An evaluation of HER2-targeting antibody-drug conjugate (ADC) sensitivity and exploration of molecular characteristics of HER2-targeting ADC sensitive groups in HER2-low/non gastric cancer cell lines

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An evaluation of HER2-targeting antibody-drug conjugate (ADC) sensitivity and exploration of molecular characteristics of HER2-targeting ADC sensitive groups in HER2-low/non gastric cancer cell lines

Directed by Professor Sun Young Rha

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Seo Young Yu

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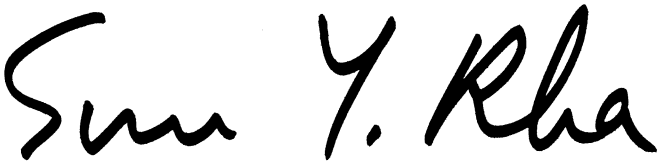
This certifies that the Master's Thesis of
Seo Young Yu is approved.



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풍부한 경험으로 저의 부족한 부분을 채워주신 김태수 선생님, 많은 조언을 주신 권우선 선생님, 처음으로 실험이 무엇인지 알려주신 영원한 사수 강선경 선생님, 학위 계획을 세우는데 앞장서주신 정인혜 선생님, 같은 실험실에서 많이 의지하고 배웠던 배현주 선생님, 서로 아끼고 살펴주며 동고동락한 황지현, 박주인 선생님, 하나밖에 없는 후배 조상우, 모든 분께 감사의 말을 전합니다.

학위 과정 동안 내 버팀목이 되어준 원진 오빠, 많은 격려와 위로를 해준 연정, 문영, 윤경, 경훈 그리고 같은 전공에서 이제 각자의 길을 걷고 있는 진송, 지현, 가은 친구 여러분께 감사의 말을 전합니다.

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ABSTRACT

An evaluation of HER2-targeting antibody-drug conjugate sensitivity and exploration of molecular characteristics of HER2-targeting antibody-drug conjugate sensitive groups in HER2-low/non gastric cancer cell lines

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(Directed by Professor Sun Young Rha)

Gastric cancer (GC) is the fifth-most diagnosed cancer and the third leading cause of cancer-related death worldwide. The 5-year survival rate for metastatic and advanced GC patients is less than 10%. Human epidermal growth factor receptor 2 (HER2) overexpression occurs in 10–30% of GC patients and such patients have lower survival rates than others. There are fewer drugs for HER2-positive GC, such as trastuzumab. Furthermore, HER2-targeted therapies do not address HER2-low, which accounts for a significant amount of GC. Antibody-drug conjugates that directly deliver anticancer drugs to tumor cells are being explored to overcome this limitation. T-DXd is a HER2-targeting antibody-drug conjugate in which an anti-HER2 antibody and DXd (DX-8951 derivative; topoisomerase I inhibitor) are conjugated with a cleavable peptide linker. In this study, we evaluated the effect of T-DXd on GC and identified the markers predicting its effect in HER2-low/non GC. The results showed that T-DXd was sensitive to HER2 overexpressing and HER2-low/non GC and that these GC cell lines had high levels of HER2 extracellular domain (ECD) expression and *MET* amplification. Thus, we concluded that T-DXd had

an antiproliferative effect on GC and that HER2 ECD expression and *MET* amplification could serve as markers for the expected anticancer effect in HER2-low/non GC.

Keywords: gastric cancer, HER2, antibody-drug conjugate, trastuzumab

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I. INTRODUCTION

Gastric cancer (GC) is the fifth-most diagnosed cancer and the third leading cause of cancer-related death worldwide[1]. Despite progress in developing methods to prevent and treat it, the prognosis of advanced and metastatic GC remains poor with a 5-year survival rate of less than 10%[2]. Human epidermal growth factor receptor 2 (HER2) is a member of the epidermal growth factor receptor family of receptor tyrosine kinases (RTK) that play critical roles in cancer development, including cell proliferation, migration, differentiation, and apoptosis[3]. These receptors have the same molecular structure, namely extracellular, transmembrane, and kinase domains, and usually exist as monomers that can be activated by HER ligands. However, HER2 has no known ligands, so it exists in an open formation causing it to form heterodimers with other RTKs and HER2-HER2 homodimers[4]. HER2 amplification leads to HER2 overexpression, which is found in many carcinomas, including breast cancer and ovarian cancer. HER2 overexpression enhances metastasis and enhances resistance to cancer therapies, or both[5]. Therefore, it is important to determine patients' for HER2 status to determine whether they may benefit from HER2-targeted therapies.

HER2 overexpression occurs in 10–30% of GC, causing GC to be more

aggressive and patients to have a shorter median overall survival than other patients[6]. Trastuzumab, pertuzumab, lapatinib, neratinib, and trastuzumab emtansine (T-DM1) are the main HER2-targeted therapies, but only trastuzumab is used in GC. Trastuzumab was the first United States Food and Drug Administration (FDA)-approved humanized anti-HER2 monoclonal antibody and showed remarkable clinical outcomes when combined with chemotherapy in HER2-positive GC patients. Trastuzumab directly binds to HER2 and degrades it, causes antibody-dependent cytotoxicity (ADCC), inhibits downstream signaling pathways, and inhibits tumor angiogenesis. The factors that affect trastuzumab mechanism are signaling through an alternative receptor pathway, trastuzumab recruitment due to the presence of HER2 extracellular domain (ECD), and HER2/HER3 heterodimerization induced by NRG1[7-10].

However, despite the effects of trastuzumab, it cannot affect HER2-low cancer, which accounts for a large proportion of GC[11]. There is an urgent need for a drug that can be used for HER2-low GC. Researchers have examined antibody-drug conjugates (ADC) for this purpose. ADCs consist of a monoclonal antibody and chemotherapeutic drugs that are bound by a peptide linker. They have a more potent anti-tumor effect than monoclonal antibody drug by delivering target-specific anticancer drugs directly to tumor cells[12].

Trastuzumab deruxtecan (T-DXd) is a HER2-targeting ADC that consists of a humanized anti-HER2 monoclonal antibody and DXd (DX-8951 derivative; topoisomerase I inhibitor) joined by a cleavable peptide linker[13]. The cleavable linker is degraded by the lysosomal enzymes, which causes the release of DXd. The DXd induces apoptosis in both target cells and neighboring cells due to high membrane permeability. Therefore, T-DXd induces cell death by apoptosis in adjacent tumor cells with low HER2 expression, which is called the ‘bystander killing effect’[14]. However, it is unknown which markers indicate effects of T-DXd in HER2-low GC.

In this study, we profiled HER2 and the factors that affect trastuzumab mechanism in a 56 GC cell line panel. T-DXd sensitivity was evaluated to determine whether it was correlated with HER2 expression levels in GC cell lines

and the molecular characteristics were of T-DXd sensitive HER2-low/non GC cell lines were identified. This study provides the drugable targets for T-DXd in HER2-low/non GC.

