Establishment of a new therapeutic strategy targeting cancer stem like cells in refractory gastric cancer acquired Trastuzumab resistance

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Establishment of a new therapeutic strategy targeting cancer stem like cells in refractory gastric cancer acquired Trastuzumab resistance

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ABSTRACT

Establishment of a new therapeutic strategy targeting cancer stem like cells in refractory gastric cancer acquired Trastuzumab resistance

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Background: Trastuzumab is the only approved target agent for the first-line treatment of HER-2 positive gastric cancer, but trastuzumab resistance is a major problem in clinical practice. To comprehend the mechanism of trastuzumab resistance, I focused on cancer stemness and Wnt/ß-catenin signaling pathway and how it influences the phenotypes and behaviors of trastuzumab-resistant gastric cancer cells.

Methods: Trastuzumab-resistant NCI-N87 cells (NCI-N87R) were established *in vitro* from human gastric cancer cell line NCI-N87 by dose-escalating, repeated trastuzumab treatment for one year. I investigated the phenotypes of NCI-N87R, including Wnt signaling pathway activity. Gastric cancer organoid cells were incubated with complete media or Wnt3a-depletion media, and their resistance to trastuzumab were compared.

Results: NCI-N87R exhibited stemness and epithelial-mesenchymal transition (EMT) like phenotypes, along with decreased levels of epithelial marker E-cadherin and increased levels of mesenchymal markers Vimentin and Snail. They also showed increased Wnt signaling pathway activity. When NCI-N87 cells were incubated in Wnt3a conditioned media, their Wnt signaling pathway activity and resistance to trastuzumab increased. Gastric cancer patients-derived organoid incubated in Wnt3a-depletion media were more susceptible than in complete media to dose-dependent inhibition of cell viability by trastuzumab.

Conclusions: Trastuzumab-resistant gastric cancer cells exhibit EMT-like phenotype and

trastuzumab resistance was promoted by Wnt/ ß-catenin signaling pathway. Wnt/ß-catenin pathway is a key signaling to trastuzumab resistance in gastric cancer cells.

Key words : Wnt, gastric cancer, trastuzumab, resistance, epithelial to mesenchymal transition

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I. INTRODUCTION

The incidence of gastric cancer is high in Eastern Asia [1]. Although the prognosis of patients with early gastric cancer is favorable, that for patients with advanced gastric cancer is still problematic [2]. Human epidermal growth factor receptor 2 (*HER2*) is a target gene in the treatment of gastric cancer [3]. According to the ToGA trial, the addition of trastuzumab to platinum-based chemotherapy for HER2-positive advanced gastric cancer has demonstrated improvements in the survival of patients receiving this combination therapy [3]. Amplification of the *HER2* gene is observed in approximately 20% of patients with gastric cancer, [4-9] and a high HER2 level is a poor prognostic factor [10,11]. Likewise, in breast cancer, HER2 overexpression induces aggressive tumor behavior [12,13]. The mechanism by which signaling pathways contribute to the aggressive characteristics of HER2-overexpressing breast cancer is mediated by a small subset of cancer stem cells (CSCs) that display stemness properties [14,15]. Despite the significant clinical benefit of trastuzumab, its positive response rate for HER2-positive gastric cancer was reported to be only approximately 47% [3]. Trastuzumab resistance was found to be caused by the deletion of phosphatase and tensin homolog deleted on chromosome 10 (PTEN, a tumor suppressor) and the activation of PI3K pathway [16-19]. In addition, upregulation of insulin-like growth factor receptor (IGFR) and hetero-dimerization of IGFR/HER-2[20], and accumulation of truncated HER-2 receptor (p95HER-2) have been identified to be involved in trastuzumab

resistance [21]. Recent evidence has shown that CSCs may be resistant to chemotherapy and radiation. A number of genes and signaling pathways that regulate CSCs in gastric cancer have been identified. In a previous study, Wnt expression in gastric cancer was shown to have a strong association with HER2 overexpression [22]. For this reason, I focused on Wnt signaling pathway and how it influences the phenotypes and behaviors of trastuzumab-resistant gastric cancer cells. My study aims to understand the mechanisms leading to trastuzumab resistance in HER2-overexpressing gastric cancer.

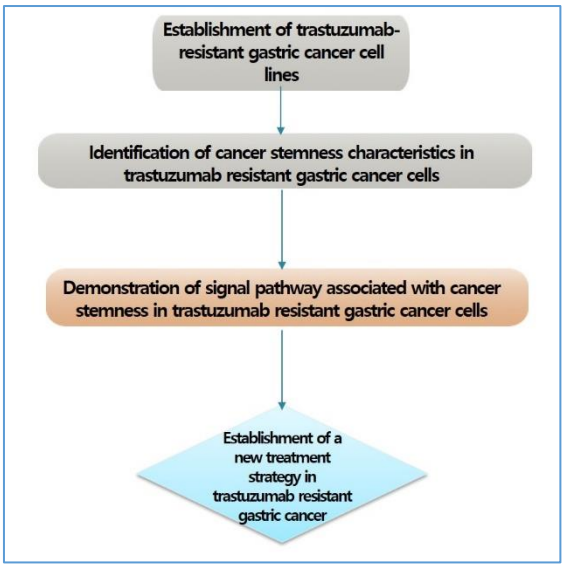

Figure 1. Hypothesis diagram

II. MATERIALS AND METHODS

1. Gastric cancer cell line and culture

Gastric cancer cell lines including MKN28, MKN45, MKN74, SNU216, SNU484, NCI-N87, and AGS were selected and obtained from the Korean Cell Line Bank. All cells were cultured in RPMI 1640 medium supplemented with 10% FBS, at 37 ° C under 5% CO2 in a humidified incubator.

