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이학석사학위논문

**A synergistic interaction between  
human epidermal growth factor  
receptor 2/neu and c-Jun N-terminal  
kinase promotes gastric cancer cell  
migration and invasion**

Human epidermal growth factor receptor 2/neu와  
c-Jun N-terminal kinase의 상호작용이 위암세포의  
이동과 침윤에 미치는 영향

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서울대학교 대학원

의학과 (협) 중앙생물학 전공

최이슬

# **A synergistic interaction between human epidermal growth factor receptor 2/neu and c-Jun N-terminal kinase promotes gastric cancer cell migration and invasion**

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지도교수 이 병 란

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서울대학교 대학원  
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최 이 슬

최이슬의 의학박사 학위논문을 인준함.  
2016년 1월

위원장	<u>김우호</u>	(인)
부위원장	<u>이병란</u>	(인)
위원	<u>전양숙</u>	(인)

## Abstract

# A synergistic interaction between human epidermal growth factor receptor 2/neu and c-Jun N-terminal kinase promotes gastric cancer cell migration and invasion

Yiseul Choi

Department of Cancer Biology

College of Medicine

Seoul National University

**Purpose:** Human epidermal growth factor receptor2 (HER2) is a crucial regulator of tumor progression, but the underlying molecular mechanisms remain unclear. Recent studies reported the association between HER2 and c-Jun N-terminal kinase (JNK) in breast cancer; however, little is studied in gastric cancer (GC). The present study investigated the relationship between HER2 and JNK in relation to the metastatic potential in GC cells.

**Methods:** HER2-overexpressing human GC cell lines SNU-216 and NCI-N87 were used. JNK activation was suppressed by treatment with SP600125 and

HER2 expression was silenced by RNA interference. Western blot and semi-quantitative reverse transcription-PCR were used to detect the expressions of pHER2, HER2, pJNK, JNK and epithelial mesenchymal transition (EMT) markers. Cell growth was determined by crystal violet assay. Cell migration and invasion were assessed by a Transwell assay.

**Results:** In both GC cell lines, pharmacological inhibition of JNK using SP600125 reduced cancer cell growth, migration, invasion and actin cytoskeleton organization. It also upregulated E-cadherin and downregulated Snail and Vimentin. HER2 silencing by lentivirus-mediated HER2 shRNA transfection blocked JNK activation. On the other hand, JNK inhibition reduced HER2 expression at the protein and mRNA levels in GC cells. Moreover, JNK inhibition in HER2-silenced GC cells induced further decrease in GC cell growth, migration and invasion compared to HER2 silencing alone.

**Conclusions:** The interaction between HER2 and JNK synergistically contributes to the GC cell growth and metastatic potential in HER2-overexpressing GC cells. Thus, JNK may be an attractive target for the treatment of GC patients with a HER2-overexpressing GC.

**Keywords:** gastric cancer; HER2; JNK; metastatic potential; EMT

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## List of Abbreviations

DMSO	Dimethylsulfoxide
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GC	Gastric cancer
HER2	Human epidermal growth factor receptor 2/neu
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
mRNA	Messenger RNA
PBS	phosphoate-buffered saline
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA

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## **Introduction**

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer death worldwide. Cases of GC in Eastern Asia account for half of the world total, and the highest estimated mortality rates are reported in those countries (Oh et al. 2015). Although metastasis is the main cause of the poor outcome of GC patients, the underlying molecular mechanisms responsible for GC metastasis still remain unclear.

Human epidermal growth factor receptor 2 (HER2/neu/ERBB2) is a member of the epidermal growth factor receptor family of receptor tyrosine kinases (Matsui et al. 2005). HER2 is overexpressed in 6-35% of GC cases (Lin et al. 2000) and in 15-59% of advanced GC cases (Bang. 2012). Since previous studies demonstrated that HER2 overexpression contributes to tumor formation and progression of GC (Ko et al. 2015; Janjigian et al. 2015), targeting HER2 combined with chemotherapy has

been the first-line treatment for HER2-overexpressing advanced gastric cancer (Bang et al. 2010). However, chronic exposure to anti-HER2 therapy eventually develop acquired resistance (Kim et al. 2014), indicating the importance of elucidating the molecular mechanism underlying this resistance in HER2-overexpressing GC.

The c-Jun N-terminal kinases (JNK)/stress-activated protein kinase (SAPK) belong to the mitogen-activated protein kinase (MAPK) family (Xiao et al. 2012). JNK is activated by phosphorylation at Thr183 and Tyr185 by the upstream MAP2K protein kinases, and can translocate to the nucleus followed by regulation of transcription factors (Kyriakis and Avruch. 2001). Since JNK is involved in a diverse range of tumor-related functions, including proliferation, survival, tumorigenesis and metastasis (Bubici and Papa. 2014), the JNK signaling mechanisms in cancer cells is the subject of the intense ongoing research. Aberrant expression and activation of JNK have been found in a variety of different cancers,

including prostate cancer (Vivanco et al. 2007), hepatocarcinoma (Hui et al. 2008), breast cancer (Han and Crowe. 2010) and esophageal squamous cell carcinoma (Qin et al. 2014). However, the role of JNK in tumor development and progression has been inconsistent depending on the cancer cell type and cell context (Khatalani et al. 2007; Ono et al. 2008; Wang et al. 2010; Wei et al. 2013; Chen et al. 2015; Sahu et al. 2015).

A previous study (Han and Creowe. 2010) revealed an association between HER2 and JNK in HER2-overexpressing breast cancer cells with respect to cancer cell proliferation and survival; however, there has been no such report on this association for any other cancer. In the present study, we extended previous studies to confirm the role of JNK in the metastatic potential in HER2-overexpressing GC cells (SNU-216 and NCI-N87) and investigated the association between HER2 and JNK in relation to cancer cell migration and invasion.

## **Materials and methods**

### **Cell culture**

Human GC cell lines SNU-216 and NCI-N87 were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mg/mL sodium bicarbonate, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technoloiges) at 37 °C in a humidified 95% air and 5 % CO<sub>2</sub> atmosphere.

### **Pharmacological inhibition of JNK**

Cells were seeded and allowed to attach for 24 h. To inhibit endogenous JNK activity, cancer cells were treated with the indicated concentrations of a specific JNK inhibitor SP600125 (Cell Signaling Technology, Beverly, MA, USA), dissolved in dimethylsulfoxide (DMSO).

## **Western blot**

Cell lysates were prepared in 100-200  $\mu$ L of 1 x sodium dodecyl sulfate (SDS) lysis buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 0.004% bromophenol blue, and 20% glycerol]. Protein contents were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated on an 8% discontinuous SDS-polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA) blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS)-Tween 20 (0.1%, v/v) for 1 h. The membranes were then incubated at 4°C overnight. The primary antibodies used were against phospho-JNKThr183/Tyr185 (1:1000, Cell Signaling Technology), JNK (1:1000, Cell Signaling Technology), phospho-HER2 Tyr1221/1222 (1:1000, Cell Signaling Technology), HER2 (1:1000, Cell Signaling Technology), E-cadherin (1:1000, BD Biosciences, San Jose, USA), Snail (1:1000, Santa Cruz Biotechnology), Vimentin (1:1000, Neomarkers) and  $\beta$ -actin (1:1000, Santa Cruz Biotechnology). Horse-radish peroxidase-conjugated anti-rabbit IgG (1:4000, Santa Cruz Biotechnology) or anti-mouse IgG (1:4000,

Santa Cruz Biotechnology) was used as a secondary antibody. Enhanced chemiluminescence was used to detect the immunoreactive proteins. Equal protein loading was confirmed by  $\beta$ -actin.