II. MATERIALS AND METHODS

1. Cell lines and materials

Fifty-six GC cell lines were used in this study. Three cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), 10 from the Korean Cell Line Bank (Seoul, Republic of Korea), 10 from the Japanese Cancer Research Resources Bank (Osaka, Japan), and 33 from the Songdang Institute for Cancer Research in the Yonsei University College of Medicine (Seoul, Republic of Korea) which were obtained from metastatic GC patients using ascites or pleural fluids. Cells were cultured in Eagle's minimum essential medium, Roswell Park Memorial Institute-1640 medium, or Dulbecco's modified Eagle's medium that contained 10% fetal bovine serum (Serana, Pessin, Germany), 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Lonza, Basel, Switzerland). Cultured cells were incubated at 37°C in an atmosphere with 5% CO_2. Three materials were used in this study. T-DXd and trastuzumab were provided by Daiichi Sankyo, Inc. (Tokyo, Japan) and Celltrion (Incheon, Republic of Korea), respectively. SN-38, which is a DNA topoisomerase I inhibitor and a metabolite of irinotecan, was purchased from Selleckchem (Houston, TX, USA).

2. In-house panel-targeted deep sequencing and RNA sequencing data analysis

In-house panel-targeted deep sequencing CancerMaster panel V2 data and RNA sequencing data of 56 GC cell lines were obtained from the genome database of the Songdang Institute for Cancer Research in the Yonsei University College of Medicine[15]. Copy number variants (CNV) and single nucleotide variants (SNV) were evaluated using CancerMaster data. mRNA expression levels were measured in fragments per kilobase of transcript per million mapped reads (FPKM) without normalization.

3. Cell viability assay

Drug sensitivity screening was conducted by seeding 2,000–8,000 cells into a 96-well plate and then incubating them at 37°C overnight. Then they were treated

with T-DXd, trastuzumab, and SN-38. The cells treated with T-DXd and trastuzumab were incubated for six days and those treated with SN-38 were incubated for three. After incubation, Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) solution was added and the plates were incubated at 37°C for 2–4 hr. Absorbance was measured at 450 nm by Sunrise microplate reader (Tecan, Männedorf, Switzerland), and the results were analyzed using CalcuSyn software (Biosoft, Cambridge, UK). The sensitivity of T-DXd and trastuzumab were measured by their inhibition rates at the effective doses of 10 and 200 μg/ml, respectively. SN-38 sensitivity was also calculated at a concentration of 70 nM, which is known as Cmax[16].

4. Flow cytometry

Cells were grown in growth media in a 100 mm dish until they reached the desired confluency at which point they were washed twice in ice-cold phosphate-buffered saline (PBS), treated with 0.25% trypsin/EDTA (Gibco, Gaithersburg, MD, USA) to detach the cells, and centrifuged at 400×g for 10 min. Then they were washed twice with a mixture of 0.1% BSA and 0.01% sodium azide in 1X PBS and resuspended in 0.1% BSA and 0.01% sodium azide in 1X PBS to produce 2×10^7 cells/ml. Then 5×10^5 cells in a reaction volume of 25 μl were taken from this solution and added to a 96-well plate. HER2 staining was conducted using APC anti-human CD340 (erbB2/HER-2) antibody (BioLegend, San Diego, CA, USA) and APC Mouse IgG1, κ Isotype Ctrl antibody (BioLegend) for isotype control. The antibodies were diluted at a ratio of 1:20 with 0.1% BSA and 0.01% sodium azide in 1X PBS and incubated with the cells for 30 min on ice. The cells were washed twice with 0.1% BSA and 0.01% sodium azide in 1X PBS and resuspended in 200 μl of 0.1% BSA and 0.01% sodium azide in 1X PBS for flow cytometry analysis. The cells were analyzed using an LSR II (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to measure the fluorescence signal. All data points for each cell line were normalized with respect to the data for untreated cells. SK-BR-3 (3+), ZR-75-1 (2+), MCF-7 (1+), and HCC1937 (0) breast cancer cells, which have known HER2 IHC statuses, were used to

determine the order of HER2 expression levels across the 56 GC cell lines[17,18]. As shown in Figure 2A, cell lines that had higher mean fluorescence intensities (MFI) than ZR-75-1 cells were categorized as HER2 overexpressing, those that had higher MFI than HCC1937 cells but lower MFI than ZR-75-1 cells were categorized as HER2 moderate/low-expression, and those that had lower MFI than HCC1937 cells were categorized as HER2 non-expression.

5. Immunoblot analysis

Total protein extracts were prepared with M-PER Reagent (Thermo Fisher Scientific, Waltham, MA, USA) with complete protease and phosphatase inhibitors (Roche, Basel, Switzerland). Then, 20–30 μg of proteins were separated on SDS polyacrylamide gel and transferred to a PVDF membrane (Millipore Co., Billerica, MA, USA). HER2 (ab16901) antibody was purchased from Abcam (Cambridge, UK). The EGFR (#2232) and FGFR2 (#11835) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and MET (sc-514148) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Peroxidase-conjugated anti-mouse or anti-rabbit antibodies were used as secondary antibodies. Protein blots were developed using enhanced chemiluminescent reagent (Amersham, Buckinghamshire, UK). Every target band was normalized with α-tubulin (Sigma-Aldrich, St. Louis, MO, USA) and the proteins' intensities were quantified using ImageJ software (National Institute of Health, Bethesda, MD, USA).

6. Immunofluorescence staining

Cells were cultured on a cell culture slide (SPL, Gyeonggi-do, Republic of Korea) and incubated at 37°C overnight. Then the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 1X PBS and blocked with a mixture of 0.2% BSA and 0.1% Triton X-100 in 1X PBS. Immunostaining was performed with HER2 (#2165) (Cell Signaling Technology, Inc.) antibody and MET (sc-514148) (Santa Cruz Biotechnology, Inc.) antibody at 4°C overnight, followed by incubation with Alexa Fluor 488 (111-545-144) and

594 (115-585-003) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 25°C for 1 hr. Then DAPI nuclear staining (Sigma-Aldrich) was conducted for 10 min. Confocal microscopy images (five fields) were acquired with an LSM710 (Carl Zeiss Microscopy, Oberkochen, Germany) with a X40 objective lens and analyzed using IMARIS software (Bitplane AG, Zurich, Switzerland). Analysis was conducted with the HER2 channel overlaid on the MET channel, and the percentage of HER2 that colocalized with MET was measured ± SD.