2. Establishment of trastuzumab-resistant gastric cancer cell lines

I used Western blot to detect the HER-2 expression in all seven gastric cancer cell lines, including MKN28, MKN45, MKN74, SNU216, SNU484, NCI-N87, and AGS. SNU216 and NCI-N87 cells showed the highest levels of HER-2 expression (**Figure 2a**). To simulate the in vivo mode of resistance, I exposed SNU216 and NCI-N87 cells by stepwise exposure to increasing doses of trastuzumab over a year. SNU216 cells were unable to produce resistant cells. NCI-N87 cells grew steadily in medium containing trastuzumab and were successfully sub-cultured, earning the name NCI-N87R. **Figure 2b** shows the evidence of trastuzumab resistance of NCI-N87R cells by the cell viability assay, while inhibition of trastuzumab on cell viability increased in a dose-dependent manner in NCI-N87 cells.

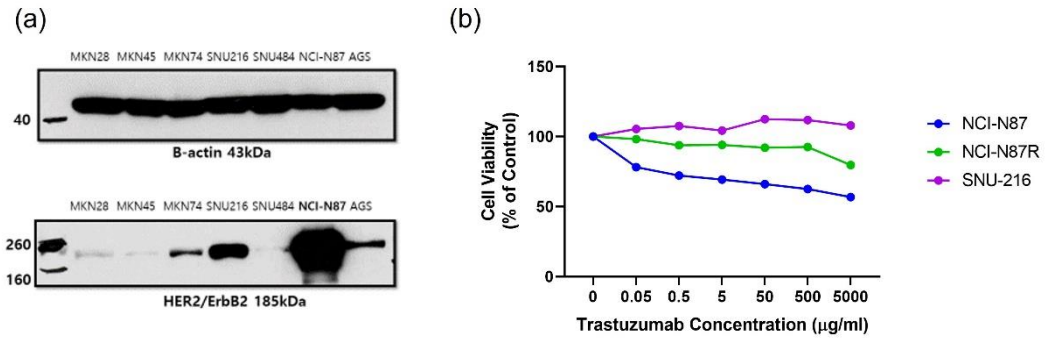

Figure 2. (a) SNU216 and NCI-N87 cells showed the highest levels of HER-2 expression. (b) Trastuzumab resistance of NCI-N87R cells revealed by cell viability assay while SNU 216 cells were unable to produce resistant cells.

3. Cell viability assay

At a density of 3-7.5 × 10^3 cells per well, cells were seeded in 96-well plates, incubated overnight at 37 °C, and then exposed to various concentrations of trastuzumab for 72 hours. Each well received a 50 μl aliquot of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA), and incubation was carried out 37 °C for an additional 4 hours. After removing the medium, 150 μl of dimethyl

sulfoxide (DMSO) was added to each well and mixed. A VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure absorbance at 540 nm.

4. Spheroid Colony Formation Assay

RPMI 1640 serum-free medium, 20 ng/mL human recombinant epidermal growth factor (Invitrogen, Carlsbad, CA, USA), 20 ng/mL human recombinant basic fibroblast growth factor (Invitrogen), and supplements B-27 and N2 were added to the trypsin-EDTA-isolated NCI-N87 and NCI-N87R cells before they were seeded in each well of an ultralow-attachment 96-well plate (Corning Life Sciences, Acton, MA, USA). Every four days, 20 μL of the medium were replaced. Each well was looked at under a light microscope after 5, 14 and 21 days and the spheroid cells' size were measured and compared with those of wild-type cells.

5. Western Blotting Analysis

Whole cells were centrifuged at 15,000 rpm for 10 minutes at 4 ° C after being lysed in RIPA lysis buffer for over 45 min. Using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA) or BCA protein assay kit (Thermo scientific, Rockford, MR, USA), the supernatant's protein concentration was determined. Each sample's 30 g of denatured protein was then transferred to a PVDF membrane (Millipore, Billerica, MA, USA) after being separated on a 10 percent SDS-PAGE gel. 5 percent skim milk or BSA was used to block the membrane for 1 hour at RT. After that, the membrane was incubated overnight at 4 °C with rabbit anti-HER2 (1:1,000; #2165; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-polycomb complex protein BMI-1 (BMI1) (1:500; #ab135713; Abcam, Cambridge, UK), rabbit anti-octamer-binding transcription factor 4 (Oct4) (1:1,000; #2750; Cell Signaling Technology, Massachusetts, USA), rabbit anti-Snail (1:500; #3895; Cell Signaling Technology, Massachusetts, USA), and rabbit anti-GAPDH (1:2,000; #2118; Cell Signaling Technology, Massachusetts, USA) primary antibodies. The membrane was incubated for 1 hour at RT with HRP-conjugated anti-rabbit or anti-mouse IgG (1:5,000;