### **Assessment of cell growth**

SNU-216 ( $1 \times 10^4$  cells/each well) and NCI-N87 cells ( $5 \times 10^4$  cells/each well) were seeded into 24-well plates and were allowed to grow for 48 h. Cell numbers were measured indirectly using the method reported by Kim et al. (Kim et al. 1994). Briefly, cells were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min, dissolved in 10% SDS, transferred into 96-well plates, and the absorbance was measured at 570 nm using an ELISA reader (Bio-Rad, Hercules, CA, USA).

### **Cell invasion and migration assay**

A 24-well Insert System with an 8  $\mu$ m pore size polyethylene terephthalate membrane was purchased from BD Biosciences. Transwell inserts were coated

with Matrigel, followed by rehydration with medium for 2 h. Ten percent FBS-containing medium was placed in the lower chambers to be used as a chemoattractant. SNU-216 cells ( $1 \times 10^4$  cells/insert) or NCI-N87 cells ( $5 \times 10^4$  cells/insert) in 300  $\mu$ L volume of 1% FBS-containing medium. After incubation for 48 h at 37°C, non-invasive cells were removed with a cotton swab. Non-invasive cells were removed from the top of each insert with a cotton swab. Invasive cells on the bottom surface of the insert were stained with 0.2% crystal violet in 20% methanol for 30 min and were photographed with an inverted microscope. Stained cells were lysed with 10% SDS for 30 min, and absorbance was measured at 570 nm using an ELISA reader (Bio-Rad) as described previously. Migration assays were performed the same way as the invasion assays, using transwell compartment except that Matrigel was not included (Zhang et al. 2013).

### **Immunofluorescence staining**

SNU-216 cells ( $1 \times 10^4$  cells/well) were cultured on 4-well chamber slide (Thermo Scientific, Rockford, IL, USA). After 24 h, cells were fixed with 4%

paraformaldehyde for 10 min, and blocked with 5% normal donkey serum containing 0.5% Triton X-100 for 5 min. Cells were incubated overnight at 4°C with mixture of the following primary antibodies: rabbit anti-HER2 (1:200; Cell Signaling Technology) and mouse anti-pJNK (1:200, Santa Cruz Biotechnology). Alexa fluor-555-conjugated anti-rabbit IgG (1:200, Life Technologies) and -488-conjugated anti-mouse IgG (1:200, Life Technologies) were used as secondary antibodies. To examine whether JNK inhibition reorganizes cytoskeleton, filamentous actin (F-actin) was visualized. Cells were incubated with 165 nM Alexa Fluor-633-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) for 10 min, followed by DAPI staining. Immunofluorescence was observed under a fluorescence microscope as described previously (Yoon et al. 2013).

### **Lentivirus-mediated shRNA silencing of HER2**

Lentiviral particles containing non-targeting shRNA or HER2 shRNA were purchased (Sigma, St. Louis, MO, USA). The sequence of HER2 shRNA was 5'-CCGGTGTCAGTATCCAGGCTTTGTACTIONCGAGTACAAAGCCTGGATACTGAC

ATTTTTG-3'. The control shRNA particles contain four base pair mismatches within the short hairpin sequence to any known human or mouse gene. Viral infection was performed by incubating GC cells in the culture medium containing lentiviral particles for 12 h in the presence of 5 µg/mL Polybrene (Santa Cruz Biotechnology). Pooled puromycin (2 µg/mL)-resistant cells were used for further analysis.

### **Semi-quantitative reverse transcription-polymerase chain reaction (SQ RT-PCR)**

To quantify mRNA levels of HER2, we used a highly sensitive SQ RT-PCR method, as previously described (Ko et al. 2015). Total RNAs were isolated using TRIzol (Invitrogen) and 1 µg RNA was reverse-transcribed at 48°C for 30 min. Complementary DNAs were amplified over 20 PCR cycles (denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 70°C for 30 s) in a reaction mixture containing 5 µCi ( $\alpha$ -<sup>32</sup>P)dCTP (NEN, Boston, MA, USA). The resulting PCR fragments (5 µl) were electrophoresed on a 2% agarose gel at 100 V in 1 X

TAE, and the gels were dried and autoradiographed. Primer sequences were 5'-GGGAGAGAGTTCTGAGGATT-3' and 5'-CGTCCGTAGAAAGGTAGTTG-3' for HER2 and 5'-ACACCTTCTACAATGAGCTG-3' and 5'-CATGATGGAGTTGAAGGTAG-3' for  $\beta$ -actin.

### **Statistical analysis**

Data were analyzed using GraphPad Prism software for Windows 7 (version 4; GraphPad Software, San Diego, CA, USA) and the significances of the results was determined by the two-tailed Student's t-test. P values of < 0.05 were considered statistically significant for all statistical analyses.

## **Results**

### **JNK activation was pharmacologically inhibited by treatment with**

#### **SP600125**

Since HER2 protein expression in GC cell lines varied, GC cell lines SNU-216 and NCI-N87 showing a high level of HER2 expression were selected. In order to observe the role of JNK in HER2-overexpressing GC cells, cells were treated with a specific JNK inhibitor SP600125 for 24 h. Western blot confirmed that JNK activation (manifested by pJNK expression) was inhibited in both GC cell lines in a dose-dependent manner (Fig. 1).

### **Pharmacological inhibition of JNK reduces the cell growth, migration and**

#### **invasion**

Since the effects of JNK on cell growth (Shibata et al. 2008; Ohsawa et al. 2010; Hong et al. 2015) and metastatic potential in GC have been inconsistent (Fu et al. 2007; Mishra et al. 2010; Li et al. 2012), we first confirmed the effect of JNK on

GC cell growth. Treatment of GC cells with SP600125 for 48 h decreased cell growth by 68% in SNU-216 and by 54% in NCI-N87 compared to control cells (Fig. 2a, d). These findings indicate that JNK activation induces cell proliferation in HER2-overexpressing GC cells.

Next, to determine the effects of JNK activation on the cell motility and invasion in GC cells were determined by transwell migration assay. We found that SP600125 treatment for 48 h significantly suppressed the cancer cell migration by 43% ( $P = 0.0288$ ) in SNU-216 cells and by 38% ( $P = 0.0033$ ) in NCI-N87 cells (Fig. 2b, e) compared to control cells. Consistently, cell invasion assay showed that JNK inhibition decreased cancer cell invasion by 49% ( $P = 0.005$ ) in SNU-216 cells and by 43% ( $P = 0.0132$ ) in NCI-N87 cells (Fig. 2c, f) compared to control cells.

### **JNK inhibition regulates the expressions of epithelial-mesenchymal transition (EMT) markers**

In the initial steps of metastasis of carcinoma cells, epithelial cancer cells change their phenotype to mesenchymal phenotype and become motile and invasive by a process called EMT (Guarino et al. 2007). This process includes downregulation of epithelial markers and upregulation of mesenchymal markers (Guarino et al. 2007). In the present study, Western blot showed that the expression of the representative epithelial marker E-cadherin was enhanced, whereas the expressions of mesenchymal markers Snail and Vimentin were reduced after SP600125 treatment for 24 h (Fig. 3a, c). These results indicate the stimulatory role of JNK in the EMT in GC cells. To further confirm these results, we examined actin organization because actin-dependent membrane protrusions are regarded as a critical determinant of EMT (Shankar et al. 2010). Staining of F-actin with FITC-conjugated phalloidin revealed the presence of many filopodia-like extensions containing actin-rich bundles in control cells with a more elongated shape, whereas they were absent or less obvious in SP600125-treated cells with cuboidal morphology (Fig 3b, d).