7. Proximity ligation assay

Cells were cultured on a cell culture slide (SPL) and incubated at 37°C overnight. Then cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 1X PBS. Proximity ligation assay (PLA) was performed using the Duolink PLA Starter Kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Confocal microscopy images (Texas red and DAPI signal) were acquired with an LSM710 (Carl Zeiss Microscopy) with a X40 objective lens. Image analysis was performed using ZEN software (Carl Zeiss Microscopy). The mean PLA signal/cell intensity ± SD from at least 50 cells in each group were calculated.

8. Conditioned media collection and detection of secreted NRG1

In order to investigate NRG1 in cell culture supernatants, 10^6 cells were seeded into a 6-well plate and incubated at 37°C overnight. Then they were washed twice in an unsupplemented growth medium and serum-starved in 1 ml of medium for 24 hr after which the culture supernatants were collected. The concentration of NRG1 in the cell culture supernatants was measured using human NRG1-beta 1 ELISA (RayBiotech Life, Inc., Peachtree Corners, GA, USA) according to the manufacturer's instructions. First, each standard and sample was added to a 96-well plate and incubated at 25°C for 2.5 hr. The plate was washed, biotinylated antibody was added, and the solution was incubated at 25°C for 1 hr. The plate was washed again, streptavidin solution was added, and the solution was incubated

at 25°C for 45 min. The plate was washed again, TMB reagent was added, and the solution was incubated at 25°C for 30 min in the dark. Finally, reactions were stopped by adding a stop solution and the absorbance was read at 450 nm. The standard curve of NRG1 was plotted using the log/log method. We defined NRG1 levels >300 pg/ml as overexpression following Ogier et al[19].

9. Detection of HER2 shedding

HER2 ECD shedding analysis was conducted by first seeding 10^6 cells into a 6-well plate and incubating them at 37°C overnight. Then they were washed in ice-cold PBS and refreshed with 1.5 ml of the appropriate growth medium. After 72 hr, culture supernatants were collected. HER2 ECD was detected in the cell culture supernatants using a human ErbB2/Her2 quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. First, assay diluent RD1W was added into 96-well plate and the standard and samples were added and incubated at 25°C for 2 hr. Then the plate was washed, Ag conjugate was added, and the solution was incubated at 25°C for 2 hr. The plate was washed again, a substrate solution was added, and the solution was incubated at 25°C for 30 min in the dark. Finally, reactions were stopped by adding a stop solution and the absorbance was read at 450 nm. The background absorbance measurement of 570 nm was subtracted from the 450 nm readings. The standard curve of HER2 ECD was plotted using the linear method.

10. Statistical analysis

Analyses were conducted using IBM SPSS version 25 software (IBM, Armonk, NY, USA). Data were analyzed using an unpaired Student's t-test. Pearson's correlation analysis was performed to determine the correlation between T-DXd sensitivity and trastuzumab sensitivity, between T-DXd sensitivity and SN-38 sensitivity, and between T-DXd sensitivity and HER2 expression level. Differences were defined as statistically significant if $p<0.05$.

III. RESULTS

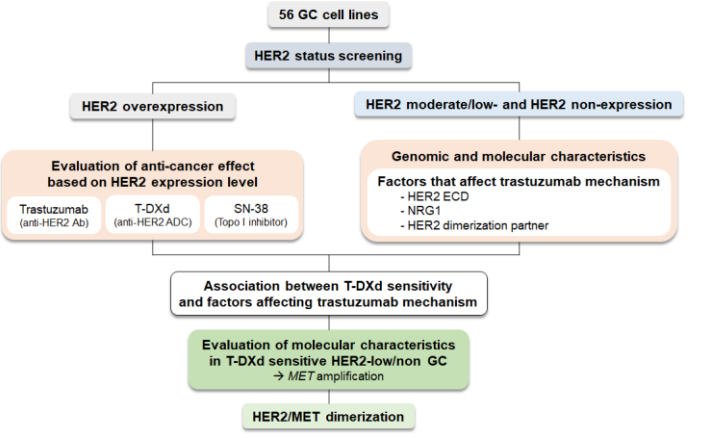


Figure 1. Experimental design.

We profiled HER2 expression in 56 GC cell lines and classified them as HER2 overexpressing, HER2 moderate/low-expressing, or HER2 non-expressing group (Figure 1). Each group's drug sensitivity and the expression levels of factors affecting the trastuzumab mechanism were evaluated. Then we identified which of these factors also affected T-DXd sensitivity in HER2 moderate/low-expressing, and HER2 non-expressing groups. The results showed that *MET* amplification was correlated with T-DXd sensitivity and that the association of HER2 and MET were confirmed by immunofluorescence and PLA.

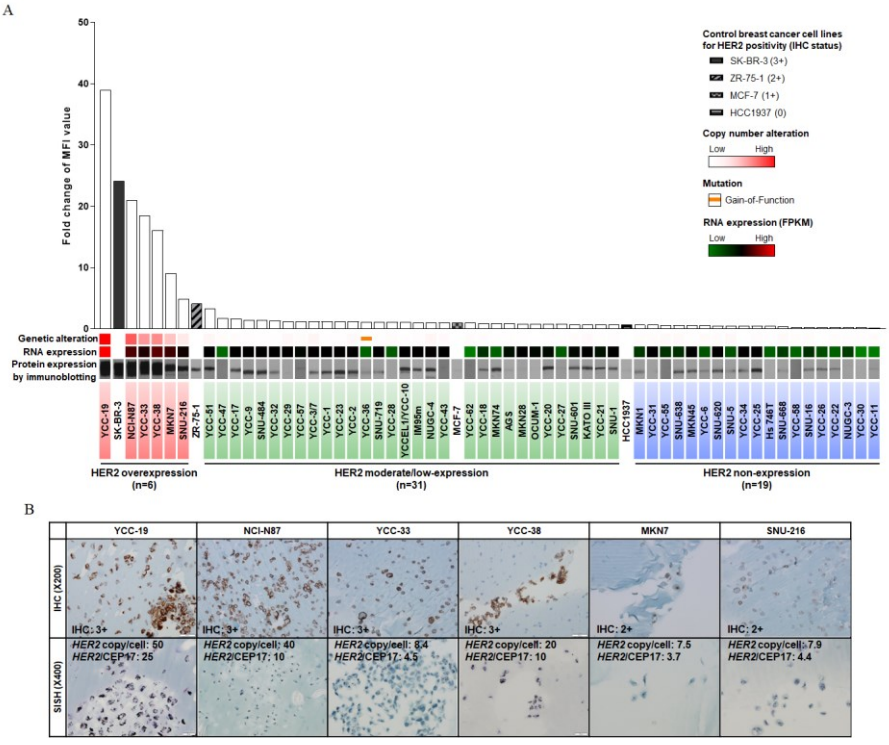


Figure 2. Integrated HER2 status profiles. A. Integrated HER2 expression level profiles were generated for 56 gastric cancer cell lines. Of the 56 gastric cancer cell lines, 6 were classified as HER2 overexpression, 31 as HER2 moderate/low-expression, and 19 as HER2 non-expression. B. HER2 IHC and SISH status of HER2 amplified/overexpressed cell lines.

