이학석사 학위논문

Lapatinb에 대한 내성을 획득한 유방암 세포주에서 Src과 *RUNX3*를 통한 내성 기전에 관한 연구

Mechanism of acquired resistance to lapatinib via Src and *RUNX3* in breast cancer

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ABSTRACT

Mechanism of acquired resistance to lapatinib via Src and *RUNX3* in breast cancer

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Background: Lapatinib is an effective EGFR and HER2 targeting small molecular tyrosine kinase inhibitor, which is one of the standard of care medicine for HER2 positive metastatic breast cancer patients. Primary and acquired resistance to lapatinib developed. Despite several mechanisms of resistance to lapatinib have been suggested,

still the mechanisms of developing lapatinib resistant remain as a question to solve. Thus, I tried to find out there is a novel mechanism which related to lapatinib resistant, and any specified molecules were involved in this process.

Methods: Acquired resistant SK-BR-3 cells were established by chronic exposure to lapatinib. Lapatinib and saracatinib sensitivity were confirmed by MTT assay. Western blotting was used to determine signal transduction molecule changes. Wound healing assay and Boyden chamber assay were conducted for verifying invasive ability. Whole exome sequencing (WES) and siRNA knock-down system were used for further analysis.

Results: Generation of Laptinib resistant (LR) cell lines confirmed by MTT assay. LR cell lines showed down-regulation of pHER2, pAkt, and pERK. The activity of Src family kinase was increased in LR cells. Vimentin, an EMT marker, is also up-regulated in LR cells. Migration and invasion were significantly increased in LR cells. Saracatinib inhibited activation of Src family kinase, cell migration and cell invasion in LR cells. Correlated with a missense mutation of RUNX3, which identified by WES, expression of RUNX3 was decreased in LR cells. Moreover, si-RUNX3 knock-down parental cells showed more resistance to lapatinib.

Conclusion: The increase of Src activation, cell migration, and invasion

was observed in LR cells. RUNX3, which identified by WES, affected to lapatinib sensitivity in SK-BR-3 cells. Based on our data, activation of Src contributes resistance to lapatinib and RUNX3 might be a potential marker, which partially contributes resistance to lapatinib.

Keywords: HER2, Lapatinib, Resistance, Src, RUNX3

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Introduction

The human epidermal growth factor receptor 2 (HER2) is a transmembrane receptor tyrosine kinase (RTK) member of HER family, which included with EGFR, HER2, HER3 and HER4. HER family activated by ligand binding, however, HER2 has no known natural ligands, it is activated by heterodimerization with other RTK members. HER2 signaling is mediated by various downstream pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) and regulate cell proliferation, differentiation, and cell survival [1-3]. HER2 is amplified in 20-25 % of breast cancer and correlated with poor prognosis [4]. Thus, HER2 has been regarded as an important therapeutic target in breast cancer and various HER2 targeted drugs were developed.

Lapatinib is an effective tyrosine kinase inhibitor targeting EGFR and HER2. It is widely used for HER2 positive breast cancer patients who failed to respond trastuzumab. However, it has limiting to use for some patients who are not responsive initially or acquired resistance [3, 5]. Multiple mechanisms of resistance to lapatinib have been reported. For example, overexpression of RTKs and activation of downstream molecules such as Akt, ERK and Src [2-3, 6].

Src is a non-receptor tyrosine kinase that belongs to the Src family kinases and its increased activity have been observed in various

tumors including colon, liver, lung and breast cancer [7]. Src interacts with multiple RTKs including EGFR, HER2, insulin-like growth factor-1 receptor (IGF-1R) and c-Met. As a result, it can regulate cell growth and survival mediated by activation of PI3K/Akt, MAPK and signal transducer and activator of transcription 3 (STAT3) [8]. Moreover, during tumor metastasis, Src promotes the cell migration and invasion through interaction with focal adhesion kinase (FAK) and p120 catenin [9]. Several studies suggested that activation of Src contributes resistance to HER2 targeted drug including trastuzumab and lapatinib, so Src is seems to be a possible target to overcome resistance to HER2 targeted drug [3, 9, 10-11].

The human runt-related transcription factor 3 (RUNX3) is a transcription factor, which binds to DNA by interaction with the cofactor, CBFβ/PEBP2β (core-binding factor-beta subunit/polymavirus enhancer-binding protein 2 beta subunit) [12]. Naturally, RUNX3 localized in the nucleus and functions as a tumor suppressor in various cancers including gastric and breast cancer [13-15]. However, recent studies revealed the oncogenic role of RUNX3, which localized to cytoplasm [16-17]. Correlated these facts, RUNX3 is usually deleted, hypermethylated or mislocalized in most of the breast cancer, it cannot function as a tumor suppressor [15]. Moreover, recently, loss of RUNX3 induce resistance to gemcitabine

was demonstrated [18]. But it has not revealed that loss of RUNX3 related with resistance to HER2 targeted drugs.

In this study, I explored the novel mechanism of resistance to lapatinib. The increase of Src family kinase activation and RUNX3 missense mutation was identified in SK-BR-3 lapatinib resistant (LR) breast cancer cells. And I confirmed that down-regulation of RUNX3 contributes resistance to lapatinib in HER2 amplified SK-BR-3 cells. This is the first report showed that down-regulation of RUNX3 contributes resistance to lapatinib which is helpful to understand resistance mechanism of lapatinib.

MATERIALS AND METHODS

1. Antibodies and reagents

Lapatinib was provided by Novartis. (Basel, Switzerland). The compound was initially dissolved in dimethyl sulfoxide (DMSO) and stored at -80 ℃. Antibodies against pSrc (Y416), FAK, pERK (T202/Y204), ERK (p44/p42), pAkt (S473), Akt were purchased from Cell signalling Technology (Danvers, MA, USA). Antibody against pFAK (Y397) was purchased from BD Biosciences (San Jose, CA, USA). Antibodies against RUNX3, pYes(Y537), Yes, Lyn, pLyn (Y418), Fyn and pFyn(Y530) were purchased from Abcam (Cambridge, UK). Antibody against Src and Testican-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against Actin was purchased from Sigma Aldrich (St. Louis, MO, USA).

2. Cell lines and cell culture

The HER2 amplified human breast cancer cell line (SK-BR-3) was purchased from the American Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI 1640 (Thermo Fisher

Scientific Inc., Waltham, MA, USA) containing 10 % fetal bovine serum (Welgene; Gyeongsan-si, Korea) and 10μg/mL gentamicin (Cellgro; Manassas, VA, USA) at 37 ℃ in a 5 % CO_2 atmosphere.