### **Crosstalk between HER2 and JNK exists in GC cell lines**

We previously reported that HER2 downregulation in GC cell lines decreased cancer cell growth, migration and invasion as well as EMT markers (Ko et al. 2015). We found consistent results in present study (data is not shown). Then, we investigate the relationship between HER2 and JNK. First, we determined the effect of HER2 silencing on JNK activation (manifested by pJNK expression) and expression. Western blot showed that JNK activation and expression were markedly reduced in HER2 shRNA transfectants compared to control shRNA transfectants (Fig. 4a, d). On the other hand, Western blot and RT-PCR analysis showed that the expressions of HER2 protein and mRNA were substantially decreased by treatment with SP600125 (Fig. 4b, e).

In addition, double immunofluorescence staining for pJNK and HER2 was performed. We found that, in both cell lines, control cells with pJNK staining in the nucleus (Fig. 5a, i) showed strong immunofluorescence at the plasma membrane (Fig. 5b, j). After SP600125 treatment, which drastically reduced the nuclear

immunofluorescence for pJNK (Fig. 5e, m), reduced the immunofluorescence for HER2 at the plasma membrane to a negligible level (Fig. 5f, n). These results indicate that there is a positive reciprocal regulatory loop between JNK and HER2.

### **JNK inhibitor exerts synergistic effect when combined with HER2 shRNA**

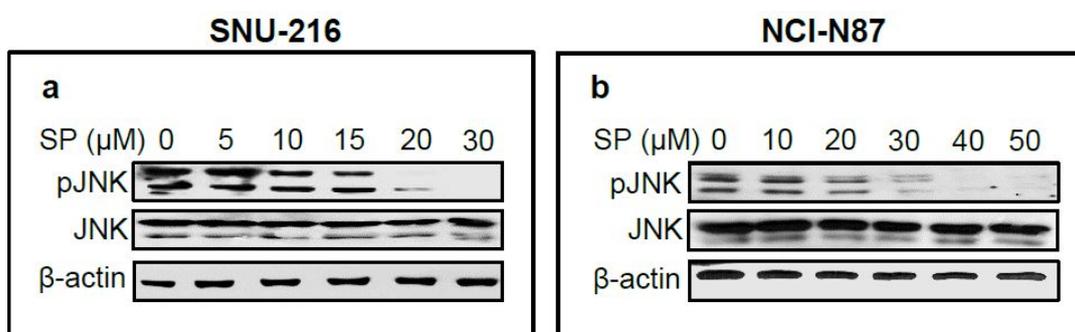
#### **transfection**

The above results showed that both HER2 and JNK enhanced metastatic potential in GC cells and that a positive-crosstalk exists between HER2 and JNK.

Fig. 6a showed that the combination of HER2 silencing and pharmacological inhibition of JNK markedly reduced the expressions of pHER2 and pJNK. To investigate whether combination of these two molecules has synergistic effects on GC cell malignancy, we examined the effect of double inhibition of these molecules on the cell growth, migration and invasion (Fig. 6b-d). Cell growth curves (Fig. 6b) showed that HER2 silencing and JNK inhibition reduced cell number to similar extent (63% reduction in HER2 silencing and 70% reduction in JNK inhibition, respectively), which was further suppressed by inhibition of both

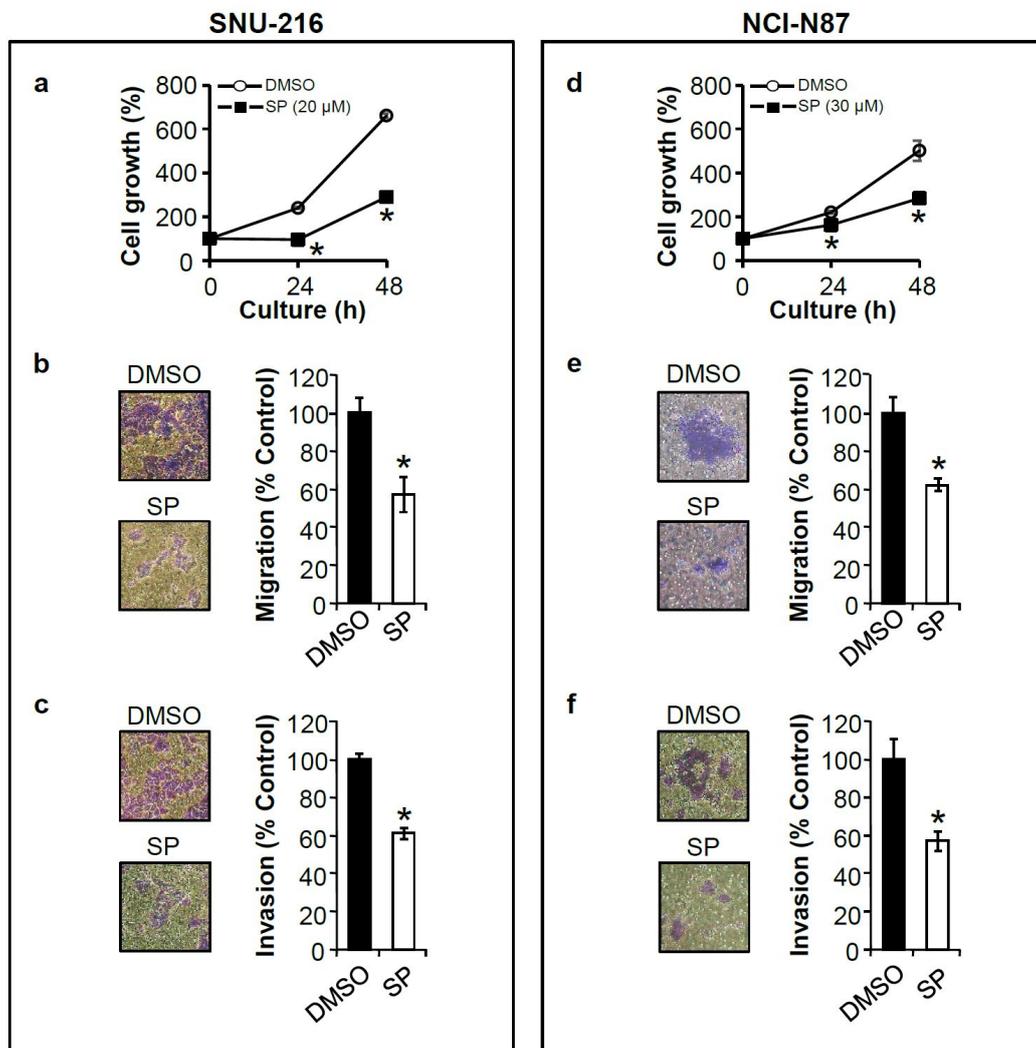
molecules (91% reduction in control). Furthermore, the cell migration capacity decreased by 49% in cells treated with SP600125, and by 47% in HER2 shRNA transfected cells compared with control cells. The combination of HER2 down-regulation and JNK inhibition showed lower migration capacity (61% lower than that of the control) than those with suppression of either alone ( $P < 0.005$ ) (Fig. 6c). Similar results were shown in the invasion assay (63% lower invasion ability in the combined treatment than that of the control) (Fig. 6d). Consistent results were shown in the experiments using NCI-N87 cells (Fig. 6e-h).