HER2 expression levels in each of the 56 GC cell lines were determined in several ways (Figure 2A). First, IHC was performed, which showed that 47 cell lines were HER2-negative (HER2 1+ ~ 0), 4 cell lines were HER2 2+, and 5 cell lines were IHC 3+. SISH was conducted on the HER2 IHC 2+ and 3+ cell lines to determine whether *HER2* genes had been amplified (Figure 2B). The results showed that 6 cell lines, namely YCC-19, NCI-N87, YCC-33, YCC-38, MKN7, and SNU-216, were HER2 amplified/overexpressed and 2 HER2 IHC 2+ cell lines, namely YCC-29 and NUGC-4, were SISH-negative (data not shown).

Comprehensive analysis of all of the experimental results showed that 6 of the cell lines were determined as HER2 overexpressing. However, the tests gave differing results for the other 50 cell lines. Therefore, HER2 expression levels were determined by flow cytometry analysis because HER2 is an RTK that locates and functions on the cell surface. The results showed that 6 cell lines were classified as HER2 overexpressing, 31 were as HER2 moderate/low-expressing, and 19 were as HER2 non-expressing. All experiments identified the same cell lines as HER2 overexpressing.

Detecting genetic variants of HER2 using CancerMaster data showed that 1/56 (1.79%) cell line, namely YCC-36, had a V842I mutation. According to cBioPortal database (https://www.cbioportal.org/), the V842I variant is an activating HER2 mutation. It is found in several types of cancer, including colorectal cancer, endometrial cancer, and GC[20,21]. Although the copy number of YCC-36 cells was not higher that of NUGC-4 cells, their HER2 expression levels were. In summary, we determined that 6 GC cell lines were HER2 overexpressing, 31 were HER2 moderate/low-expressing, and 19 were HER2 non-expressing.

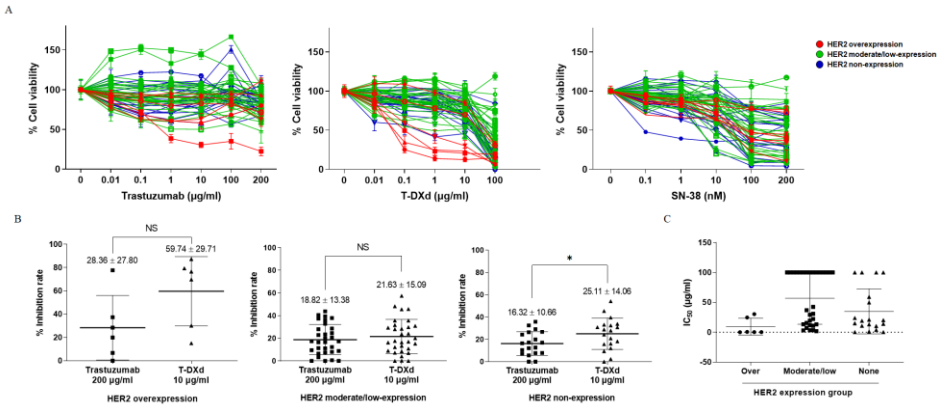


Figure 3. Drug sensitivity of 56 gastric cancer cell lines. A. The dose response curve for HER2 overexpressing cell lines is shown in red, for HER2 moderate/low-expressing cell lines in green, and for HER2 non-expressing cell lines in blue. B. Dot plots of the drug inhibition rate for each HER2 expression group when treated with 200 μg/ml of trastuzumab and 10 μg/ml of T-DXd. C. Dot plots represent IC_{50} values of T-DXd for each cell line. * p<0.05.

We evaluated the antiproliferative effects of trastuzumab, T-DXd, and SN-38 by HER2 expression group (Figure 3A). Trastuzumab and T-DXd showed cytostatic and cytotoxic effects, respectively. Regardless of the HER2 expression level, SN-38, topoisomerase I inhibitor, showed cytotoxic effects. T-DXd inhibited cell proliferation at lower concentrations than trastuzumab. In the HER2 overexpressing group, 4/6 (66.7%) cell lines were sensitive to trastuzumab and 5/6 (83.3%) cell lines were sensitive to T-DXd (Figure 3B). Based on the inhibition rate at 200 μg/ml of trastuzumab and 10 μg/ml of T-DXd, in the HER2 moderate/low-expressing group, 14/31 (45.2%) cell lines were sensitive to trastuzumab and 15/31 (48.4%) cell lines were sensitive to T-DXd. In the HER2 non-expressing group, 7/19 (36.8%) cell lines were sensitive to trastuzumab and 12/19 (63.2%) cell lines were sensitive to T-DXd.

The IC_{50} value of trastuzumab was only calculated for NCI-N87 (data not shown). IC_{50} values of T-DXd can be calculated because it shown a cytotoxic

effect (Figure 3C). Based on the 122 μg/ml of T-DXd, which was determined to be a clinically achievable concentration, 37/56 (66.1%) cell lines were sensitive (IC_{50} range: 0.08–59.13 μg/ml), and 19/56 (33.9%) cell lines were resistant (IC_{50}≥100 μg/ml).

Taken together, these results showed that the HER2 overexpressing group was sensitive to T-DXd. Interestingly, 15/19 (78.9%) cell lines in the HER2 non-expressing group (IC_{50} range: 0.15–59.13 μg/ml) were sensitive to T-DXd, which was higher percentage than the 16/31 (51.6%) cell lines in the HER2 moderate/low-expressing group (IC_{50} range: 1.67–42.35 μg/ml).

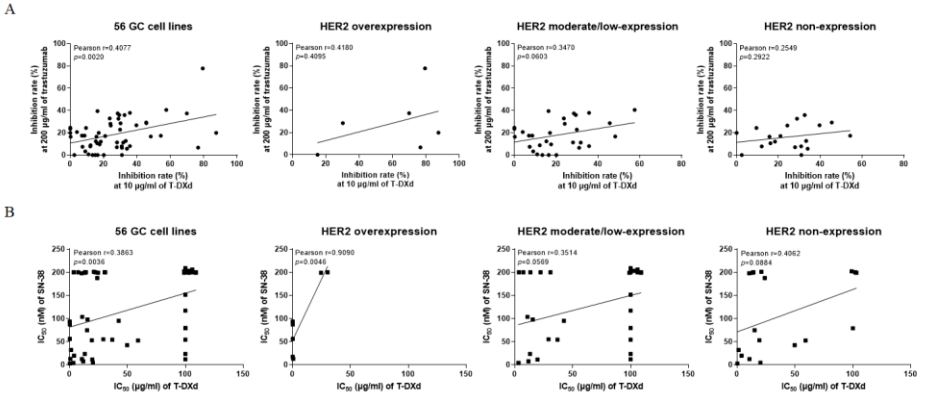


Figure 4. The association between T-DXd sensitivity with trastuzumab and SN-38 sensitivity. Dot plots represent each cell line for the association between T-DXd sensitivity and trastuzumab (A) and SN-38 (B) sensitivity in a 56 gastric cancer cell panel and each HER2 expression group.