3. Generations of lapatinib resistant SK-BR-3 cells

LR cells were established by continuously exposing cells to lapatinib, starting with 30 nmol/L and incrementally increasing to 10 μM over 7 months. Clonal selection was done by serial dilution. Two lapatinib resistant SK-BR-3 clones (LR#2, LR#5), which have similar sensitivity to lapatinib and characteristcs with pools of lapatinib resistant, were selected. Cells (pools of resistant cells and resistant clones) were expanded in RPMI-1640 medium containing 10% fetal bovine serum and 1 μM Lapatinib.

4. Cell growth inhibitory assay

Cells (0.8-6.5 × 10^3 in 100 μℓ/well) were seeded in 96-well plates and incubated overnight at 37 ℃ in 5 % CO_2. The cells were exposed

to increasing concentrations of lapatinib (doses ranged from 0-5 μmol/L) or saracatinib (doses ranged from 0-1 μmol/L) for 3 days. After drug treatment, 50 μℓ of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide solution (Sigma Aldrich) was added to each well and the plates were incubated for 4 hours at 37 ℃. The media was removed and dissolving the formazan crystals with 150 μℓ of DMSO. The absorbance of each well was measured at 540 nm with a VersaMax microplate reader (Molecular Devices; Sunnyvale, CA, USA). The absorbance and IC_{50} of lapatinib were analyzed using Sigma Plot software (Statistical Package for the Social Sciences, Inc. (SPSs); Chicago, IL, USA). Six replicate wells were included in each analysis and at least three independent experiments were conducted.

5. Receptor tyrosine kinase (RTK) antibody assay

Cells were collected, washed with ice-cold PBS, and incubated in extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % NP40, 0.1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 2 mM phenyl methylsulfonyl fluoride, 1 mg/mL pepstain A, 0.2 mM leupeptin, 10 μg/mL aprotinin, 1 mM sodium vanadate, 1 mM nitrophenylphosphate,

and 5 mM benzamidine] on ice for 30min. The lysates were cleared by centrifugation at 13,000 rpm for 20 min. Cell lysates were incubated on profiler slides (Cell Signaling Technology; Danvers, MA, USA) and slides were imaged via chemiluminescence on film according to the manufacturer's

6. Western blot analysis

Equal amounts of proteins were separated on an 8-12 % SDS-polyacrylamide gel. The resolved proteins were transferred onto nitrocellulose membranes, and the blots were probed with primary antibodies overnight at 4 ℃. Antibody binding was detected using an enhanced chemiluminescence system according to the manufacturer's protocol (GE Healthcare life science; Chicago, IL, USA) [19].

7. Wound healing assay

Cells ($4-8\times10^5$) were seeded in 6-well plates and incubated overnight at 37 ℃ in a 5 % CO_2. Cells were scratched with yellow tip and washed with DPBS. Cells were incubated with medium containing

DMSO or saracatinib (1 μM). The images were captured using image J program (National Institutes of Health (NIH); Bethesda, MD, USA) at 0 h, 24 h and 36 h.

8. Invasion assay

The invasive ability of parental (PR) SK-BR-3 cells and lapatinib resistant (LR) SK-BR-3 cells was evaluated using the Boyden chamber based cell invasion assay kit (Corning, Inc; New York, USA). Cells were harvested and resuspended in serum free medium, 5×10^4 cells were seeded into the upper 24-well chambers and treated with saracatinib(1 μM) or DMSO. Medium with 10 % FBS was added to the lower chamber as a chemoattractant. After 24 h, the invasive ability of cells was evaluated according to the manufacturer's instructions.

9. Whole exome sequencing (WES)

Genomic DNA was extracted using the DNeasy purification kit (QIAGEN; Hilden, Germany) and according to the manufacturer's

instructions. Exome captures were performed using SureSelect V5+ UTR-post (Macrogen, Inc; Seoul, Korea) according to preparation guide. Sequencing was performed with the Illumina Hiseq 2000 as paired-end reads following the manufacturer's instructions. Raw fastq files were aligned to hg19, and SNPs and indels were called using two separate pipelines. Fastq files were aligned to the hg19 using Burrows-Wheeler Alignment tools (BWA) and variants were called using the Genome Analysis Toolkit (GATK). Variants identification was performed with SnpEff software (http://snpeff.sourceforge.net) [20]

10. siRNA transfection

The siRNA specific for RUNX3 was obtained from Genolution (Seoul, Korea). SK-BR-3 parental cells were initially transfected with siRNA at a final concentration of 80 nmol/L using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h of

incubation, cells were re-transfected with siRNA at the same concentration. Thereafter, transfected cells were seeded and subjected to other analysis. The sequence of the RUNX3 specific siRNA was 5′-UGACGAGAACUACUCCGCUUU-3′. The sequence of the control (non-specific) siRNA was 5′-AAUUCUCCGAACGUGUCACGUUU-3′

11. Statistical analysis

Statistical analyses were performed using SigmaPlot version 10.0 (SPSs). A two-sided Student's t-test was used when appropriate. The results are expressed as the mean ± standard deviation (S.D.) or standard error (S.E.). A P value less than 0.05 was considered to be statistically significant. All experiments were conducted in duplicate or triplicated and repeated at least twice.

Results

1. Establishment and characterization of lapatinib resistant SK-BR-3 (LR) cells.

LR cell lines were established from the HER2 amplified SK-BR-3 breast cancer cell line, which has a high sensitivity to the lapatinib. Two lapatinib resistant SK-BR-3 clones (LR clone #2, LR clone #5), which has similar characteristic with pools of LR cells, were selected. Resistance to lapatinib of LR cells was confirmed compared with parental (PR) cells (Fig. 1, Table 1). To investigate the difference between PR cells and LR cells, the basal expression of protein associated with HER2 signal transduction pathways were examined (Fig. 2). In LR cells, protein expression of EGFR was not significantly different compared with PR cells, however, expression of pEGFR, HER2, pHER2, HER3 and pHER3 was down-regulated.

Akt and ERK protein expression were down-regulated in LR cells,

although p85 expression was increased. These results suggest that LR cells are independent to HER family signal transduction, and other signal transduction molecules excepting Akt and ERK induce resistance to lapatinib in LR cells.

Therefore, receptor tyrosine kinase (RTKs) array was performed to explore signal transduction pathway associated with lapatinib resistance (Fig. 3). Likewise the results of western blot, down-regulation of HER2, Akt and ERK was observed, Src was up-regulated in LR cells. Thus, I anticipated that lapatinib resistance occurs by Src pathway activation.