#7074S or #7076S; Cell Signaling Technology, Massachusetts, USA) secondary antibodies after being washed with wash buffer (10 mM Tris-HCl, 70 mM NaCl, and 0.05 percent Tween 20). The membrane was then washed triple with wash buffer for ten minutes, and ECL (Amersham Biosciences, GE Healthcare, Arlington Heights, IL, USA) was used to detect it.

6. Immunofluorescence Staining of E-Cadherin and β-Catenin

NCI-N87 and NCI-N87R cells labeled with rabbit monoclonal antibodies against E-cadherin (1:1,000; #sc-7870; Santa Cruz Biotechnology, Dallas, TX, USA) and β-catenin (1:50; #sc-7199; Santa Cruz Biotechnology). An FITC-labeled secondary antibody was used to detect the bound antibodies. The cells were observed under a laser-scanning confocal microscope after the nuclei were stained with 1 g/mL DAPI (Sigma-Aldrich, St. Louis, MO, USA) (LSM 780; ZEISS, Oberkochen, Germany).

7. Luciferase Assay

NCI-N87 and NCI-N87R cells were transfected with pTA-Luc and TCF/LEF luciferase reporter vectors (Promega, Madison, WI, USA). Cells were co-transfected with the TopFlash firefly luciferase reporter vector and pRL-SV40-Renilla luciferase vector (Promega) and incubated for 72 hours to detect Wnt signaling pathway activity, The dual-luciferase reporter method (Promega) was then used to determine the relative luciferase activity

8. Organoid culture

Clinical samples for organoid establishment and biologic analyses were obtained from patients at Gangnam Severance Hospital with informed consent after study approval by the ethical committees (IRB 3-2018-0209). Gastric cancer specimens were collected by surgical resection or biopsy. Surgical specimens were washed with phosphate-buffered saline (PBS) and minced into 1-mm^3 fragments. The fragments were digested with

CollgenaseI (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 hour, and undigested pellets were separated by pressing with a plastic stick. Before plating, collected epithelia were washed with PBS supplemented with 1% bovine serum albumin to inactivate digestive enzymes. Advanced Dulbecco's modified Eagle's medium/F12 was supplemented with antibiotic/antimycotic, 10 mM HEPES, 2 mM GlutaMAX, 1 × B27 (Thermo Fisher Scientific) for basal culture medium. Complete medium was prepared by supplementing the basal culture medium with the niche factors. Plated organoids were maintained in a 37°C incubator with 5% CO_2, and media were changed every 3–4 days.

9. Statistical analysis

All statistical analyses were performed using SPSS 26.0. To determine the difference between subgroups, one-way ANOVA was used. A statistically significant difference was defined as a P value < 0.05.

III. RESULTS

1. Trastuzumab-resistant gastric cancer cells exhibit stemness and EMT-like phenotypes

To evaluate the stemness of trastuzumab resistant gastric cancer cells, parental NCI-N87 cells and NCI-N87R cells were cultured in suspension for 21 days. Both types of cells produced nonadherent spherical colonies known as spheres. Compared with NCI-N87 cells, NCI-N87R cells demonstrate significantly larger sphere size and higher cell counts of spheres larger than 50 μm (**Figure 3a**). In order to confirm EMT, I evaluated the expression of E-cadherin. E-cadherin was downregulated in NCI-N87R cells, as confirmed by immunofluorescence. Stem cell markers including CD44s, CD54, BMI1, OCT4, Vimentin and Snail were significantly upregulated in NCI-N87R cells compared to their parental cells, according to Western blot analysis (**Figure 3b**). These results suggest that trastuzumab-resistant gastric cancer cells have stemness and molecular characteristics with

EMT cells.