**Figure 1.**



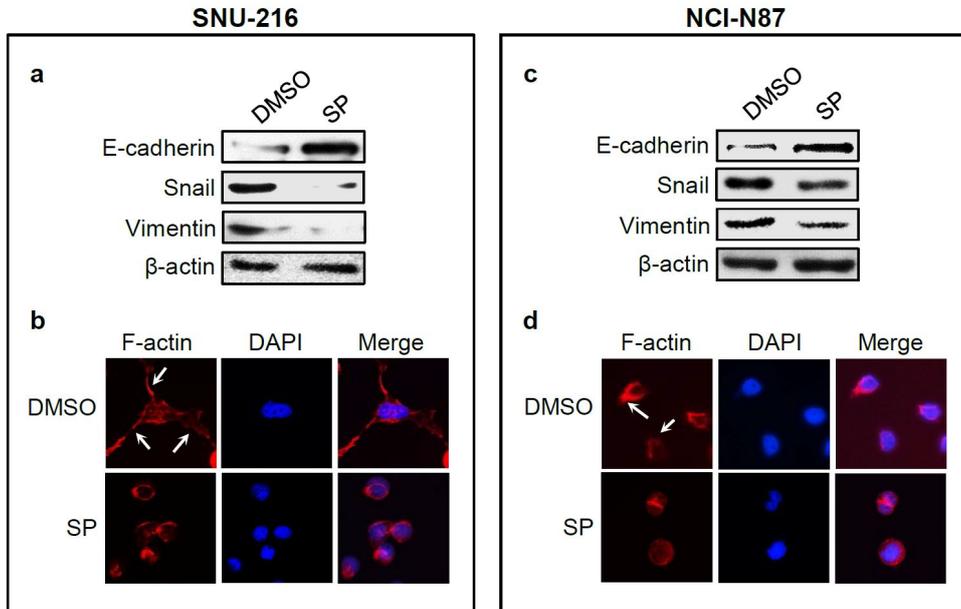
**Figure 1. Effect of pharmacological inhibition of JNK in GC cell lines SNU-216 and NCI-N87 cells.** Cells were treated with various concentrations of SP600125 (SP) for 24 h. Protein expressions of pJNK and total JNK were determined by Western blot.

Figure 2.



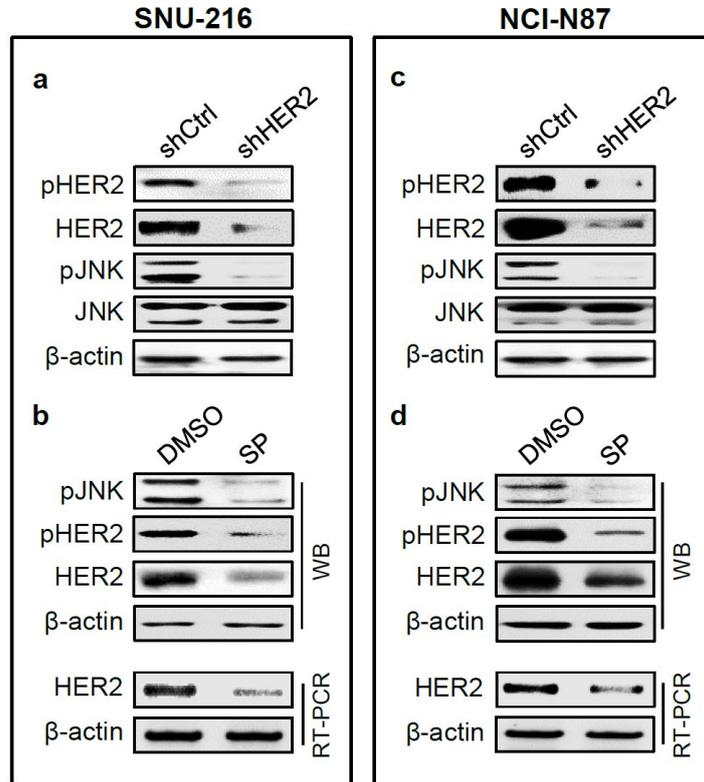
**Figure 2. Effect of pharmacological inhibition of JNK on cell growth, migration and invasion in GC cell lines.** GC cells were cultured in the presence or absence of SP600125 (SP). **a, d** Cells were treated with SP600125 (SP) (20  $\mu$ M for SNU-216 and 30  $\mu$ M for NCI-N87) for 48 h. Cell growth was analyzed using crystal violet assay on the indicated times, and absorbance was measured at 570 nm. **b, e** The effect of treatment with SP (20  $\mu$ M for SNU-216 and 30  $\mu$ M for NCI-N87) on the cell migration was analyzed after plating for 48 h by Transwell migration assay. **c, f** The effect of treatment with SP on the cell invasion was analyzed 48 h after plating by cell invasion assay. Results were calculated as percentages relative to control cells. Each bar represents the mean  $\pm$  SD ( $n = 4$  per each group). \*  $P < 0.05$  vs control cells.

**Figure 3.**



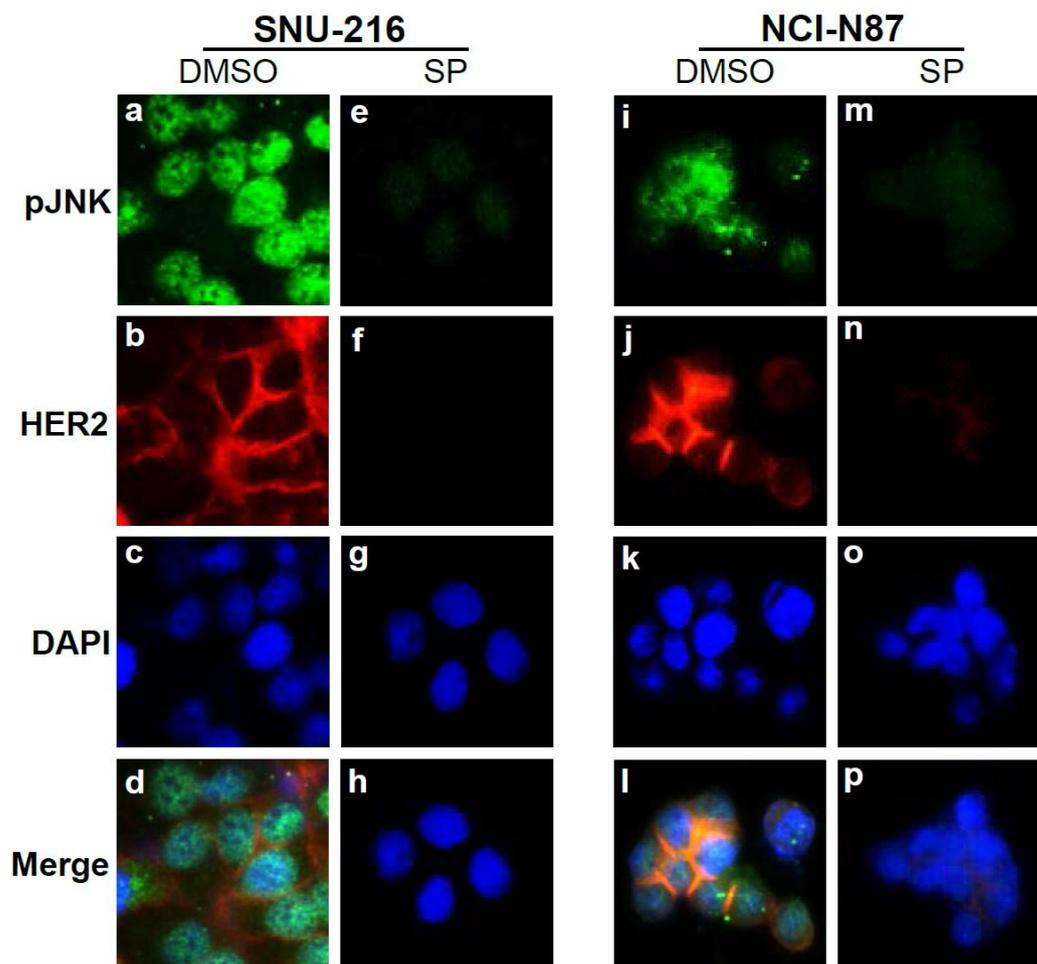
**Figure 3. Effect of JNK inhibition on EMT marker expressions and actin cytoskeleton organization.** SNU-216 and NCI-N87 GC cells were treated with SP600125 (SP) for 24 h. **a, c** The effect of SP (20  $\mu$ M for SNU-216 and 30  $\mu$ M for NCI-N87) treatment for 24 h on EMT marker expressions were analyzed by Western blot for E-cadherin, Snail and Vimentin. **b, d** Changes in the organization of the actin cytoskeleton. Cells were stained with Alexa Fluor 633-conjugated phalloidin to visualize F-actin (red), and the cell nuclei were visualized by DAPI staining (blue). Photographs were taken with a fluorescence microscope (x 400 magnification).