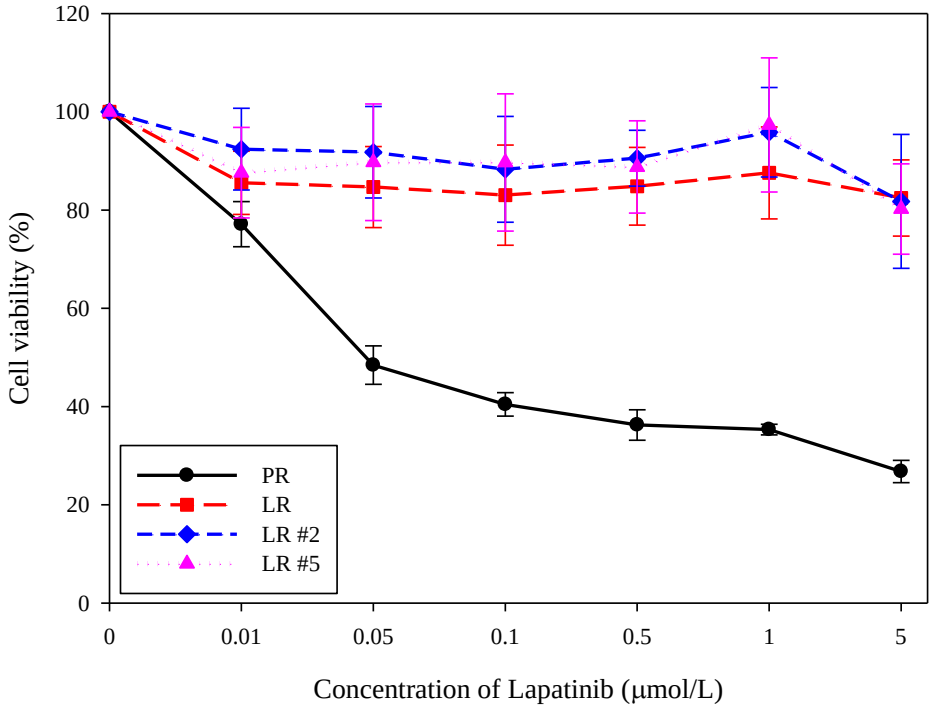


Figure 1. Lapatninb resistant cells generated from SK-BR-3 cells.

Growth inhibition was analyzed by the MTT assay. Cells were treated with lapatinib for 72 hours as indicated lapatinib concentrations.

Cell line	Lapatinib IC_{50} (means±SD, μmol/L)
PR	0.48 ± 0.18
LR	>5
LR clone #2	>5
LR clone #5	>5

Table 1. IC_{50} of lapatinib in PR and LR cells.

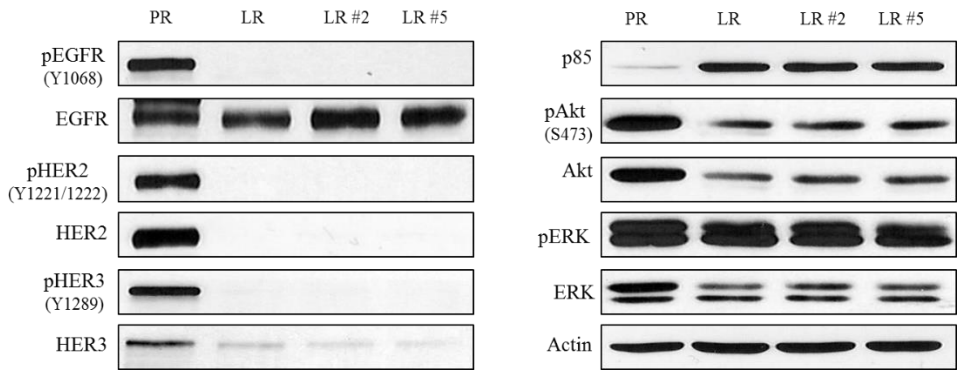


Figure 2. Western blot analysis of signal transduction molecules associated with HER2 pathway.

The expression of HER family and signal transduction molecules were analyzed by western blotting. The activity of pEGFR, HER2, pHER2, HER3, pHER3 and pAkt was reduced in LR cells.

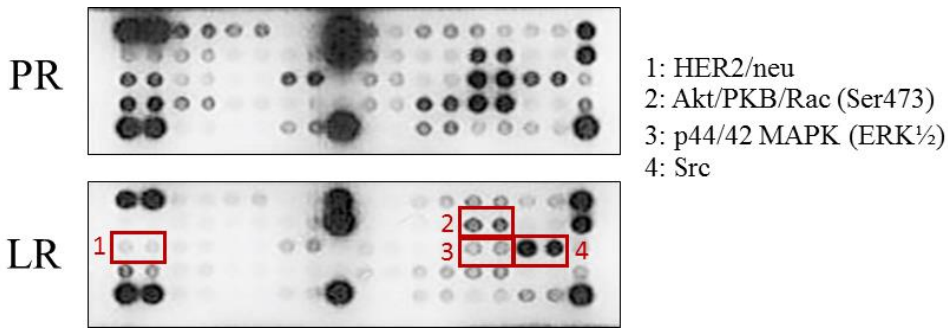


Figure 3. Expression of receptor tyrosine kinases (RTKs).

The basal expression of RTKs were evaluated by RTK antibody array. HER2/neu, Akt/PKB/Rac (Ser473) and p44/42 MAPK (ERK) were downregulated, and Src was upregulated in LR cells.

2. Activation of Src contributes resistance to lapatinib in LR cells.

To confirm the results of RTK antibody assay, Src family kinase expression in LR cells were examined (Fig. 4). The expression of Yes was not changed, but Src, Lyn and Fyn were increased in LR cells. Moreover, FAK, which interact with Src was up-regulated in LR cells. Src is an important molecule regulating cell migration. Therefore, cell migration was compared by wound healing assay (Fig. 6). Cell migration was significantly increased in LR cells.

Activation of Src increase invasion as well as migration, and this contributes to epithelial-mesenchymal transition (EMT). Thus, expression of Snail, Vimentin and Testican-1 which were associated with EMT were explored. LR cells showed down-regulation of Testican-1 which induce EMT. However, expression of Vimentin and Snail, which were well known as EMT markers were increased in LR cells (Fig. 4). Following these results, cell invasion was noticeably increased in LR cells (Fig. 7).

To confirm activation of Src increased cell migration and invasion, analysis were performed after inhibition of Src by Src inhibitor (Saracatinib). After treatment of saracatinib, activation of Src, Yes and Lyn were decreased (Fig 5). In addition, cell migration and invasion

were significantly decreased in LR cells (Fig. 6, 7). However, inhibition of Src by saracatinib was not affect to cell proliferation (Fig. 8). Based on these results, increase of cell migration and invasion via Src signaling was one of the mechanism, which generates lapatinib resistance.