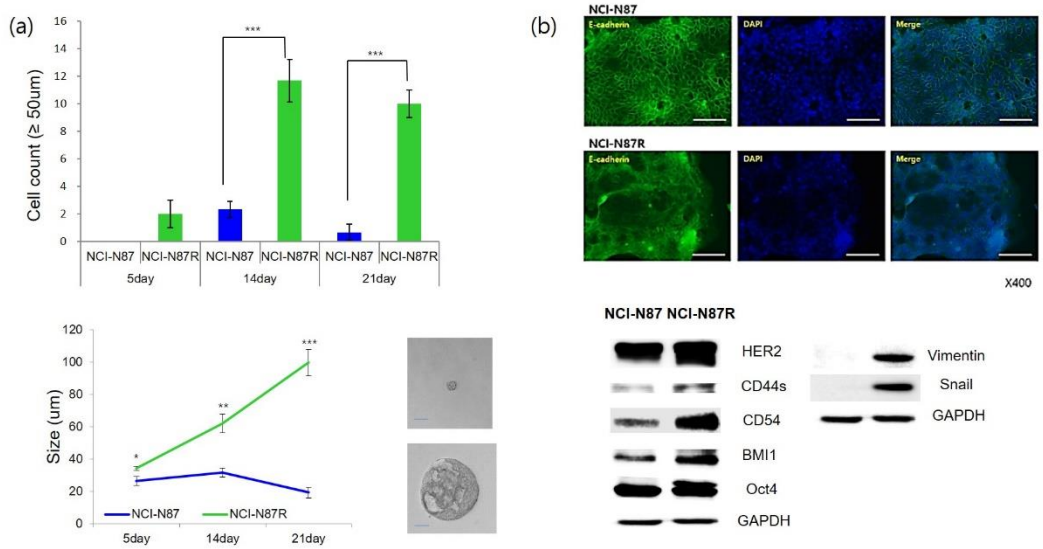

Figure 3. (a) NCI-N87R cells had significantly larger sphere size and higher cell counts of spheres larger than 50 μm than NCI-N87 cells ($^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.005). (b) E-cadherin was downregulated in NCI-N87R cells, as confirmed by immunofluorescence compared with parental cells. Stem cell markers including CD44s, CD54, BMI1, Oct4, Vimentin and Snail were significantly upregulated in NCI-N87R cells compared with parental cells, according to Western blot analysis.

2. Trastuzumab-resistant gastric cancer cells exhibit increased activity of Wnt signaling pathway

As trastuzumab-resistant gastric cancer cells showed EMT-like phenotype change, I analyzed the activity of the Wnt signaling pathway, well known signal transduction pathway that plays a crucial role in EMT. Previous report showed that the expression of Wnt3 activated Wnt/ β-catenin pathway and promoted EMT-like phenotype in trastuzumab resistant breast cancer cells and other study showed that activation of Wnt/ β-catenin contributed to trastuzumab resistance in gastric cancer [23,24]. Therefore, I analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to identify altered

pathways in trastuzumab resistant NCI-N87 cells. The output data (Gene Expression Omnibus Series accession number GSE 77346) was used to determine recurrent copy number changes. Thirteen genes that are part of the Wnt pathway were either up- or downregulated ≥2-fold in trastuzumab resistant NCI-N87 cells compared with parental NCI-N87 cells (**Figure 4**).

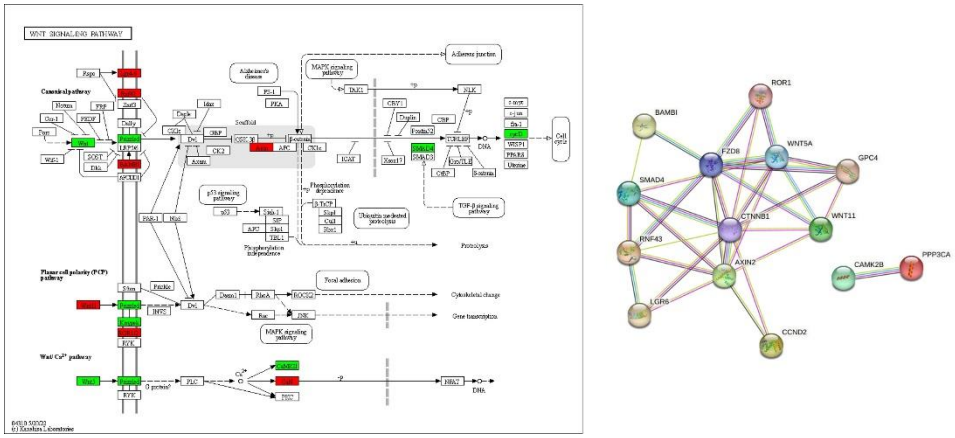

Figure 4. KEGG pathway analysis in trastuzumab resistant NCI-N87 cells using GSE77346

TCF/LEF reporter kit was used to assess the activity of the Wnt signaling pathway. NCI-N87R cells showed significantly higher activity of the Wnt signaling pathway compared to that of parental cells (**Figure 5a**). Likewise, nucleus β-catenin expression was evident and significantly upregulated in NCI-N87R cells compared with parental cells in immunofluorescence staining (**Figure 5b**).