**Figure 4.**



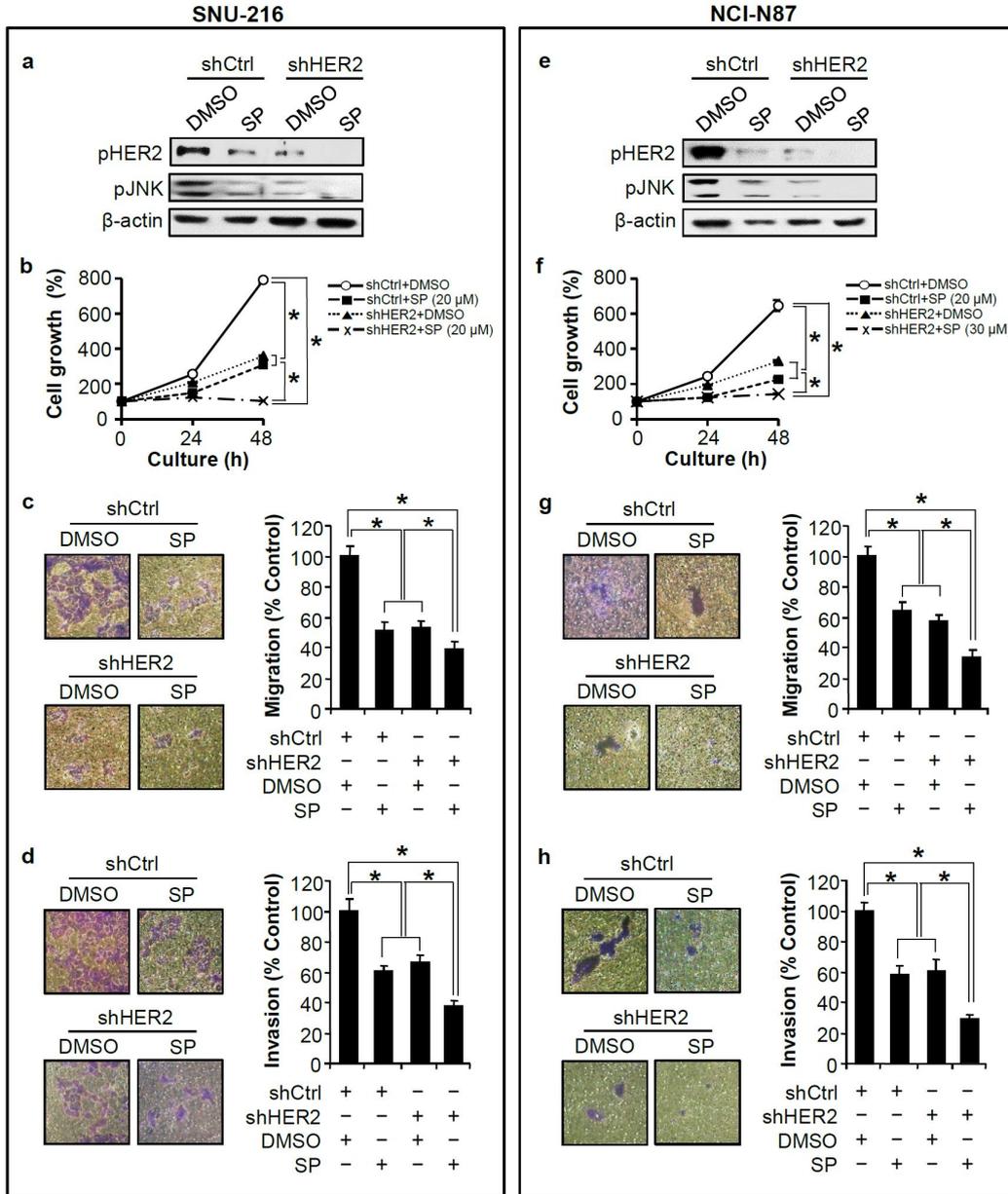
**Figure 4. The relationship between HER2 and JNK in GC cell lines.** SNU-216 cells (a, b) and NCI-N87 cells (c, d) were transfected with HER2 shRNA (a, c) or treated with SP600125 (SP) (b, d). **a, c** The effects of HER2 shRNA transfection on expressions of pHER2, HER2, pJNK and JNK were determined by Western blot. **b, d** The effects of SP treatment (20  $\mu$ M for SNU-216 and 30  $\mu$ M for NCI-N87) on the expressions of pJNK, pHER2 and HER2 were determined by Western blot (WB) and the expression of HER2 were determined by RT-PCR.

Figure 5.



**Figure 5. Immunofluorescence staining showing the effect of SP600125 on HER2 expression.** SNU-216 cells (a-h) and NCI-N87 cells (i-p) were cultured with vehicle control (DMSO) or SP600125 (SP). The effect of JNK inhibition on the subcellular localization of HER2 was observed by double immunofluorescence staining for pJNK (green) and HER2 (red). 4',6'-diamidino-2-phenylindole (DAPI) staining was performed for nucleus (blue). (x 400 magnification).

**Figure 6.**



**Figure 6. Synergistic effects of HER 2 and JNK on metastatic potential of GC cells.** SNU-216 (a-d) and NCI-N87 (e-h) GC cell lines were transfected with either control shRNA (shCtrl) or HER2 shRNA (shHER2). Then, cells were treated with vehicle control (DMSO) or SP600125 (SP) (20  $\mu$ M for SNU-216 and 30  $\mu$ M for NCI-N87). **a, e** Transfected cells were treated with SP for 24 h. Protein expression of pHER2 and pJNK were determined by Western blot. **b, f** Transfected cells were treated with SP for 48 h after plating. Cell growth rates were analyzed using the crystal violet assay on the indicated times, and absorbance was measured. **c, g** Transfected cells were treated with SP for 48 h after plating. Effect of interaction of HER2 and JNK on cell migration capacity was evaluated by Trans well migration assay. **d, h** Transfected cells were treated with SP for 48 h after plating. Effect of interaction of HER2 and JNK on cell invasion capacity was evaluated by cell invasion assay. Results were calculated as percentages relative to control cells. Each bar represents the mean  $\pm$  SD (n = 4 per each group). \* P < 0.05 vs control cells.