We tested whether there was an association between T-DXd sensitivity and trastuzumab sensitivity and between T-DXd sensitivity and SN-38 sensitivity in a 56 GC cell line panel and each HER2 expression group. T-DXd sensitivity was only showed a trend following trastuzumab sensitivity in the HER2 moderate/low-expressing group (Figure 4A). However, T-DXd sensitivity was tended to follow SN-38 sensitivity in all HER2 expression groups (Figure 4B). These results indicated that T-DXd sensitivity dependent on both HER2 expression levels and effects of DXd.

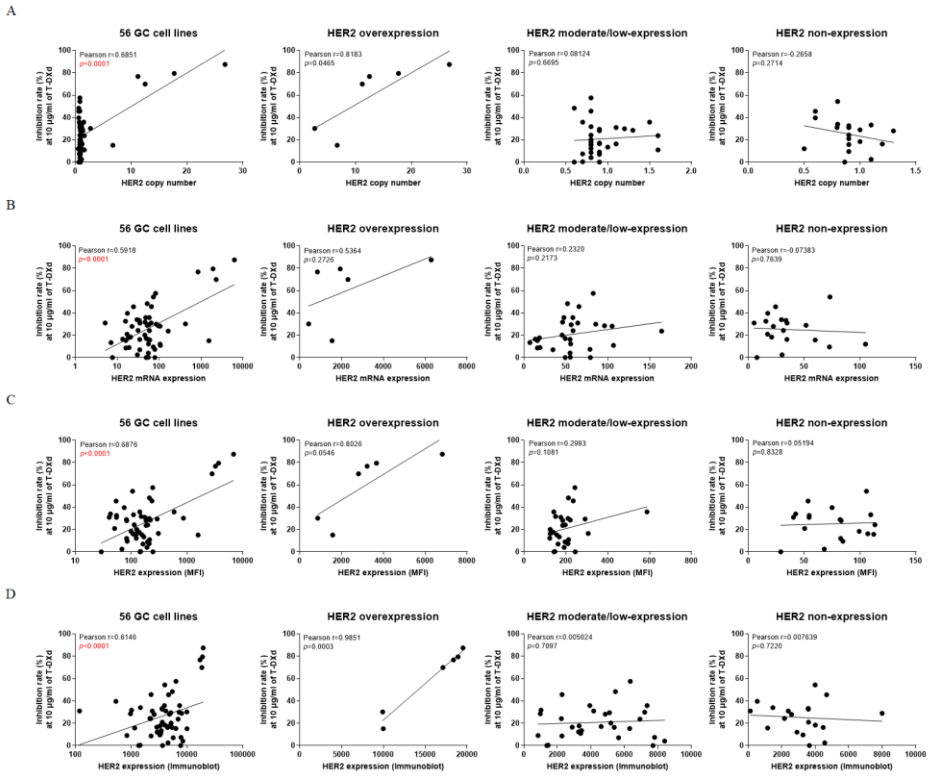


Figure 5. Association between HER2 expression level and T-DXd sensitivity. HER2 expression levels as measured by (A) targeted sequencing for copy number variation (CNV), (B) RNA-Seq to determine mRNA expression levels, (C) flow cytometry analysis, and (D) immunoblot to determine protein expression levels. All data represent in a 56 gastric cancer cell line panel and each HER2 expression group.

We identified the association between HER2 expression levels, as measured by DNA, RNA, and protein levels, and T-DXd sensitivity. (Figure 5). T-DXd was statistically significantly correlated with HER2 expression levels as measured by DNA, RNA, and protein levels. In the HER2 overexpressing group, T-DXd sensitivity was positively correlated with HER2 expression levels as determined by copy number and protein levels. In the HER2 moderate/low-expressing group,

T-DXd was not correlated with HER2 expression levels. In the HER2 non-expressing group, T-DXd was not correlated with HER2 expression levels. These results indicated that T-DXd sensitivity was depended on HER2 expression levels as measured by flow cytometry analysis in the HER2 moderate/low-expressing group.

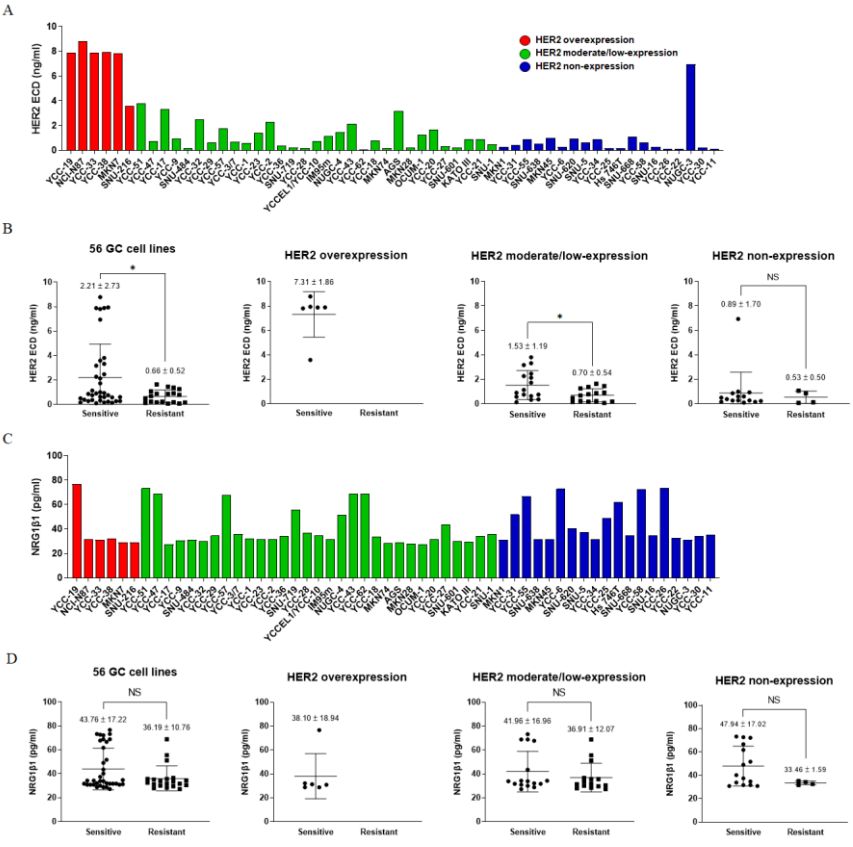


Figure 6. HER2 ECD and NRG1 expression levels. A, C. Bar plots presenting HER2 ECD and NRG1 expression levels in the HER2 overexpressing group indicated by red, the HER2 moderate/low-expressing group indicated by green, and the HER2 non-expressing group indicated by blue. The results are displayed in decreasing order of HER2 expression level. B, D. Dot plots presenting HER2 ECD and NRG1 expression levels according to T-DXd sensitivity for each HER2 expression group. * p<0.05.