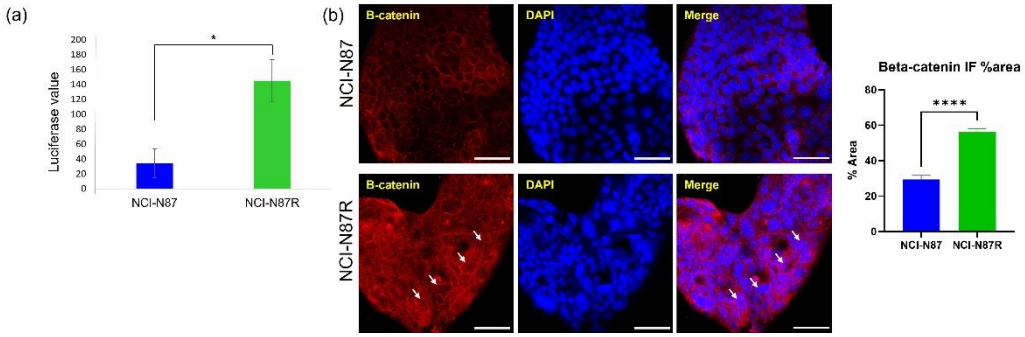

Figure 5. (a) NCI-N87R cells showed significantly higher activity of the Wnt signaling pathway compared with that of parental cells ($^{*}P < 0.05$). (b) Nucleus expression of β-catenin was significantly upregulated in NCI-N87R cells compared with parental cells in immunofluorescence staining. White arrows denote nucleus expression of β-catenin ($^{****}P < 0.0001$).

3. Gastric cancer cells incubated in Wnt3a conditioned media exhibit increased activity of Wnt signaling pathway

The activity of Wnt signaling pathway, which is important role in EMT, was found to be increased in trastuzumab-resistant gastric cancer cells. Next, I investigated whether Wnt signaling pathway can be activated when parental gastric cancer cells are incubated in Wnt3a conditioned media. When the NCI-N87 cells which were incubated in Wnt3a conditioned media (NCI-N87_WNT), a significantly increased activity of the Wnt signaling pathway was evident (**Figure 6a**). In immunofluorescence staining, NCI-N87_WNT cells showed markedly higher levels of β-catenin expression than NCI-N87 cells (**Figure 6b**). Additionally, E-cadherin was downregulated in NCI-N87_WNT cells, and this was similar to that of NCI-N87R cells (**Figure 6c**).

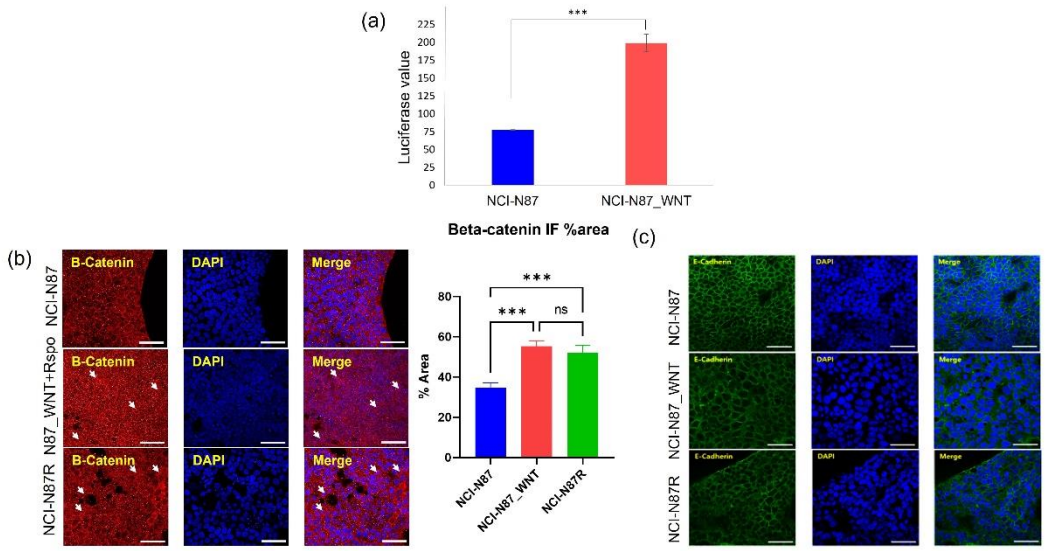

Figure 6. (a) NCI-N87_WNT cells showed significantly increased activity of the Wnt signaling pathway ($^{***}P$ <0.005). (b) NCI-N87_WNT cells showed markedly higher levels of nucleus β-catenin expression than NCI-N87 cells. White arrows denote nucleus expression of β-catenin ($^{***}P$ <0.005). (c) E-cadherin was downregulated in NCI-N87_WNT cells, and this was similar in NCI-N87R cells.

4. Gastric cancer cells incubated in Wnt3a conditioned media acquired trastuzumab resistance

Gastric cancer cells incubated in Wnt3a conditioned media showed increased activity of the Wnt signaling pathway and decreased E-cadherin expression level, like trastuzumab-resistant gastric cancer cells. I, nextly compared the trastuzumab resistance ability of each cell by trastuzumab concentration by cell viability assay. NCI-N87_WNT cells showed lower inhibition of cell proliferation than the NCI-N87 cell (the parental cells without conditioned Wnt media) (**Figure 7**).

