



## Pyrotinib shows a potent antitumor effect on HER2-positive esophageal cancer cells by promoting HER2 proteasomal degradation

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### ABSTRACT

Pan-HER TKIs (pyrotinib, lapatinib) are potent HER2 inhibitors, however, their anti-tumor efficacy on esophageal cancer remains to be elucidated. Using two HER2-positive esophageal cancer cell lines, we observed that both pyrotinib and lapatinib could significantly suppress the activation of HER2 and its downstream signaling. However, pyrotinib showed a potent inhibitory effect at 0.1  $\mu\text{M}$  treatment relative to 1  $\mu\text{M}$  of lapatinib. Moreover, treatment with pyrotinib, but not lapatinib, markedly reduced the protein level of HER2 through enhancing HER2 ubiquitination level and proteasomal degradation. In vitro and in vivo experiments further revealed that pyrotinib effectively suppresses cancer cell invasion and migration, as well as the growth of tumors in nude mice. Overall, our results suggest that pyrotinib is a superior TKI over lapatinib in inhibiting esophageal cancer cell proliferation and tumorigenic potential, and can be chosen as a neo-adjuvant for esophageal cancer treatment.

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### Introduction

Squamous cell carcinoma and adenocarcinoma are the two main types of esophageal cancer, which is ranked as the sixth highest contributor to cancer-related fatalities globally (1). The development of esophageal cancer is linked to both genetic and environmental influences. Especially along with the rapid advance of the high-throughput analysis techniques, many genetic and epigenetic alterations have been identified in EC specimens, among which HER2, also known as HER2/neu encoded by ERBB2, has been found to be amplified or overexpressed in 10% of esophageal squamous and 30% of adenocarcinoma (2, 3). HER2 belongs to the family of tyrosine protein kinases and can dimerize with HER family receptors to activate multiple intracellular signaling pathways, such as RAS/MAPK and PI3K/AKT, which are responsible for uncontrolled cell growth and the advancement of tumors (4, 5).

HER2 amplification/overexpression was associated with a poor prognosis in patients with operable esophageal cancer (6). Adjuvant blockage of HER2 by a humanized monoclonal antibody against HER2 (trastuzumab, also named Herceptin) has yielded promising outcomes in various human cancers including esophageal cancer, with a higher median overall survival (mOS) than chemotherapeutic drug alone (7, 8). Lapatinib, another class of HER2 blockers through targeting the HER2 tyrosine kinase ac-

tivity, has been clinically tested as an adjuvant agent to chemotherapy. The results (9) did show an improved PFS in metastatic HER2-positive breast cancer patients, but not in the overall trial population with HER2-positive upper gastrointestinal (UGI) adenocarcinoma where only a subgroup of Asian UGI cancer patients showed a much improved PFS (10, 11). Meanwhile, the adverse cytotoxic effect also limits its clinical use (9, 12).

In 2018, China conditionally approved pyrotinib as an adjuvant medication for advanced or metastatic HER2-positive breast cancer, in combination with capecitabine. Pyrotinib is an irreversible pan-HER TKI, and it was the first drug to receive this approval (13, 14). Its efficacy has been clinically tested in metastatic breast cancer, advanced NSCLC, and gastric cancer, and all patients receiving pyrotinib showed favourable benefits in terms of objective response rate (ORR), median progression-free survival (PFS), and median overall survival (OS) (15-17). Meanwhile, adjuvant pyrotinib exhibited a much better efficacy than lapatinib (18). However, the anti-tumor effect of pyrotinib against esophageal cancer is still not thoroughly investigated. In this research paper, we chose two HER2-positive esophageal cancer cell lines to compare the potency of pyrotinib vs. lapatinib in suppressing the HER2 activity and tumorigenicity. The findings will be useful in the guidance of pan-HER TKI neo-adjuvant use in esophageal cancer treatment.

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## Materials and Methods

### Cell lines and mice

The TE-1 and KYSE-150 cell lines, obtained as a gift from the Beijing Institute of Genomics, Chinese Academy of Sciences, were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were incubated in a humidified chamber at 37°C with 5% CO<sub>2</sub>.

BALB/c nude mice were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained in a specific pathogen-free facility at the Bethune International Peace Hospital. All animal experiments were approved by the Institutional Animal Care and Use Committee of Bethune International Peace Hospital approval.

### Antibodies and chemical reagents

The following chemical reagents were used: Pyrotinib from Jiangsu Hengrui Pharmaceuticals Co, Ltd. (Lianyungang, China); Lapatinib; MG-132, Bortezomib(PS-341) were from Selleck Chemicals (Houston, TX, USA); Bafilomycin A1 was from MedChemExpress (Monmouth Junction, NJ, USA); TB Green Premix Ex Taq™ II, PrimeScript™ RT reagent Kit with gDNA Eraser from TaKaRa (Tokyo, Japan);

Antibodies: phospho-HER2 (Tyr1221/1222), HER2, phospho-AKT (Ser473), phospho- ERK (Thr202/Tyr204), ubiquitin (E4I2J) from Cell Signaling (Danvers, MA, USA); Erk1/2, Protein A/G PLUS-Agarose, Normal mouse IgG, HER2 mouse mAb from Santa Cruz (Santa Cruz, CA, USA); AKT, GAPDH, HSP70 from Proteintech Group (Rosemont, IL, USA).