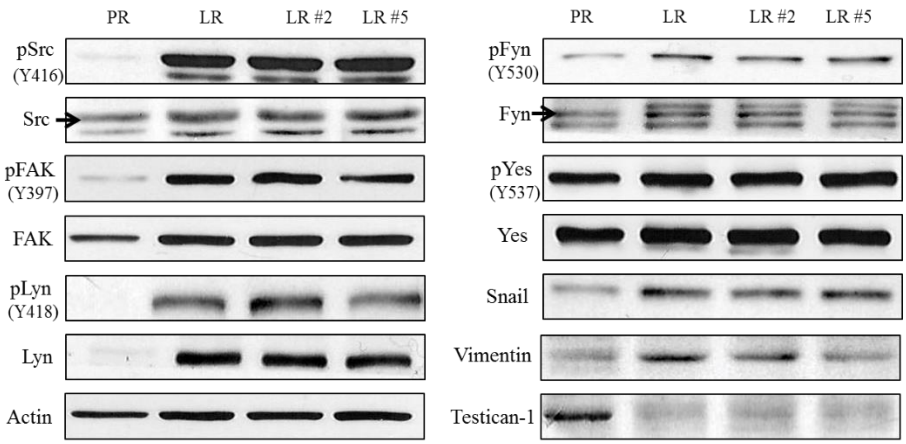


Figure 4. Expression of Src family kinase and EMT markers increased in LR cells.

Results of western blotting show difference between PR cells and LR cells. Active form of Src, FAK, Lyn, Fyn as well as EMT markers, such as Vimentin and Snail, levels were up-regulated in LR cells.

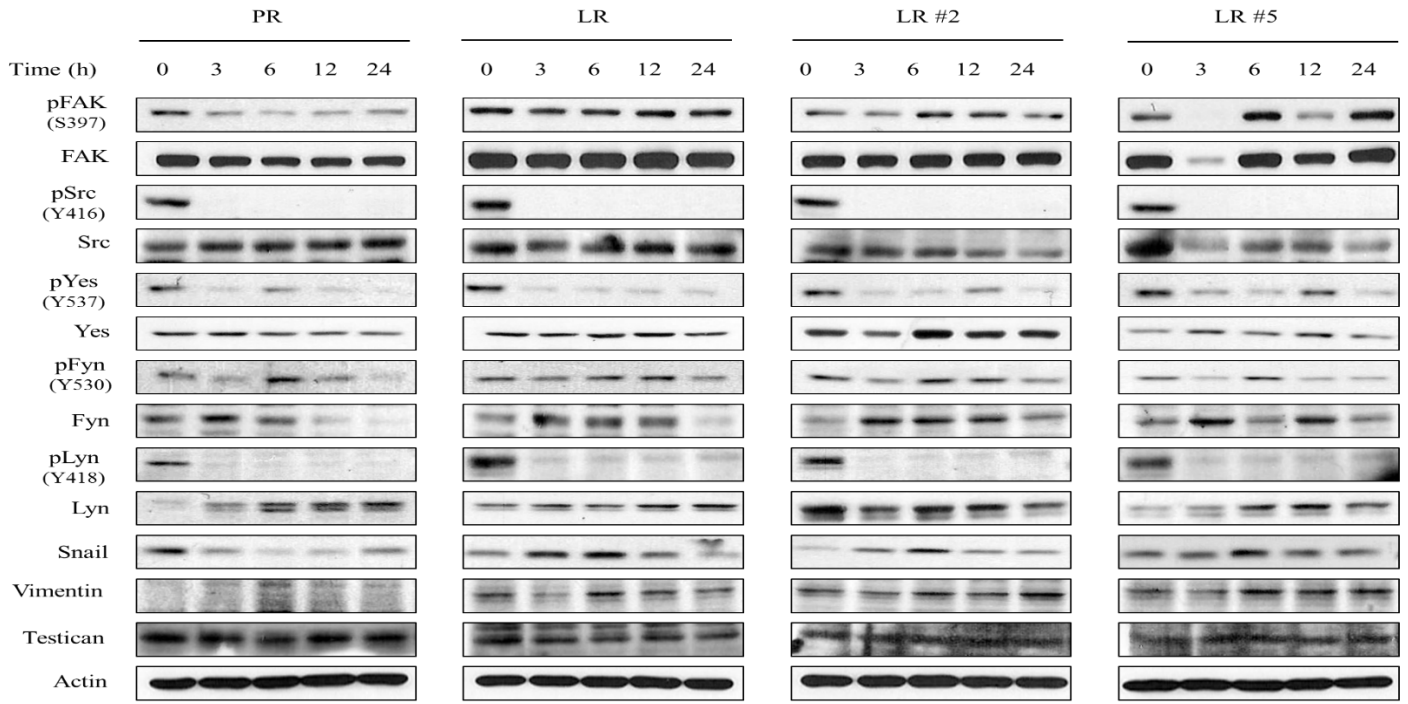
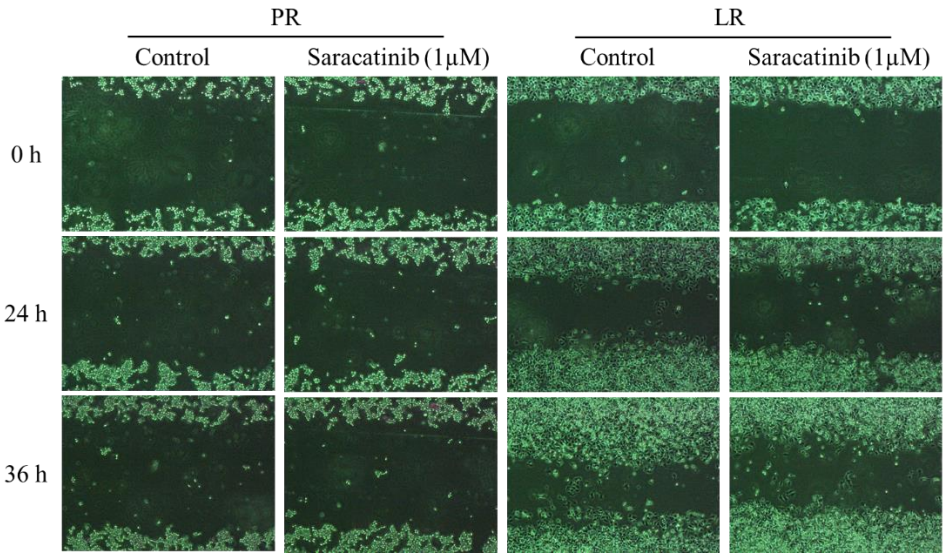


Figure 5. Saracatinib inhibits activation of Src family kinase.