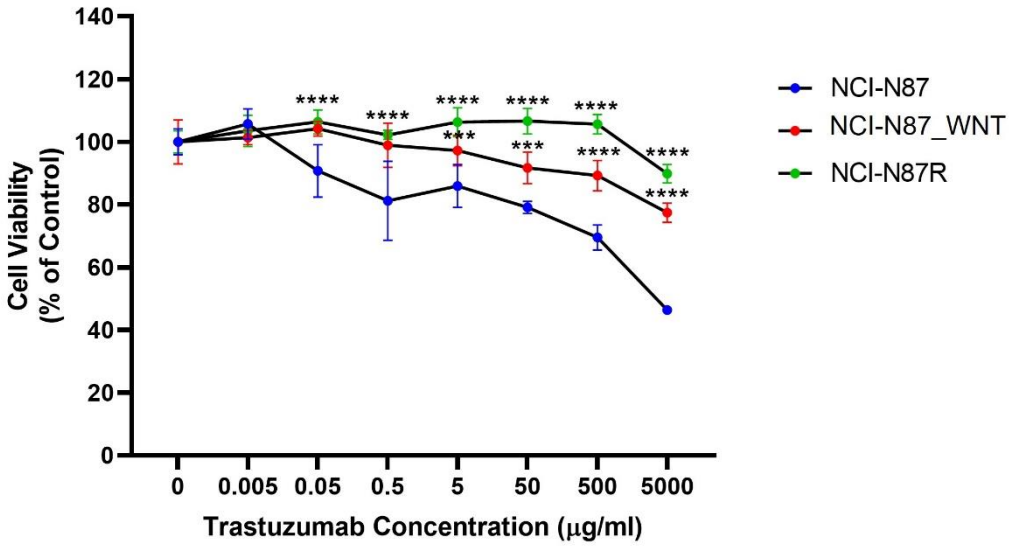

Figure 7. NCI-N87_WNT cells showed lower inhibition of cell proliferation than parental cells (***P <0.001, ****P <0.0001).

5. Wnt signaling pathway affected trastuzumab resistance of gastric cancer in a patients-derived organoids

Next, I investigated whether Wnt signaling pathway affected trastuzumab resistance on cell viability in a patients-derived organoid. Organoid cells were incubated in Wnt3a-depletion media and evaluated cell viability by stepwise exposure to increasing doses of trastuzumab. Organoids with conditioned media were more susceptible to dose-dependent inhibition of cell viability by trastuzumab than organoids with complete media (**Figure 8**).

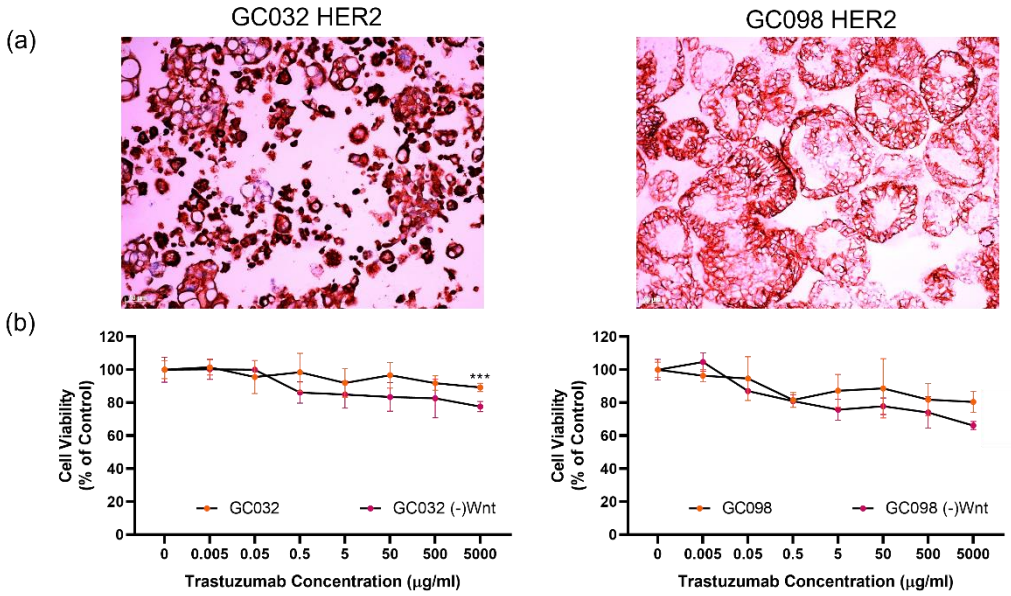

Figure 8. (a) Representative immunohistochemical analysis of the HER2 in patients-derived organoid. (b) Gastric cancer organoid cells incubated in Wnt3a-depletion media were more susceptible to dose-dependent inhibition of cell viability by trastuzumab than in complete media ($^{***}P$ <0.001).

IV. DISCUSSION

I established the trastuzumab-resistant gastric cancer cell lines using NCI-N87 cells, and demonstrated the stemness and EMT-like phenotypes in trastuzumab resistant gastric cancer cells. In addition, I confirmed that trastuzumab-resistant gastric cancer cells exhibit increased activity of Wnt signaling pathway and when parental gastric cancer cells are incubated in Wnt3a conditioned media, parental gastric cancer cells showed the same effect which is a significantly increased activity of the Wnt signaling pathway.