To determine whether factors that affect trastuzumab mechanism affected the antitumor effect of T-DXd, we measured HER2 ECD and NRG1 expression levels by ELISA. The results showed that HER2 ECD expression levels were correlated with T-DXd sensitivity. HER2 ECD was the most expressed in HER2

overexpressing group, followed in decreasing order of magnitude by the HER2 moderate/low-expressing and HER2 non-expressing groups (Figure 6A). The NUGC-3 cell line had the third-lowest HER2 expression levels, but expressed as many HER2 ECD as of the cell lines in the HER2 overexpressing group.

Then, we compared HER2 ECD expression levels according to T-DXd sensitivity in each HER2 expression group. The cell lines that had high HER2 ECD expression levels in the HER2 moderate/low-expressing group were relatively sensitive to T-DXd (Figure 6B). However, in the HER2 non-expressing group, HER2 ECD expression levels were not statistically significantly correlated with T-DXd sensitivity. NRG1 expression levels did not vary significantly between cell lines, but they were highest in the HER2 non-expressing group, followed in decreasing order of magnitude by the HER2 moderate/low-expressing and HER2 overexpressing groups (Figure 6C). NRG1 expression was not correlated with T-DXd sensitivity (Figure 6D). In summary, HER2 ECD was highly expressed in the HER2 overexpressing group and the NUGC-3 cell line and the cell lines that had high HER2 ECD expression levels were sensitive to T-DXd (IC_{50} = 0.20 μg/ml). However, T-DXd sensitivity was not correlated with NRG1 expression levels.

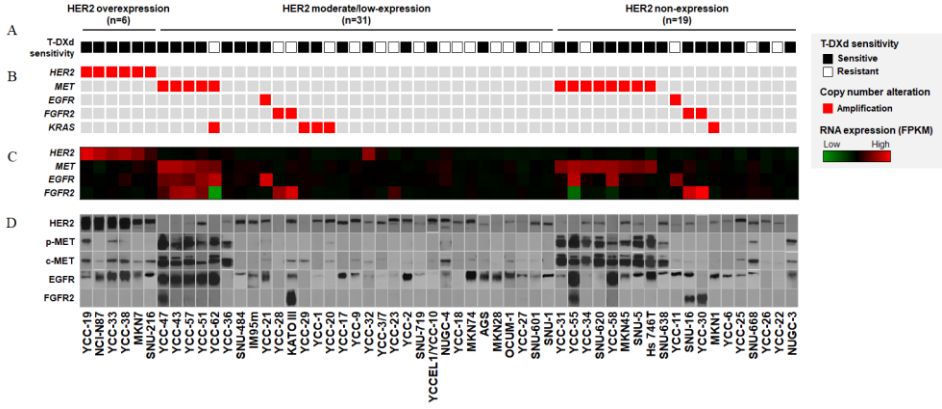
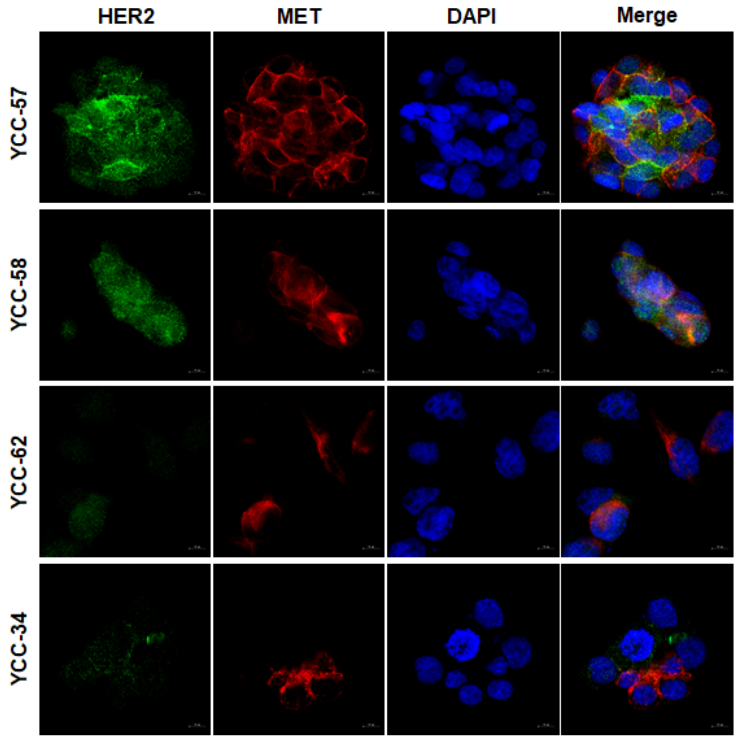


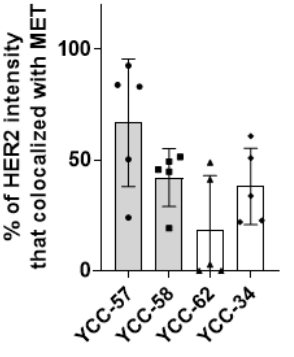
Figure 7. T-DXd sensitivity in RTK and *KRAS* amplified cell lines. A. T-DXd sensitivity was presented in IC_{50}. B. Copy number alterations for *HER2*, *MET*, *EGFR*, *FGFR2*, and *KRAS* were determined by analyzing CancerMaster data. C. mRNA expression was determined by analyzing RNA sequencing data. D. Protein expression levels were determined by immunoblotting.

Another factor that we hypothesized would affect trastuzumab mechanism was the dimerization partner receptors. We thought that they might act with HER2 receptors because T-DXd was sensitive to the cell lines in the HER2 moderate/low-expressing and the HER2 non-expressing groups. T-DXd sensitivity was shown to identify its effect in RTK and *RAS* amplified cell lines (Figure 7A). *MET*, *EGFR*, *FGFR2*, and *KRAS* were amplified in 23.21% (13/56), 3.57% (2/56), 7.14% (4/56), and 8.93% (5/56), respectively. (Figure 7B). RTK and *RAS* amplifications were mutually exclusive in 56 GC cell lines. RNA and protein expression levels were showed a similar pattern (Figure 7C and D). T-DXd sensitivity was observed in 11/13 (84.6%) of *MET* amplified cell lines, 1/2 (50.0%) of *EGFR* amplified cell lines, 2/4 (50.0%) of *FGFR2* amplified cell lines, and 3/5 (60.0%) of *KRAS* amplified cell lines. However, 7 of *MET* amplified cell lines belonged to the HER2 non-expressing group and were sensitive to T-DXd. In summary, most *MET* amplified cell lines were sensitive to T-DXd except for those in the HER2 overexpressing group.