Cells were treated with saracatinib (1 μM) for indicated times. The expression of Src family kinase and EMT markers were analyzed by western blotting. Saracatinib inhibits activation of Src, Yes and Lyn.


PR
LR
Control
Saracatinib (1μM)
Control
Saracatinib (1μM)
0 h
24 h
36 h

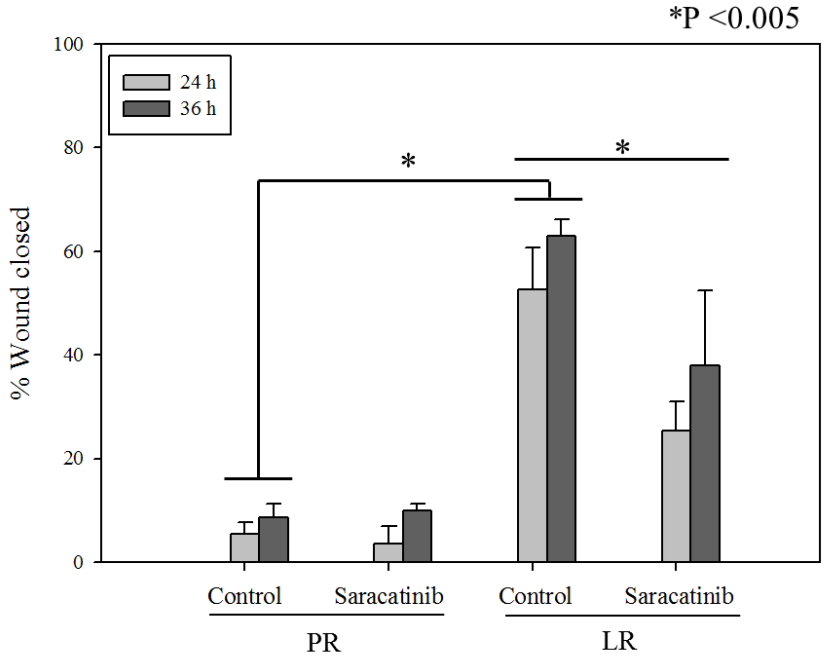
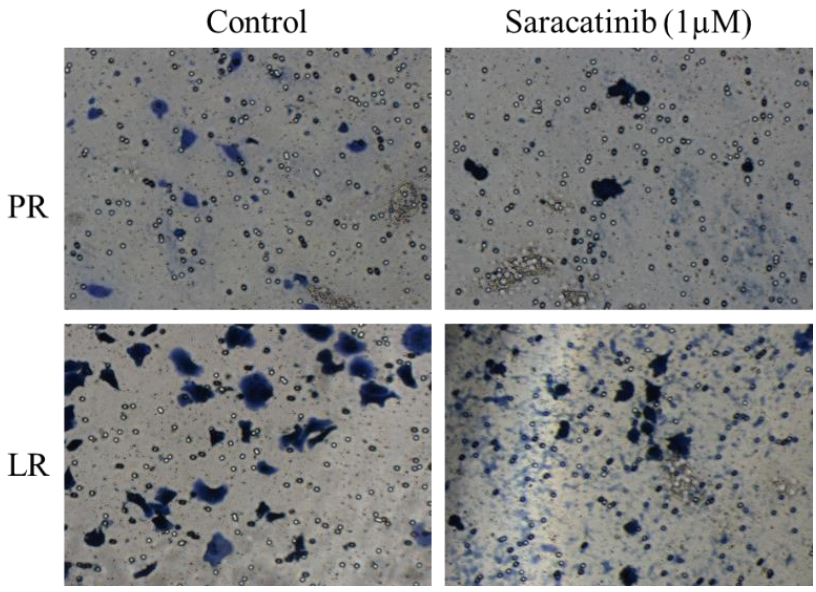

*P <0.005
24 h
36 h
% Wound closed
100
80
60
40
20
0
*
*
Control
Saracatinib
Control
Saracatinib
PR
LR

Figure 6. Cell motility was noticeably increased in LR cells and saracatinib inhibits cell migration.

Cell motility was examined by wound healing assay. Cells were treated with DMSO or saracatinib (1 μM). At the indicated time points, cells were examined with microscopy and the length of gap was analyzed using Image J software (NIH).


Control
Saracatinib (1μM)
PR
LR

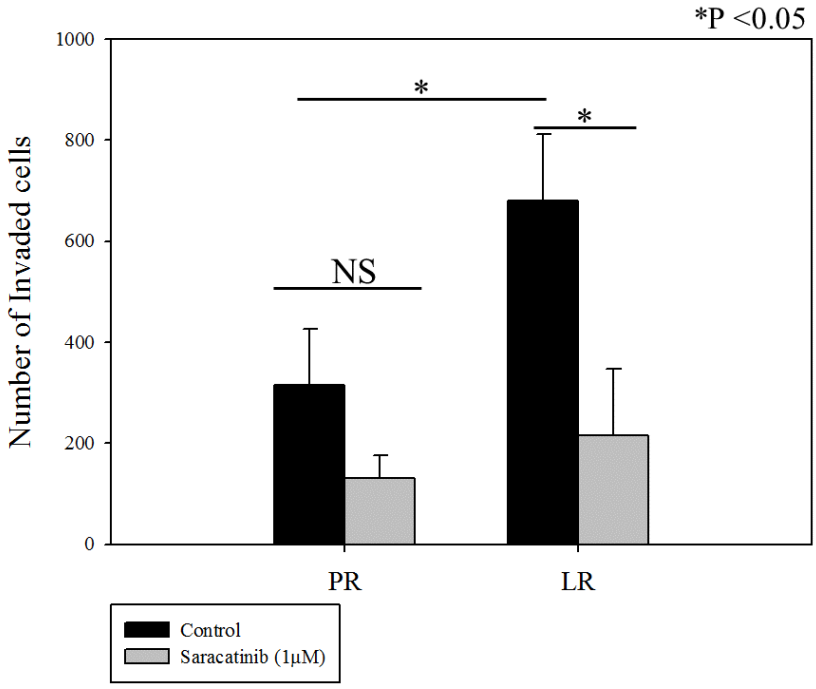

*P <0.05
Number of Invaded cells
1000
800
600
400
200
0
*
*
NS
PR
LR
Control
Saracatinib (1μM)

Figure 7. LR cells were more invasive than PR cells.

The invasive ability was evaluated by the Boyden chamber based Cell invasion assay. Before analysis, cells were starved for 18 h. Cells were resuspended with serum free medium and treated with DMSO or saracatinib (1 μM). Medium containing 10 % fetal bovine serum used for chemoattractant. Invasion cells were stained with coomassie brilliant blue and counted with microscopy.