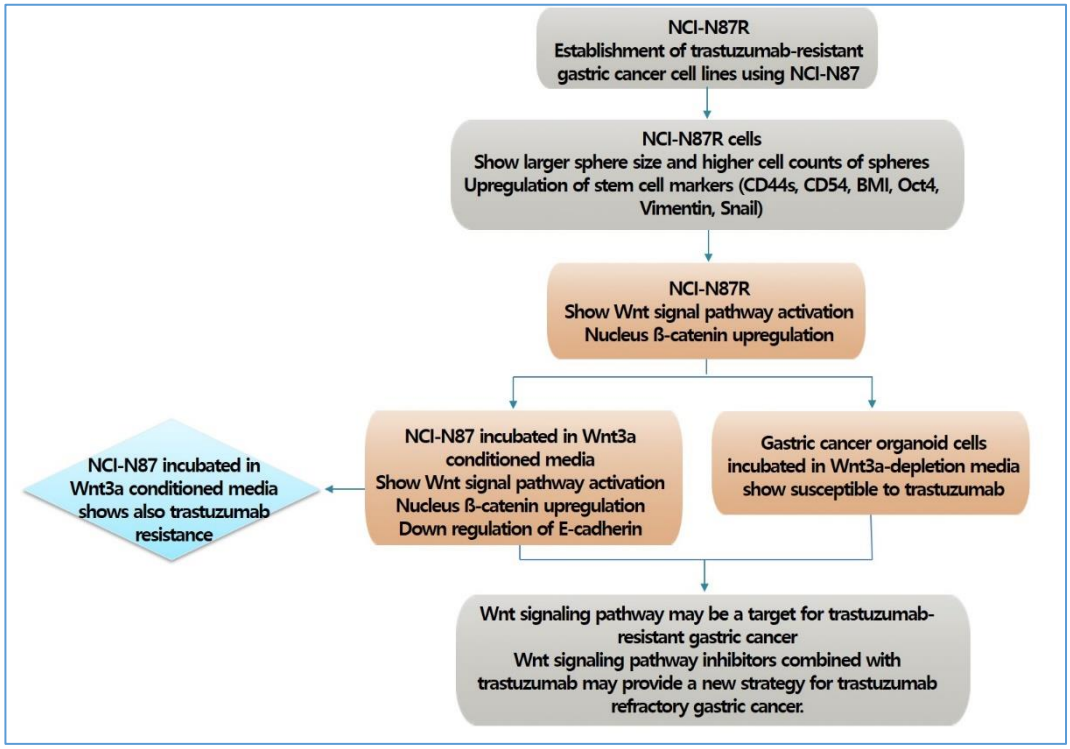

Figure 9. Result diagram

The theory that abnormal CSCs participate in tumor invasion as well as tumorigenesis explains the causes of cancer recurrence and anticancer drug resistance. In this study, using trastuzumab-resistant gastric cancer cells, I demonstrated the CSC activity and the signaling pathway that was associated with the expression of stem cell- and EMT-related genes in the trastuzumab-resistant cells.

Trastuzumab, one of the most effective anti-HER2 antibodies for breast and gastric cancer and has been used in clinical therapy for long time, but the development of resistance is a major obstacle to trastuzumab-based treatment in both HER2-overexpressing breast and gastric cancers. Although numerous trastuzumab resistance mechanisms have been proposed for breast cancer, it is unclear whether similar mechanisms apply to gastric cancer. Therefore, it is crucial to understand the mechanisms and identify the phenotype of trastuzumab resistance in gastric cancer for the development of novel therapeutic

approaches.

In the current study, trastuzumab-resistant cells were obtained in vitro from human gastric cancer cell lines NCI-N87 through repeated, dose-escalating trastuzumab treatment. Trastuzumab-resistant gastric cancer cells showed higher level of stemness and EMT characteristics. Trastuzumab resistant cells showed obvious acquisition of the mesenchymal morphology, decreased levels of epithelial marker and increased levels of mesenchymal markers. Self-renewal (forming spheres), increased clonogenicity, and tumorigenicity were also observed in trastuzumab resistant gastric cancer cells. EMT can trigger reversion to CSC. CSC, which can initiate tumorigenesis and have a high metastatic potential tend to be resistant to chemotherapeutic agents [16]. These findings suggest that prolonged trastuzumab treatment induces stemness and EMT-like phenotype in gastric cancer cells, leading to trastuzumab resistance.

