Xuan, 빠른이지만 친구하기로 해서 편하게 지내는, 동갑이지만 박사과정인 전자기기 신제품 소식이 빠른 얼리어답터 지환이, 그리고 새로 들어온 후배지만

친구 혁진이까지 힘들기도 했지만 lee lab 모두가 있어서 보람찬 대학원 생활을 할 수 있었습니다. 처음 들어왔을 때 다들 친절하게 많이 알려주시고 도움을 주셔서 이렇게 무사히 졸업할 수 있었습니다.

그리고 무엇보다도 대학교 졸업하고 또 대학원 가서 공부하겠다는 딸을 위해 지원을 아끼지 않으신 부모님께 감사드립니다. 아침잠이 많고, 아침에 먹는 것 챙기기 귀찮아하는 저를 위해 본인도 출근하시면서 늘 깨워주고 과일을 챙겨 주시고, 늘 걱정해 주시는 엄마와 퇴근하시고 집에 가고 싶으실 텐데 자주, 특히 요새는 코로나라고, 늦게 퇴근하니까 힘들다고 거의 매일 데리러 와 주시는 아빠 감사드립니다. 두 분 덕분에 무사히 졸업할 수 있었습니다. 그리고 가끔 티격대며 싸우지만 동생 공부한다고 가끔 성과급 나오면 용돈 주던 오빠도 고마워. 늘 나보고 힘 얻는다던(ㅎ), 갑자기 힘듦을 토로해도 잘 받아주는 지연이 시은이, 대학원 가니까 시간이 잘 안 맞아서 자주는 못 볼지라도 만나면 늘 즐거운, 기분전환 시켜주는 의생명 동기들, 동아리 친구들, 그리고 뜬금없이 전화해도 저의 투정을 받아주고 위로해준 모든 지인들 덕분에 무사히 졸업할 수 있었습니다. 감사합니다.

이렇게 많은 분들의 도움으로 이 논문이 완성될 수 있었습니다. 앞으로 나아감에 있어 받은 많은 도움들을 잊지 못할 것입니다. 석사 과정을 마쳤지만, 이는 끝이 아니라 새로운 시작이 될 것입니다. 앞으로 더 좋은 모습, 좋은 연구자가 되도록 노력하겠습니다. 다시 한 번 모든 분들께 감사드립니다.