A



B



	YCC-57	YCC-58	YCC-62	YCC-34
HER2	0.1	-0.2	0.1	-0.5
MET	4.3	4.2	3.1	4.2

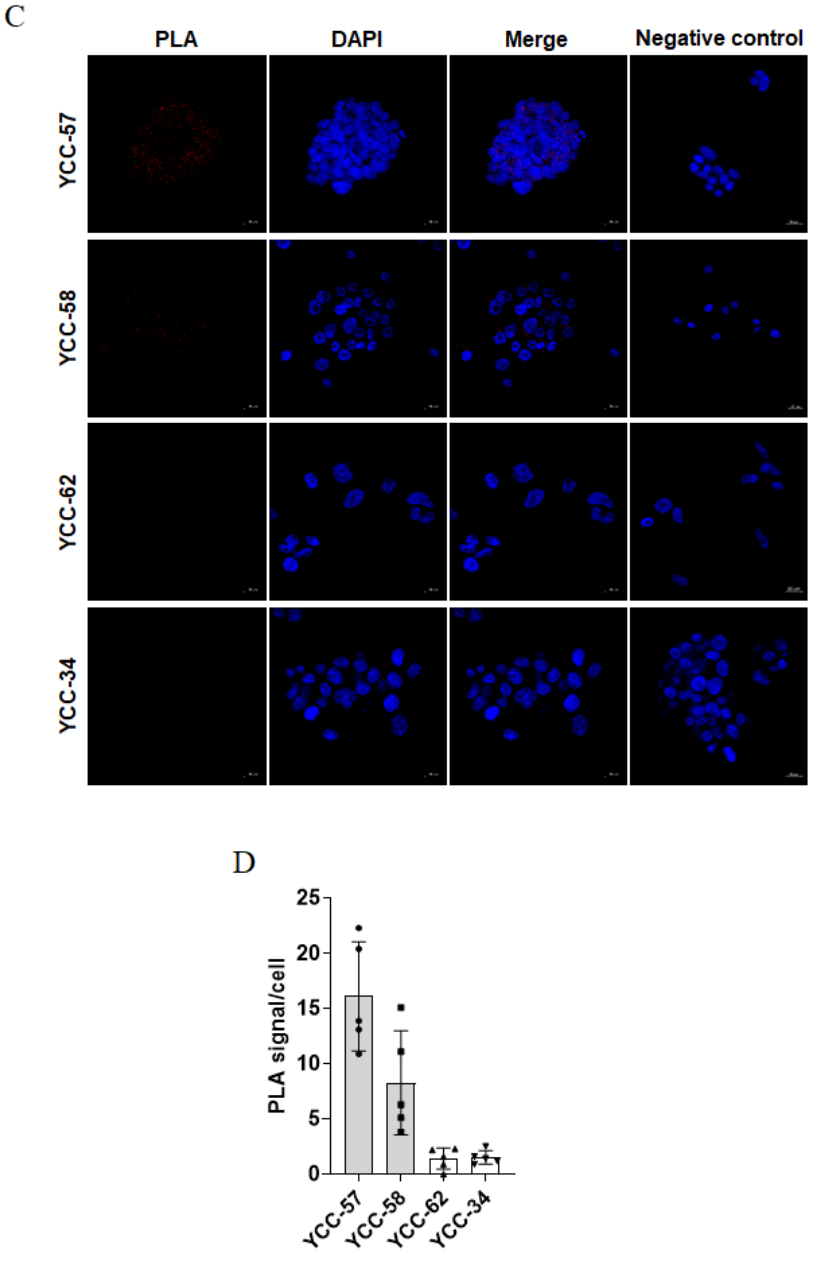


Figure 8. Interaction of HER2 and MET in *MET* amplified cell lines. A. Confocal images of immunofluorescence for HER2 (green), MET (red), DAPI (blue), and merge in YCC-57, YCC-58, YCC-62, and YCC-34 cells. Scale bars represent 10 μm. B. (Left) HER2 intensities colocalized with MET were measured

in T-DXd sensitive cell lines, represented by grey bars, and resistant cell lines, represented by white bars. (Right) Copy numbers of *HER2* and *MET*, which were $\log_2$ converted. C. Confocal images for HER2/MET dimerization were obtained by PLA in YCC-57, YCC-58, YCC-62, and YCC-34 cells. Scale bars: 20 μm. D. PLA signals calculated by the number of cells (at least >50) for in T-DXd sensitive cell lines, represented by grey bars, and resistant cell lines, represented by white bars.

We hypothesized that *MET* amplified cell lines with low HER2 expression levels were sensitive to T-DXd was due to T-DXd internalization caused by HER2/MET dimerization. T-DXd sensitive (YCC-57 and YCC-58) and T-DXd resistant (YCC-62 and YCC-34) cell lines from the HER2 moderate/low-expressing and HER2 non-expressing groups were selected to identify what we hypothesized. Before determining whether our hypothesis was supported, we first determined that it dimerization was possible by identifying the position of both receptors through immunofluorescence (Figure 8A). The results showed that HER2 and MET were located in the membranes of T-DXd sensitive cell lines, but only MET was in the T-DXd resistant cell lines while HER2 was located in the cytoplasm. We quantified HER2 intensities that was colocalized with MET. The results showed that colocalization was higher in T-DXd sensitive cell lines than T-DXd resistant cell lines (Figure 8B).

Then, we characterized HER2/MET dimerization by PLA. PLA signals, which represented HER2/MET dimerization, were detected in all four cell lines, but dimerization was the highest in the YCC-57 cell line and second-highest in the YCC-58 cell line (Figure 8C). The intensities of PLA signal were also stronger in T-DXd sensitive cell lines than T-DXd resistant cell lines (Figure 8D). In summary, T-DXd sensitive cell lines had more HER2 in their membranes, and a higher the HER2/MET dimerization ratio than T-DXd resistant cell lines.

IV. DISCUSSION

Targeting HER2 is a promising treatment option for gastric cancer GC because it plays a pivotal role in the growth and progression of HER2-positive GC[22]. The ToGA trials demonstrated that trastuzumab was the first biological therapy that had clinical benefits in advanced HER2-positive GC[23]. However, T-DM1 was the first FDA-approved ADC, but it failed clinical trials in HER2-positive GC and was tried in HER2-low breast cancer since ADC has a bystander killing effect, but the overall outcome was poor[24,25]. There are limited drugs available for GC generally and even fewer for HER2-low GC in particular. Thus, it is important to develop ADCs that deliver anticancer drugs to tumor cells that are not limited by existing drugs' shortcomings and to identify markers to predict patient response.