Through EMT induction, pathophysiological conditions such as tissue injury or tumorigenesis can cause differentiated cells to acquire a multipotent stem cell-like phenotype. This could be similar to developmentally regulated EMT signaling pathways like Wnt, Notch, and Hedgehog, which are responsible for both normal and CSC renewal and maintenance [25,26]. Wnt is a highly conserved signaling pathway that plays a critical role in controlling embryonic and organ development, as well as human cancer progression including breast cancer, colorectal cancer, ovarian cancer and prostate cancer [27]. Recently rapid developing genome-wide sequencing and gene expression profile analyses have demonstrated that Wnt signaling is involved mainly in the processes of cancer proliferation and metastasis. The most recent studies have indicated that Wnt signaling is crucial in breast cancer immune microenvironment regulation, stemness maintenance, therapeutic resistance and phenotype shaping [28-30]. Wu Et al. showed Wnt3 overexpression in trastuzumab-resistant breast cancer cells activates Wnt signaling pathway that leads to transactivation of EGFR and promotes EMT-like transition [31]. The EMT-like transition in cancer cells could promote tumor invasion and metastases, as well as mediate drug resistance. Data from my current study also indicated that trastuzumab-resistant gastric

cancer cells exhibit increased activity of Wnt signaling pathway. Additionally gastric cancer cells incubated in Wnt3a conditioned media exhibit increased activity of Wnt signaling pathway and trastuzumab resistance. I also investigate how Wnt signaling pathway affected trastuzumab resistance on cell viability in a patients-derived preclinical model. Organoid cells from HER-2 positive gastric cancer patients which were incubated in Wnt3a-free media were more susceptible to dose-dependent inhibition of cell viability by trastuzumab than parental cells. In several types of tumors, preclinical data of which the agents targeting the Wnt pathway were used accumulated. Zhi et al[22] demonstrated that salinomycin could effectively kill CSCs in gastric cancer [32]. Lu et al[23] showed that salinomycin inhibited Wnt signaling by inducing ionic changes that interfere with the phosphorylation of the Wnt coreceptor lipoprotein receptor-related protein 6 (LRP6) [33]. Therefore, the combination of Wnt inhibitors with trastuzumab has potential as an effective therapeutic strategy to reduce CSC activity in HER2-overexpressing gastric cancer.

My study revealed that trastuzumab-resistant gastric cancer cells exhibit EMT-like phenotype by promoting Wnt signaling pathway. Because most of cancers are heterogenous, future efforts to find new treatment option for improving patient survival will undoubtedly need to consider the plasticity of cancer cells. EMT induction and the emergence of CSCs relate to the plasticity, as well as drug resistance. Several key signaling pathways, including Wnt, which are known inducers of EMT and promoters of stem cell maintenance, contribute to this process [34].

V. CONCLUSION

I demonstrated that Wnt signaling pathway may be a target for trastuzumab-resistant gastric cancer, and Wnt signaling pathway inhibitors combined with trastuzumab may provide a promising strategy for patients with trastuzumab refractory gastric cancer. Further research is required to understand trastuzumab-resistant mechanism for individualized and precise gastric cancer treatment.

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ABSTRACT(IN KOREAN)

트라스투주맙 내성을 획득한 난치성 위암에서 암줄기양 세포를 표적으로 하는 새로운 치료 전략 수립

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정다현

배경: 트라스투주맙은 HER-2 양성 위암의 1차 치료제로 승인된 유일한 표적 약제이나 트라스투주맙 내성을 보이는 경우 위암 치료의 큰 난관이 되고 있다. 트라스투주맙 내성의 기전을 이해하기 위해 Wnt/ß-카테닌 신호전달 경로와 이것이 트라스투주맙 내성 위암 세포의 표현형 및 행동에 미치는 영향에 초점을 맞추어 연구하고자 하였다.

방법: 인간 위암 세포주 NCI-N87에 실험적으로 트라스투주맙의 용량을 증량하며 반복 처리하여 트라스투주맙 내성 세포인 NCI-N87R을 확립하였다. 그리고, Wnt 신호 전달 경로 활동을 포함하여 NCI-N87R의 표현형을 조사하였다. 게대가 위암 오르가노이드 세포를 완전배지 또는 Wnt3a 고갈배지에 함께 배양하고 트라스투주맙에 대한 내성을 비교하였다.

결과: NCI-N87R은 상피 마커인 E-cadherin의 발현 감소 및 간엽 마커인 Vimentin 및 Snail의 발현 증가와 함께 줄기세포능 및 상피-간엽 이행(EMT) 유사 표현형을 나타냈다. 그리고 Wnt 신호전달 경로를 활성화 시켰다. 트라스트주맙 내성이 없는 위암 세포를 Wnt3a 조절 배지에서 배양하였더니 Wnt 신호 경로가 활성화되고 트라스투주맙에 대한 내성이 증가하였다. 또한

위암 환자 유래 오르가노이드를 Wnt3a 고갈배지에서 배양하였을 때 완전 배지에서 배양했을때보다 트라스투주맙을 처리하였을 때 용량 의존적으로 세포 생존성이 감소하는 것을 알 수 있었다.

결론: Trastuzumab 내성 위암 세포는 상피-간엽 이행 유사 표현형을 나타내며 trastuzumab 내성은 Wnt/ß-catenin 신호 전달 경로에 의해 촉진된다. Wnt/ß-카테닌 경로는 위암 세포에서 트라스투주맙 내성에 대한 핵심 신호 경로이다.

핵심되는 말 : Wnt, 위암, 트라스트주맙, 내성, 상피-간엽 이행