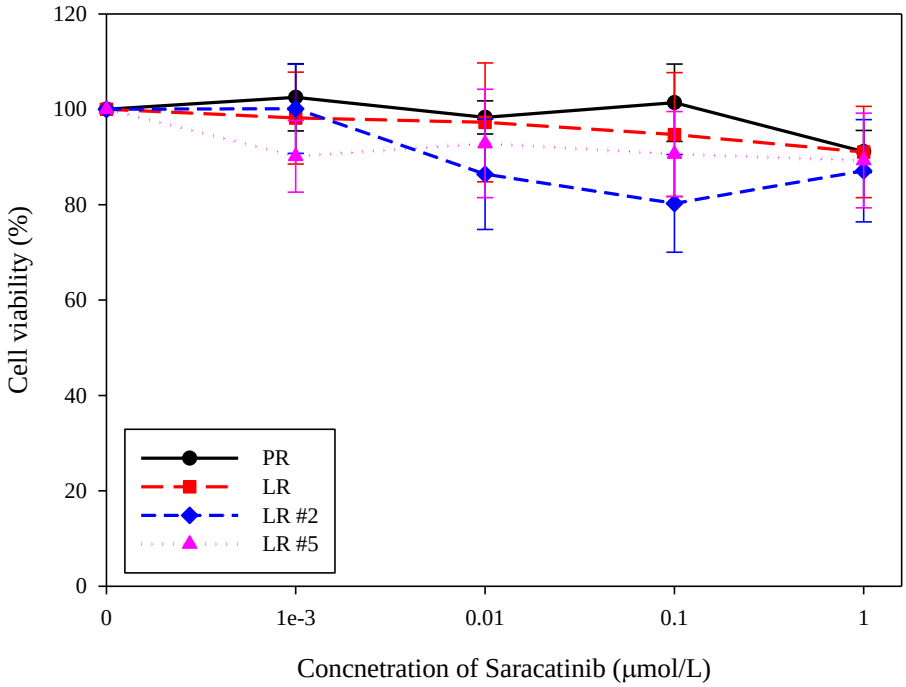


Figure 8. The effect of saracatinib on the growth of PR cells and LR cells.

Growth inhibition was analyzed by the MTT assay. Cells were treated with saracatinib for 72 hours as indicated lapatinib concentrations.

3. Whole exome sequencing showed RUNX3 missense mutation in LR cells and RUNX3 was down-regulated in LR cells.

To investigate the difference of PR cells and LR cells and to explore novel marker of resistance to lapatinib, whole exome sequencing (WES) of SK-BR-3 parental cells and LR cells were performed. Mutation of RUNX3 was detected: a missense mutation ($ATC^{Ile} \rightarrow AAC^{Asn}$) at exon 1 (Fig. 9, 10). RUNX3 is a transcription factor, well known as tumor suppressor in breast cancer [13]. Therefore, I focused on RUNX3 and hypothesized that RUNX3 affects the sensitivity of lapatinib by regulating expression of HER2 and Src. Down-regulation of RUNX3 was observed through Western blot assay. In addition, estrogen receptor α (ERα), which was regulated by RUNX3, was increased in LR cells (Fig. 11). RUNX3 is known to act as a tumor suppressor in the nucleus, but recent studies reported that it functions as an oncogenic molecule in the cytoplasm [13-17]. Based on this fact, localization of RUNX3 was examined to determine whether RUNX3 actually acts as a tumor suppressor in SK-BR-3 cells. RUNX3 expression was observed in the cytosol and nucleus of PR cells with predominantly in the nucleus, but in LR cells, RUNX3 expression was not observed in both of the nucleus and cytosol (Fig. 12).

Consequently, LR cells showed down-regulation of RUNX3 that was regarded as a tumor suppressor in SK-BR-3 cells.

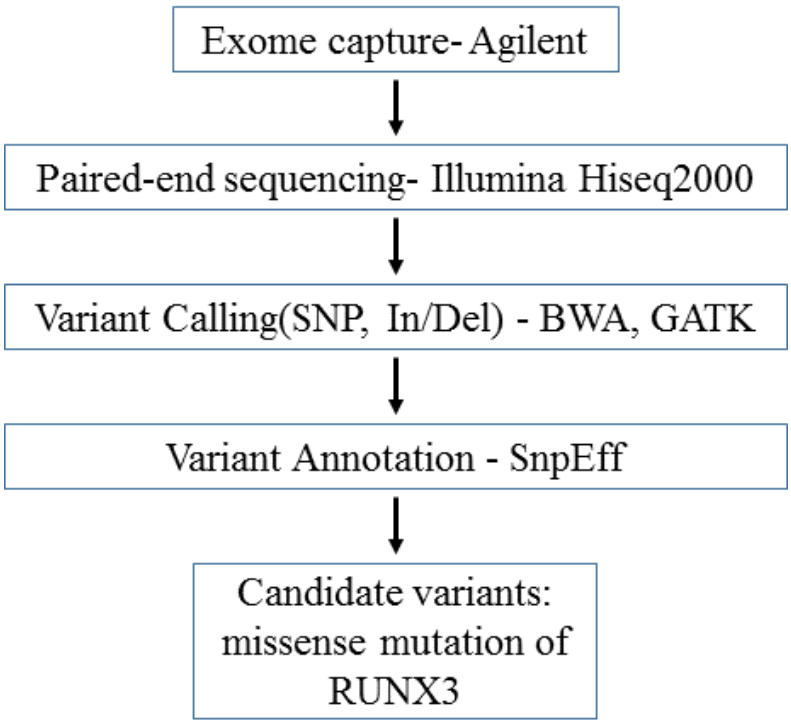


Figure 9. Scheme of Whole exome sequencing (WES)

Exome captures were performed using SureSelect V5+ UTR-post and sequencing was performed with the Illumina Hiseq 2000. Raw fastq files were aligned to hg19, and SNPs and indels were called using BWA and GATK. Variants annotation was performed with SnpEff software.