In this study, we classified 56 GC cell lines according to their HER2 expression level and analyzed the molecular characteristics of T-DXd sensitive HER2 moderate/low-expressing and HER2 non-expressing GC cell lines. The cell line model system was used to predict clinical responses. There have been many studies on HER2-positive cell lines in breast cancer research, but much fewer in GC research[26-30]. Thus, this study can be used to further characterize GC according to HER2 expression level and to discover novel T-DXd targets.

T-DXd is an ADC in which an anti-HER2 antibody and DXd are joined via a cleavable linker. Trastuzumab and SN-38 were tested together in 56 GC cell lines to compare their effect to that of anti-HER2 antibody and topoisomerase I inhibitor separately. Like other antibody drugs, trastuzumab exhibits cytostatic activity, such as inhibiting HER2 signaling, preventing ECD cleavage, and inhibiting angiogenesis in preclinical study[31]. However, in this study T-DXd showed a greater antitumor effect than conventional HER2-targeted therapies for GCs with all levels of HER2 expression. It was also cytotoxic effect, indicating that DXd plays a pivotal role in the antiproliferative effect of T-DXd. The HER2 moderate/low-expressing group was somewhat depend on HER2 expression levels and sensitive to DXd. DXd appeared to have been delivered to cells by an anti-HER2 antibody where it directly damaged cells in HER2 non-expressing group. However, it is not clear why the HER2 moderate/low-expressing group had

a lower proportion of T-DXd sensitive cell lines than HER2 non-expressing group.

HER2 ECD is highly expressed in HER2 overexpressing breast cancers[32,33]. In this study, it was highly expressed in the HER2 overexpressing group. Notably, NUGC-3 cell line from the HER2 non-expressing group expressed as many HER2 ECD as the HER2 overexpressing group and were also sensitive to T-DXd. Some studies have found that HER2 ECD recruits trastuzumab and increases susceptibility to trastuzumab[34]. T-DXd also has an anti-HER2 antibody, so it induced cell death in the cell lines that had high HER2 ECD expression. However, NUGC-3 cell line was HER2-negative with an IHC status of 0 and RNA levels, flow cytometry, and immunoblotting showed that they had low HER2 expression levels. Additional studies are needed to determine why HER2 ECD expression levels are higher in cell lines with low HER2 expression levels.

NRG1 is a well-known trastuzumab resistance mechanism that induces HER2/HER3 heterodimerization. It was universally expressed in 56 GC cell lines examined in this study and was not correlated with antitumor effect of T-DXd. NRG gene fusion was not detected and flow cytometry analysis showed that HER3 was expressed in all cell lines (data not shown).

In this study, we evaluated *MET*, *EGFR*, *FGFR2*, and *KRAS* amplification and found that 11 of 13 *MET* amplified cell lines were sensitive to T-DXd. Although HER2/MET dimerization did not occur frequently, the *MET* gene (N375S) mutation in the sema domain enables MET to interact with HER2 to form heterodimers[35]. The SNU-638 cell line was MET overexpressing and had the N375S variant and were shown to be sensitive to T-DXd. We determined whether HER2 and MET formed heterodimers in the *MET* amplified cell lines, although they had not N375S variant. The results showed that HER2 and MET were located in the membranes of the T-DXd sensitive cell lines (YCC-57 and YCC-58) and PLA analysis showed that they had higher PLA signals than the T-DXd resistant cell lines (YCC-62 and YCC-34). Flow cytometry analysis showed that the YCC-62 cell line had a higher MFI than the YCC-58 cell line but the *MET* copy number was higher in the YCC-58 cell line than the YCC-62 cell line. However, these results cannot be compared because they were obtained using different

experimental methods, so the mechanisms behind how HER2 and MET interact should be conducted. Furthermore, although HER2/MET dimerization was observed in *MET* amplified cell lines, further research is needed to determine whether T-DXd has an antitumor effect in them.

This was the first known attempt to profile HER2 expression levels in GC cell lines. This study will be helpful in further studies related to HER2 in GC. We showed that T-DXd has an antiproliferative effect in GC and that HER2 ECD expression levels and *MET* amplification are candidate markers for determining the effect of T-DXd in HER2 moderate/low-expressing and HER2 non-expressing GC. However, further research should be conducted to understand the mechanisms by which they are related to T-DXd treatment. This study provides helpful preclinical information that can be used in future studies on using T-DXd in GC.

V. CONCLUSION

Our results indicated that T-DXd has greater antiproliferative effect on GC than trastuzumab and that HER2 ECD expression levels and *MET* amplification may be able to serve as predictive markers for the effect of T-DXd in GC.

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ABSTRACT (IN KOREAN)

HER2-low/non 위암 세포주에서 HER2 표적화 항체-약물결합체의 감수성 평가 및 민감 그룹의 분자생물학적인 특성 탐색

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유 서 영

위암은 전세계적으로 다섯 번째로 높은 발병률을 나타내며 암으로 인한 사망의 주 원인이다. 전이성 또는 진행성 위암 환자의 5년 생존율은 10% 미만이며 예후가 좋지 않으며, 그 중 HER2 증폭/과발현은 위암에서 약 10-30%로 전체생존기간이 짧으며, 유방암에 비해 HER2 양성 위암에 사용할 수 있는 약물도 제한적이다. 또한, 현재 HER2 표적 치료법은 위암에서 많은 부분을 차지하는 HER2 저발현에는 사용할 수 없다. 이러한 치료상의 한계를 극복하기 위해, 종양 세포에 항암제를 전달하는 항체-약물 결합체 (antibody-drug conjugates; ADC)가 주목을 받고 있다. T-DXd는 항 HER2 항체와 DXd (DX-8951 유도체; topoisomerase I inhibitor)가 분해 가능한 펩타이드 링커를 통해 결합된 HER2 표적화 항체-약물 결합체다. 따라서, 본 연구에서는 위암에서 T-DXd의 항종양 효과를 평가하고 HER2-low/non 위암에서 약제의 효과를 대변할 수 있는 마커를 탐구하였다. 그 결과, T-DXd는 HER2 과발현 그룹은 물론 HER2-low/non 발현 그룹에서도 감수성을 가졌으며, 이들 세포주가 HER2 extracellular domain (ECD)을 많이 발현하거나 *MET* 증폭이 있다는 것을 확인했다. 그러므로 우리는 T-DXd가

위암에서 항종양 효과가 있으며, HER2 ECD 발현과 *MET* 증폭은 HER2-low/non 위암에서 T-DXd 효과를 예측할 수 있는 마커로써 가능성이 있음을 제시하고자 한다.

핵심되는 말: 위암, HER2, 항체-약물 결합체, trastuzumab