## Discussion

Understanding the oncogenic signaling pathways may lead to the development of therapeutic strategies for cancer treatment. Although the pivotal role of HER2 in gastric cancer progression has been shown, the link between HER2 and JNK has not been validated in gastric cancer. This is the first study, to the best of our knowledge, to show the association between HER2 and JNK and their significance in the cell growth and metastatic potential in gastric cancer cells.

Two key survival pathways downstream of HER2 included the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the MAPK pathway (Gschwantler-Kaulich et al. 2016). Among these pathways, PI3K/Akt pathway has gained much attention for the development of an alternative target for anti-HER2 cancer therapy (Chakrabarty et al. 2013). However, the interest has been diverged to the JNK pathway in experiments using breast cancer cells. JNK, which belongs to MAPK family, has shown to promote cell survival in HER2-

overexpressing breast cancer cells (Han and Crowe. 2010). Moreover, the current study by Gschwantler-Kaulich et al. (2016) reported that JNK mediated the anti-HER2 drug resistance in HER2-overexpressing breast cancer cells. These results suggested that JNK plays a central role in the HER2 signaling in HER2-overexpressing breast cancer cells.

Metastatic tumor relapses are characterized by rapid proliferating, drug-resistant cancers (Chakrabarty et al. 2013). With respect to gastric cancer, implications of JNK in HER2-overexpressing gastric cancer cell growth have been inconsistent. JNK activation increased the gastric cancer cell survival in BCG-823 cells (Xia et al. 2006), whereas it decreased the cell survival in SGC-7901 cells (Xiao et al. (2012). In the present study, we confirmed that JNK, like HER2, increased the cell growth of SNU-216 and NCI-N87 gastric cancer cells with HER2 overexpression, which is consistent with the finding of Xiao et al. shown in breast cancer cells.

In previous studies, the role of JNK in HER2-overexpressing gastric cancer cells still remains elusive. Wang et al. (Wang et al. 2010) reported that JNK induced gastric cancer cell migration and invasion were increased by JNK in SGC7901 cells, whereas they were suppressed in SNU-216 cells (Jung et al. 2013). Thus, the role of JNK in GC cell metastasis needs to be further elucidated.

Previously, Ko et al. (Ko et al. 2015) showed that HER2 increased cell migration, invasion in vitro and metastasis of two HER2-overexpressing gastric cancer lines (SNU-216 and NCI-N87) in nude mice. However, the association between HER2 and JNK in these cell lines has not been determined. In the present study, we investigated implications of JNK activation in HER2-overexpressing gastric cancer cell lines. We found that the pharmacological inhibition of the JNK by SP600125 resulted in decreased migration and invasion ability in both gastric cancer cell lines. Furthermore, analyses of the EMT marker expressions and actin organization revealed that JNK inhibition suppressed the expressions of mesenchymal markers (Snail and Vimentin) and increased E-

cadherin expression accompanied by appearance of epithelial morphology. Thus, these results indicate that JNK might contribute to acquisition of malignant phenotype of GC cells through promotion of EMT. Our data provide evidences for the role of JNK in the regulation of metastatic potential in GC cells through the modulation of mesenchymal phenotype.

The success of future trials of combination anti-HER2 therapy will depend on the selection of molecular biomarker selection. Major survival pathways downstream of HER2 included phosphatidylinositol 3-kinase (PI3K)/Akt pathway and MAPK pathway (Gschwantler-Kaulich et al. 2016). Among these pathways, PI3K/Akt pathway has gained much attention for the development of an alternative target for anti-HER2 cancer therapy (Chakrabarty et al. 2013). However, the interest has been diverged to the JNK pathway. JNK, which belongs to MAPK family, has shown to promote cell survival in HER2-overexpressing breast cancer cells (Han and Crowe. 2010). Moreover, the current study by Gschwantler-Kaulich et al. (Gschwantler-Kaulich et al. 2016)

showed that JNK was inhibited in lapatinib-sensitive HER2-overexpressing breast cancer cells, but not in the lapatinib-resistant cancer cells.

In the present study, we investigated the role of JNK in HER2-overexpressing gastric cancer cell lines. The pharmacological inhibition of the JNK by SP600125 resulted in decreased migration and invasion ability in HER2-overexpressing gastric cancer cell lines. Furthermore, analyses of the EMT marker expressions and actin organization revealed that JNK inhibition suppressed the expressions of mesenchymal markers (Snail and Vimentin) and increased E-cadherin expression accompanied by appearance of epithelial morphology. Thus, these results indicate that JNK might contribute to acquisition of malignant phenotype of GC cells through promotion of EMT. Our data provide direct evidence for the role of JNK in the regulation of metastatic potential in GC cells through the modulation of mesenchymal phenotype.

Since we wondered whether there is a reciprocal regulatory loop between

JNK and HER2 in GC, we further analyzed the effect of HER2 silencing on the JNK activation. We found that HER2 downregulation suppressed JNK activation (manifested by pJNK expression). In turn, JNK inhibition was accompanied by decreases in the expression of HER2 and pHER2. These results suggest that JNK controls and is controlled by HER2, and that there is a positive cross-talk between HER2 and JNK in GC cells.

Although targeted therapies may offer enhanced efficacy and improved selectivity, mostly their effects are not durable when they are used alone. For this reason, combination therapies are often needed to effectively treat many tumors. In the present study, we found that the combination of HER2 silencing and JNK inhibition further reduced migration and invasion of gastric cancer cells compared to inhibition of each molecule. Therefore, HER2 and JNK seems to act in a synergistic manner in modulating migration and invasion of gastric cancer cells.

In conclusion, the present study found that JNK activation in HER2-

overexpressing gastric cancer cell lines increased cell growth, migration and invasion as well as EMT markers. In addition, the interaction between HER2 and JNK, which showed positive crosstalk, synergistically contributes to the GC cell growth and metastatic potential in HER2-overexpressing GC cells. Given the importance of HER2 overexpression in the gastric tumor development and progression, the identification of JNK as an important downstream component of HER2 oncogenic signaling pathway represents an important finding. Further experiments using anti-HER2 drug-resistant gastric cancer cells and animal studies might give evidence that this pathway could serve as a useful target for therapeutic strategies for treatment of HER2-overexpressing gastric cancer. attractive as therapeutic strategies in gastric cancer (Chen et al. 2010).

HER2 is of particular interest as a drug target in GC therapy because it has been shown to be amplified and/or overexpressed in a subset of gastric cancers (Chen et al. 2010). However, due to development of acquired drug resistance, alternative therapeutic targets are needed to effective treatment of HER2-overexpressing GC patients. Understanding HER2 signaling pathways may lead

to the development of therapeutic strategies for cancer treatment. In present study, we investigated the relationship between HER2 and JNK in terms of proliferation and metastatic potential of GC cells. To the best of our knowledge, this is the first study to show the association between HER2 and JNK in GC.