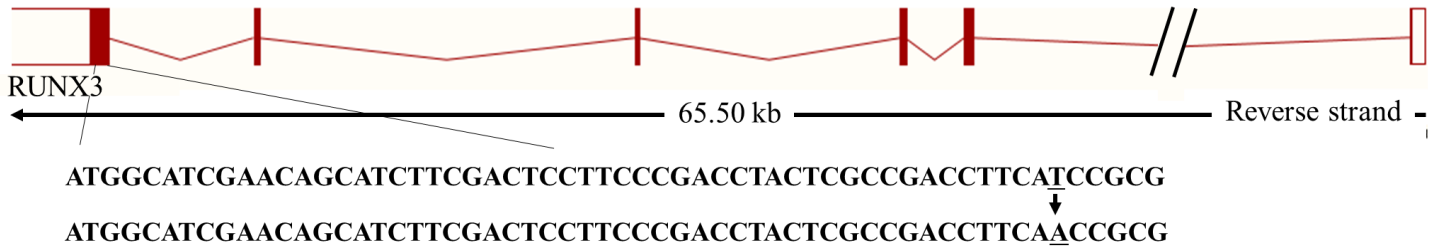


Figure 10. RUNX3 missense mutation was detected in LR cells.

RUNX3 missense mutation (c.53T>A; $ATC^{Ile} \rightarrow AAC^{Asn}$ at exon 1) was detected in LR cells.

Bold bar means exon and line means intron.

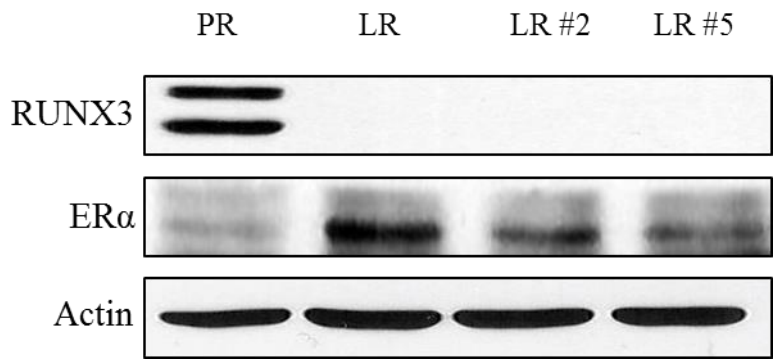


Figure 11. Expression of RUNX3 in PR cells and LR cells.

Western blot analysis of RUNX3 and ER α expression in SK-BR-3 cells and LR cells. RUNX3 was down-regulated and ER α was up-regulated in LR cells.

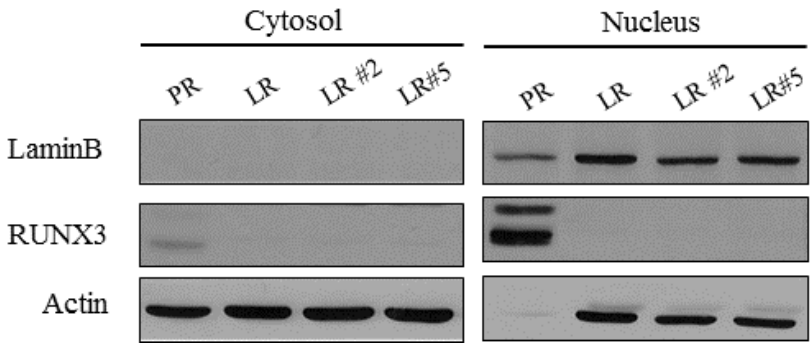


Figure 12. Localization of RUNX3 in PR cells and LR cells.

Localization of RUNX3 was confirmed by western blotting. RUNX3 was predominantly localized in Nucleus in PR cells. However, RUNX3 was down-regulated in LR cells.

4. Expression of RUNX3 affects lapatinib sensitivity in SK-BR-3 cells.

To determine whether down-regulation of RUNX3 contributes resistance to lapatinib, knock-down of RUNX3 of PR cells using siRNA was performed. The experiments were performed following the scheme (Fig. 13). Down-regulation of RUNX3 by si-RUNX3 was confirmed by western blotting (Fig. 14). PR cells transfected with si-control as well as cells transfected with si-RUNX3 showed resistance to lapatinib compared with not transfected cells. But, RUNX3 knock-down cells were more resistance to lapatinib than transfected with si-control cells (Fig. 14, Table 2). Based on this result, down-regulation of RUNX3 contribute to the lapatinib resistance.

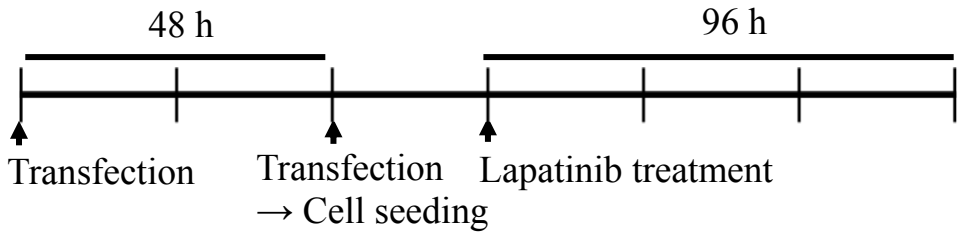


Figure 13. Scheme of transfection

PR cells were transfected with si-RUNX3 (80 nmol/L) or si-Control (80 nmol/L) according to scheme.

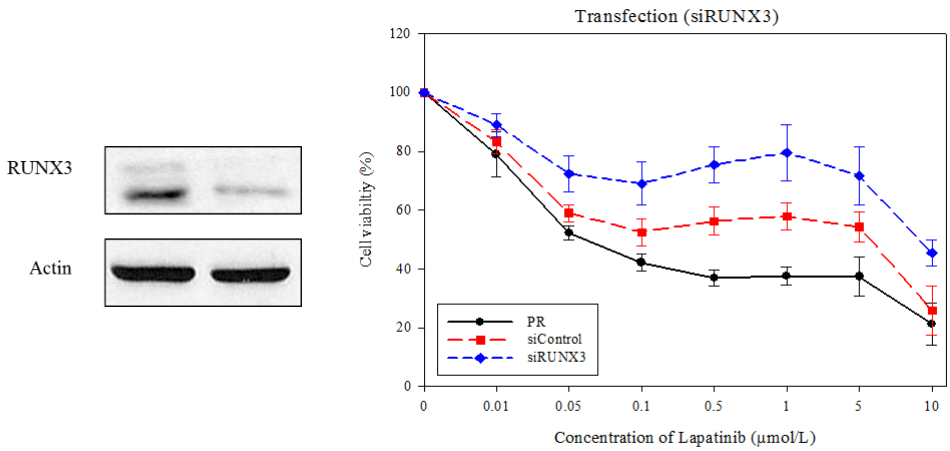


Figure 14. Knock-down of RUNX3 contributes resistance to lapatinib in SK-BR-3 cells.

Knock-down of RUNX3 was confirmed by western blotting. After transfection with siRNA, cells were harvest at the indicated time point. MTT assay using various concentration of lapatinib in PR cells without transfection, PR cells transfected with si-control RNA or si-RUNX3 were performed. Cells were incubated with lapatinib for 72 hours at the indicated concentration.