Major survival pathways downstream of HER2 included phosphatidylinositol 3-kinase (PI3K)/Akt pathway and MAPK pathway (Gschwantler-Kaulich et al. 2016). Among these pathways, PI3K/Akt pathway has gained much attention for the development of an alternative target for anti-HER2 therapy (Chakrabarty et al. 2013). However, the interest has been diverged to the JNK pathway. JNK, which belongs to MAPK family, has shown to promote cell survival in HER2-overexpressing breast cancer cells (Han and Crowe. 2010). Moreover, a recent study (Gschwantler-Kaulich et al. 2016) showed that JNK was inhibited in lapatinib-sensitive HER2-overexpressing breast cancer cells, but not in the lapatinib-resistant cells. These results indicate that lapatinib-induced apoptosis in HER2-overexpressing breast cancer cells is mediated through JNK inhibition.

Metastatic tumor relapses are characterized by rapid proliferating, drug-resistant cancers (Chakrabarty et al. 2013). With respect to GC growth, the implication of JNK has been inconsistent (Chen et al. 2002; Xia et al. 2006; Shibata et al. 2008; Xiao et al. 2012). Previously, in vitro studies (Chen et al. 2002; Xiao et al. 2012) showed that JNK activation decreased the cell survival in GC cell lines (MGC80-3, SGC-7901). In contrast, JNK activation increased the survival in GC cell lines BCG-823, MKN-45 and AGS (Xia et al. 2006). Moreover, Shibata et al. (Shibata et al. 2008) observed that loss of JNK decreased the tumorigenesis of gastric tumor in mice. Thus, the role of JNK in GC still remains elusive. In the present study, we confirmed that JNK, like HER2, increased the GC cell growth, which is consistent with the finding of Han and Crowe (Han and Crowe. 2010).

Initial studies of JNK examined their ability to induce the phosphorylation of several transcription factors, such as the oncogene c-Jun. However, recent evidence indicates that JNK have conserved their evolutionary role in cell

movement, cytoskeleton rearrangement and migration. The ability of tumor cells to migrate from the primary tumor to surrounding tissues is a prerequisite for metastasis. JNK promotes increased motility of multiple cell types including tumor cells (Ono et al. 2008). Although findings reported here support a role for JNK in GC metastasis, the mechanism by which this pathway is activated in GC have not been elucidated. To address the underlying molecular mechanism involved in the JNK pathway implicated in cancer cell motility, we used migration and invasion assay in the HER2-overexpressing GC cells. The inhibition of the JNK by treatment with SP600125 resulted in a decreased migration and invasion ability in GC cells. Furthermore, analyses of the EMT marker expressions and actin organization revealed that SP600125 treatment suppressed the expressions of mesenchymal markers (Snail and vimentin) and increased E-cadherin expression, with induction of epithelial morphology. In addition, these phenotypic changes were attenuated by inhibition of JNK activation. Thus, these results indicate that JNK might contribute to acquisition of malignant phenotype of GC cells through promotion of EMT. Our data provide direct evidence for the role

of JNK in the regulation of metastatic potential in GC cells through the modulation of mesenchymal phenotype.

Previous ours (Ko et al. 2015) showed that HER2 increased GC cell migration and invasion. However, the role of JNK in HER2-mediated cell motility is less defined. Since we wondered whether there is a reciprocal regulatory loop between JNK and HER2 in GC, we further analyzed the effect of HER2 silencing on the JNK activation. We found that HER2 downregulation suppressed JNK activation (manifested by pJNK expression). In turn, JNK inhibition was accompanied by decreases in the expression of HER2 and pHER2. These results suggest that there is a positive cross-talk between HER2 and JNK in GC cells.

Although targeted therapies may offer enhanced efficacy and improved selectivity, mostly their effects are not durable when they are used alone. For this reason, combination therapies are often needed to effectively treat many tumors (Yoon et al. 2010). In the present study, we found that the combination of HER2

silencing and JNK inhibition further reduced migration and invasion of gastric cancer cells compared to inhibition of each molecule. Therefore, HER2 and JNK seems to act in a synergistic manner in modulating migration and invasion of gastric cancer cells.

In conclusion, the present study identified HER2/JNK as a signaling pathway responsible further migration and invasion in GC for the first time. Given the importance of EMT in the tumor development in the progression the identification of JNK as a key regulator of HER2-induced EMT represents an important finding. Further studies might give evidence that this pathway could serve as a useful target for therapeutic strategies for regard to EMT reversion.

## Reference

- Bang YJ (2012) Advances in the management of HER2-positive advanced gastric and gastroesophageal junction cancer. *J Clin Gastroenterol* 46:637–648
- Bubici C, Papa S (2014) JNK signalling in cancer: in need of new, smarter therapeutic targets. *Br J Pharmacol* 171:24-37
- Chen J, Miller EM, Gallo KA (2010) MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells. *Oncogene* 29:4399-411
- Chen XY, Zhou J, Luo LP, Han B, Li F, Chen JY, Zhu YF, Chen W, Yu XP (2015) Black Rice Anthocyanins Suppress Metastasis of Breast Cancer Cells by Targeting RAS/RAF/MAPK Pathway. *Biomed Res Int* 2015:414250
- Fu P, Thompson JA, Bach LA (2007) Promotion of cancer cell migration: an insulin-like growth factor (IGF)-independent action of IGF-binding

protein-6. *Biol Chem* 282:22298-306

Guarino M, Rubino B, Ballabio G (2007) The role of epithelial-mesenchymal transition in cancer pathology. *Pathology* 39:305–318

Gschwantler-Kaulich D, Grunt TW, Muhr D, Wagner R, Kölbl H, Singer CF (2016) HER Specific TKIs Exert Their Antineoplastic Effects on Breast Cancer Cell Lines through the Involvement of STAT5 and JNK. *PLoS One* 11:e0146311

Han JS and Crowe DL (2010) Jun amino-terminal kinase 1 activation promotes cell survival in ErbB2-positive breast cancer. *Anticancer Res* 30:3407–3412

Hong NR, Park HS, Ahn TS, Jung MH, Kim BJ (2015) Association of a Methanol Extract of *Rheum undulatum* L. Mediated Cell Death in AGS Cells with an Intrinsic Apoptotic Pathway. *J Pharmacopuncture* 18:26-32

Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF (2008) Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. *J Clin Invest* 118:3943-

3953

Janjigian YY (2015) Lapatinib in Gastric Cancer: What Is the LOGiCal Next Step? *J Clin Oncol* pii:CO642892

Jung MK, Houh YK, Ha S, Yang Y, Kim D, Kim TS, Yoon SR, Bang SI, Cho BJ, Lee WJ, Park H, Cho D (2013) Recombinant Erdr1 suppresses the migration and invasion ability of human gastric cancer cells, SNU-216, through the JNK pathway. *Immunol Lett* 150:145–151

Kim HP, Han SW, Song SH, Jeong EG, Lee MY, Hwang D (2014) Testican-1-mediated epithelial-mesenchymal transition signaling confers acquired resistance to lapatinib in HER2-positive gastric cancer. *Oncogene* 33:3334-41

Kim WH, Schnaper HW, Nomizu M, Yamada Y, Kleinman HK (1994) Apoptosis in human fibrosarcoma cells is induced by a multimeric synthetic Tyr-Ile-Gly-Ser-Arg (YIGSR)-containing polypeptide from laminin. *Cancer Res* 54:5005–5010

Ko YS, Cho SJ, Park J, Kim Y, Choi YJ, Pyo JS, Jang BG, Park JW, Kim

WH, Lee BL (2015) Loss of FOXO1 promotes gastric tumour growth and metastasis through upregulation of human epidermal growth factor receptor 2/neu expression. *Br J Cancer* 113:1186-1196

Kyriakis JM and Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81:807-869

Li LH, Luo Q, Zheng MH, Pan C, Wu GY, Lu YZ, Feng B, Chen XH, Liu BY (2012) P21-activated protein kinase 1 is overexpressed in gastric cancer and induces cancer metastasis. *Oncol Rep* 27:1435-1442

Lin W, Kao HW, Robinson D, Kung HJ, Wu CW, Chen HE (2000) Tyrosin kinases and gastric cancer. *Oncogene* 19:5680-5689

Matsui Y, Inomata M, Tojigamori M, Sonoda K, Shiraishi N, Kitano S (2005) Suppression of tumor growth in human gastric cancer with HER2 overexpression by an anti-HER2 antibody in a murine model. *Int J Oncol* 27:681-685

Mishra P, Senthivinayagam S, Rangasamy V, Sondarva G, Rana B (2010)

Mixed lineage kinase-3/JNK1 axis promotes migration of human gastric cancer cells following gastrin stimulation. *Mol Endocrinol* 24:598–607

Oh DY, Doi T, Shirao K, Lee KW, Park SR, Chen Y, Yang L, Valota O, Bang YJ (2015) Phase I Study of Axitinib in Combination with Cisplatin and Capecitabine in Patients with Previously Untreated Advanced Gastric Cancer. *Cancer Res Treat* 47:687-696

Ono R, Matsuoka J, Yamatsuji T, Naomoto Y, Tanaka N, Matsui H, Matsushita M (2008) M-RIP, a novel target of JNK signaling and a requirement for human cancer cell invasion. *Int J Mol Med* 22:199-203

Ohsawa R, Miyazaki H, Niisato N, Shiozaki A, Iwasaki Y, Otsuji E, Marunaka Y (2010) Intracellular chloride regulates cell proliferation through the activation of stress-activated protein kinases in MKN28 human gastric cancer cells. *J Cell Physiol* 223:764-770

Qin X, Zheng S, Liu T, Liu Q, Liang M, Li X, Sheyhidin I, Lu X (2014) Roles of phosphorylated JNK in esophageal squamous cell carcinomas of Kazakh ethnic. *Mol Carcinog* 53:526–536

- Sahu SK, Garding A, Tiwari N, Thakurela S, Toedling J, Gebhard S, Ortega F, Schmarowski N, Berninger B, Nitsch R, Schmidt M, Tiwari VK (2015) JNK-dependent gene regulatory circuitry governs mesenchymal fate. *EMBO J* 34:2162-2181
- Shankar J, Messenberg A, Chan J, Underhill TM, Foster LJ, Nabi IR (2010) Pseudopodial actin dynamics control epithelial-mesenchymal transition in metastatic cancer cells. *Cancer Res* 70:3780–3790
- Shibata W, Maeda S, Hikiba Y, Yanai A, Sakamoto K, Nakagawa H, Ogura K, Karin M, Omata M (2008) c-Jun NH2-terminal kinase 1 is a critical regulator for the development of gastric cancer in mice. *Cancer Res* 2008 68:5031-5039
- Vivanco I, Palaskas N, Tran C, Finn SP, Getz G, Kennedy NJ, Jiao J, Rose J, Xie W, Loda M, Golub T, Mellinghoff IK, Davis RJ, Wu H, Sawyers CL (2007) Identification of the JNK signaling pathway as a functional target of the tumor suppressor PTEN. *Cancer Cell* 11:555–569
- Wang J, Kuitatse I, Lee AV, Pan J, Giuliano A, Cui X (2010) Sustained c-

Jun-NH2-kinase activity promotes epithelial-mesenchymal transition, invasion, and survival of breast cancer cells by regulating extracellular signal-regulated kinase activation. *Mol Cancer Res* 8:266-277

Wei W, Li H, Li N, Sun H, Li Q, Shen X (2013) WNT5A/JNK signaling regulates pancreatic cancer cells migration by Phosphorylating Paxillin. *Pancreatology* 13:384-392

Yoon J, Cho SJ, Ko YS, Park J, Shin DH, Hwang IC, Han SY, Nam SY, Kim MA, Chang MS, Lee HS, Kim WH, Lee BL (2013) A synergistic interaction between transcription factors nuclear factor- $\kappa$ B and signal transducers and activators of transcription 3 promotes gastric cancer cell migration and invasion. *BMC Gastroenterol* 13:29–39

Xia HH, He H, De Wang J, Gu Q, Lin MC, Zou B, Yu LF, Sun YW, Chan AO, Kung HF, Wong BC (2006) Induction of apoptosis and cell cycle arrest by a specific c-Jun NH2-terminal kinase (JNK) inhibitor, SP-600125, in gastrointestinal cancers. *Cancer Lett* 241:268-274

Xiao F, Liu B, Zhu QX (2012) c-Jun N-terminal kinase is required for

thermotherapy-induced apoptosis in human gastric cancer cells. *World J*

*Gastroenterol* 18:7348-7356

Zhang H, Liu L, Wang Y, Zhao G, Xie R, Liu C, Xiao X, Wu K, Nie Y, Zhang

H, Fan D (2013) KLF8 involves in TGF-beta-induced EMT and promotes

invasion and migration in gastric cancer cells. *J Cancer Res Clin Oncol*

139:1033-42

## 국 문 초 록

**목적:** 위암에서 표피성장인자2 (epidermal growth factor receptor2, EGFR2, HER2)의 과발현은 위암에서 악성도 증가를 초래하므로 HER2는 위암 환자에서 임상적 효과를 나타내는 유일한 치료표적이다. 그러나 획득성 내성으로 인하여 치료 효율의 향상은 제한되어 있다. c-Jun N-terminal kinase (JNK)는 위암에서 과발현되어 있으나 아직까지 확실한 기능은 알려져 있지 않다. HER2와 JNK의 관련성이 유방암에서 보고되어 있으므로 본 연구의 목적은 HER2를 과발현하는 위암세포주들인 SNU-216과 NCI-N87 세포들을 사용하여 JNK가 위암세포의 성장, 이동 및 침윤에 미치는 영향을 확인하고, HER2와의 관련성을 관찰함으로써 위암환자의 치료제 개발에 유용한 분자적 기전을 규명하고자 하였다.

**방법:** SNU-216과 NCI-N87 위암세포주를 JNK 저해제인 SP600125로 처리하여 JNK의 활성을 억제시켰고, HER2 shRNA 이입으로 HER2의 발현을 감소시켰다. Western blot를 시행하여 HER2, phospho-HER2 (pHER2), JNK, phospho-JNK (pJNK), E-cadherin, Snail 및 Vimentin의 단백 발현 변화를 관찰하였다. 세포수의 관찰을 위하여 Crystal violet assay를 시행하였고, 세포의 이동과 침윤 관찰을 위하여 Transwell migration assay와 invasion assay를 시행하였다.

**결과:** 위암세포들에서 HER2와 JNK는 위암세포의 성장과 전이능을 증가시켰으며 상호작용을 통하여 그 효과를 상승시키는 관련성을 나타내었다. 또한, 이들은 E-cadherin의 발현을 감소시키고 Snail과 Vimentin의 발현은 증가시켰으며, 형태학적으로 간엽세포와 유사한 변화를 유발시켰다.

**결론:** 위암세포에서 JNK는 HER2와 유사하게 세포성장 및 전이능의 증가를 초래하였으며, HER2와의 상호작용을 통하여 이러한 기능이 상승효과를 나타내었다.

주요어: 위암; JNK; HER2; 전이능; EMT

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