Cell line	Lapatinib IC_{50} (means±SD, μmol/L)
PR	0.054 ± 0.069
si-Control	0.12 ± 0.028
si-RUNX3	>10

Table 2. IC_{50} of lapatinib in PR cells and transfected cells.

Discussion

Lapatinib is a small molecule inhibitor, which can target both EGFR and HER2, and used for HER2 amplified breast cancer patients. However, resistance to lapatinib develops, limiting its long-term use [3, 5]. Various mechanisms of lapatinib resistance were suggested, but the factors that confer acquired resistance to lapatinib are not well characterized.

In this study, a novel mechanism of resistance to lapatinb and specified molecule associated resistance were explored. Lapatinib resistant (LR) SK-BR-3 cells were generated and characterized. Activation of EGFR/HER3 and downstream molecules of HER2 were well known mechanism of resistance to lapatinib [21]. Thus, expression of HER family and signal transduction molecules associated HER2 were examined. Several studies suggested that the activation of PI3K/Akt signal and MAPK/ERK signal promote resistance to lapatinib [22, 23]. Unlike the facts demonstrated, the data showed down-regulation of pEGFR, pHER3, pAkt and pERK in LR cells. These results imply that other mechanism contributes to resistance.

Several studies suggested that activation of Src induces resistance

to lapatinib [3, 9-10]. In parallel, LR cells showed up-regulation of Src family kinase, which involved in Src, cell migration and invasion were significantly increased in LR cells. After treatment with sarcatinib, activation of Src family kinase, cell migration and invasion were significantly decreased in LR cells. Based on these data, activation of Src contributes to lapatinib resistance in SK-BR-3 cells, and further studies regarding which mechanism involved in Src activation in LR cells are needed.

To discover a novel mechanism and specified molecule associated lapatinib resistance, whole exome sequencing (WES) of parental SK-BR-3 cells and LR cells were performed. Missense mutation (c.53T>A; $ATC^{Ile} \rightarrow AAC^{Asn}$ at exon 1) in RUNX3 gene was found in LR cells. LR cells showed down-regulation of RUNX3 because of missense mutation, which were identified by WES. RUNX3 was known as a tumor suppressor which localized in nucleus, and it does not function by hypermetylation, mislocalization and gene loss in breast cancer [13-15]. Recent studies suggested that the missense mutation(c.53T>A) of RUNX3 correlated with risk of *H. pylori* infection in gastric cancer [24]. However, it has not been reported that this mutation affects breast cancer. To examine that down-regulation of RUNX3 affects sensitivity to lapatinib, knock-down of RUNX3 was performed using

siRNA in SK-BR-3 parental (PR) cells. Sensitivity of laptinib was decreased in RUNX3 knock-down PR cells. Thus, I inferred that RUNX3 might be a potential marker of lapatinib resistance.

Src phosphorylate tyrosine residue of RUNX3 and translocalized nucleus to cytosol [25]. Correlated this fact, RUNX3 partly localized in the cytosol in PR cells. However, whether RUNX3, as a transcription factor, affects expression of Src has not been studied and needs to study. Several studies suggested that RUNX3 regulates expression of Vimentin and loss of RUNX3 promotes cancer metastasis in gastric cancer [14]. In parallel, up-regulation of Vimentin and increased cell invasion in LR cells. To confirm if down-regulated RUNX3 change signal, observation for long-term is required. However, in the cells transfected with si-Control was decreased sensitivity of lapatinb, I could not use siRNA system to down-regulate RUNX3 for long-term. Therefore, I need to RUNX3 knock-out by other systems and should be conducted further study.

In conclusion, Src activation contributes to lapatninb resistance in SK-BR-3 cells and RUNX3 might be a potential marker, which partially contributes resistance to lapatinib.

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국문 초록

Lapatinib은 EGFR과 HER2 의 tyrosine kinase domain 에 작용하여 EGFR 과 HER2 신호 전달을 억제하는 tyrosine kinase 억제제로써, HER2 양성 유방암 환자의 표준치료제 중의 하나이다. 그러나 환자에게 내성이 나타남으로 인해 사용에 한계가 있다. Lapatinib에 대한 여러 가지 내성기전이 보고되었으나, 알려지지 않은 내성기전이 여전히 존재하고, 이것은 해결해야할 문제로 남아있다. 따라서 본 논문에서는 lapatinib의 새로운 내성기전과 이와 관련된 특정 분자를 밝혀내고자 하였다.

SK-BR-3 세포주에 lapatinib을 장기간 처리하여 lapatinib 내성 세포주 (LR)를 수립하고, MTT assay를 통해 lapatinib과 saracatinib에 대한 감수성을 확인하였다. 또한, western blot을 통해 세포신호전달 분자의 변화를 확인하였다. Wound healing assay와 Boyden chamber assay를 수행하여 세포 침윤능과 이동능을 확인하였으며, whole exome sequencing (WES)과 siRNA-knock down system을 이용하여 lapatinib에 대한 감수성에 미치는 영향을 비교하였다.

실험결과 LR 세포주에서 pHER2, pAkt 및 pERK의 발현은 감소한 반면, Src family kinase의 활성은 증가하였다. EMT 마커로 잘 알려진 Vimentin 또한 LR 세포에서 증가하였고 세포의 이동능과 침윤능이 유

의하게 증가하였다. 또한, saracatnib을 처리하였을 때 LR 세포의 이동능과 침윤능이 감소한 것을 확인하였다. WES에 의해 확인된 RUNX3의 missense 돌연변이(c.53T>A; $ATC^{Ile} \rightarrow AAC^{Asn}$ at exon 1)에 의해 LR 세포에서 RUNX3의 발현이 감소된 것을 확인하였다. 또한 siRNA에 의해 RUNX3가 knock-down된 SK-BR-3 세포에서 lapatinib에 대한 내성이 증가된 것을 확인하였다.

본 연구에서 Src family kinase의 활성화와 세포 이동 및 침윤이 LR 세포에서 증가한 것을 확인하였다. 또한 WES에 의해 확인 된 RUNX3의 missense 돌연변이가 SK-BR-3 세포의 lapatinib 감수성에 영향을 미친 것을 확인할 수 있었다. 이러한 결과들을 바탕으로, Src의 활성화가 lapatinib에 대한 저항성에 기여한다는 것을 확인할 수 있었으며, SK-BR-3 세포주에서 lapatinib 내성을 유발하는 잠재적인 마커로써 RUNX3의 가능성을 확인할 수 있었다.

주요어: HER2, Lapatinib, 내성, Src, RUNX3

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