### Proliferation inhibition assay

TE-1(3000 cells/well) and KYSE-150 (2000 cells/well) cells were seeded into 96-well plates and left to attach overnight. Subsequently, lapatinib and pyrotinib were added at a concentration range of 0.1-32 µM. After incubation for 24 hours, 10 µl of CCK8 (Solarbio, Beijing, China) was performed to determine cell viability. The optical density (OD) values at 450 nm were determined by a microplate reader.

### Colony formation assay

TE-1 and KYSE-150 cells were seeded into the 35 mm dishes (500 cells/dish). The cells were cultured with or without Pyrotinib (0.1 µM). the cells underwent washing and fixation using methanol for 30 minutes. Subsequently, the cells were stained with 0.1% crystal violet and subjected to counting.

### Wound healing assay

TE-1 and KYSE-150 cells were seeded in 35 mm dishes. After the cells reached a confluency of 70-80%, a wound was scraped with a 200 µl pipette tip across the entire center. Subsequently, the cells were washed twice with PBS to eliminate the floating cells. The cells were cultured in RPMI 1640 without serum, either with or without Pyrotinib (0.1 µM). The specified time points were used to acquire the images.

### Transwell migration and invasion assays

The transwell migration assay was conducted with a transwell chamber (Corning, Corning, NY, USA). In the migration assay, 3×10<sup>4</sup> were seeded in the upper chamber with 200 µl of Pyrotinib-free RPMI 1640 or RPMI 1640 containing Pyrotinib (0.1 µM). The lower chamber was filled with 600 µl of RPMI 1640 supplemented with 10% FBS. After being incubated for 24 h, the cells were treated with methanol for 30 minutes to fix them. The stationary cells in the upper chamber were cautiously eliminated with a cotton swab. After staining with 0.1% crystal violet, the migrated cells were observed and quantified in five randomly chosen fields using a microscope. For the invasion assay, the top section was pre-covered with 100 µl of Matrigel. The remaining procedures were identical to those used in the transwell migration assay.

### Western blot analysis

Radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) was used to harvest and lyse TE-1 and KYSE-150 cells. The bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, Waltham, MA, USA) was used to measure the protein concentration. The same quantities of protein were isolated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes with a pore size of 0.45 µm (Thermo Fisher, Waltham, MA, USA). To block the membranes, either 5% skim milk or 3% bovine serum albumin (BSA) in tris buffered saline-tween (TBST) was used, followed by overnight incubation at 4°C with the designated primary antibodies. The detection reagents of enhanced chemiluminescence (ECL) were utilized to visualize protein bands.

### Co-immunoprecipitation (co-IP)

TE-1 and KYSE-150 cells were lysed in RIPA buffer (Solarbio, Beijing, China) and incubated with HER2 mouse mAb and Normal mouse IgG overnight at 4°C, then incubated with Protein A/G PLUS-Agarose for 8 hours. Immune complexes were pelleted by centrifugation at 6000 rpm for 1 min, washed twice with IP lysis buffer (50 mM Hepes, 0.1% Triton X-100) and 0.5 M NaCl in Hepes/Triton X-100. After being heated at 100°C for 10 min in 2×loading buffer, the binding complex was separated on 8% SDS-PAGE gels followed by Western blotting.

### Quantitative reverse transcription-PCR (qRT-PCR)

For mRNA level examination, total RNA from KYSE-150 and TE-1 cells was isolated by using TRIzol. Complementary deoxyribose nucleic acid (cDNA) was synthesized using the PrimeScript RT reagent kit qRT-PCR was performed using the TB Green Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Tokyo, Japan). GAPDH was used as an endogenous control for mRNA detection. The HER2 primers were as follows: forward, 5'-TGTGACTGCC-TGTCCCTACAA-3'; and reverse, 5'-CCAGACCATAG-CACACTCGG-3'. The GAPDH primers were as follows: forward, 5'-GGAGCGAGATCCCTCCAAAAT-3'; and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'. The 20 µl qPCR system contained 10 µl TB Green Premix Ex Taq II, 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM), 2 µl reverse transcription product. The reaction was performed on a LightCycler480 qPCR system

(Roche, Basel, Switzerland). The following PCR program was used: Pre-denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Then the fold change was calculated with the relative quantification method ( $2^{-\Delta\Delta Ct}$ ).

### In vivo antitumor activity

KYSE-150 cells ( $2 \times 10^6$ ) were subcutaneously injected on the right side of the dorsal area of 5-week-old female BALB/c nude mice. Tumor volumes were measured using calipers every three days:  $V = 1/2 \times (\text{length} \times \text{width}^2)$ . Once the tumor size reached about  $75 \text{ mm}^3$ , mice were randomly divided into 4 groups ( $n=6$ ): including control and three pyrotinib-treated groups (5, 20, 40 mg/kg/d). All BALB/c nude mice were sacrificed after 3 weeks and tumor tissues were weighed. Then, tumor tissues were analyzed using Western blotting and immunohistochemistry (IHC).

### Immunohistochemistry (IHC)

After the nude mice were sacrificed, tumors were dissected and fixed in 4% neutralized formaldehyde and embedded in paraffin. Immunostaining was performed on 4- $\mu\text{m}$ -thick sections using antibodies against Her-2. The IHC signals were assessed by pathologists. The staining intensity was scored in a range from 0–3 (0=none, 1=weak, 2=moderate, 3=strong).

### Statistical analysis

All data was expressed as mean  $\pm$  SEM. Measurement data were analyzed using the student's t-test. Statistical analysis was performed using Statistic Package for Social Science (SPSS) 25.0 software (IBM, Armonk, NY, USA).  $P < 0.05$  was considered statistically significant.

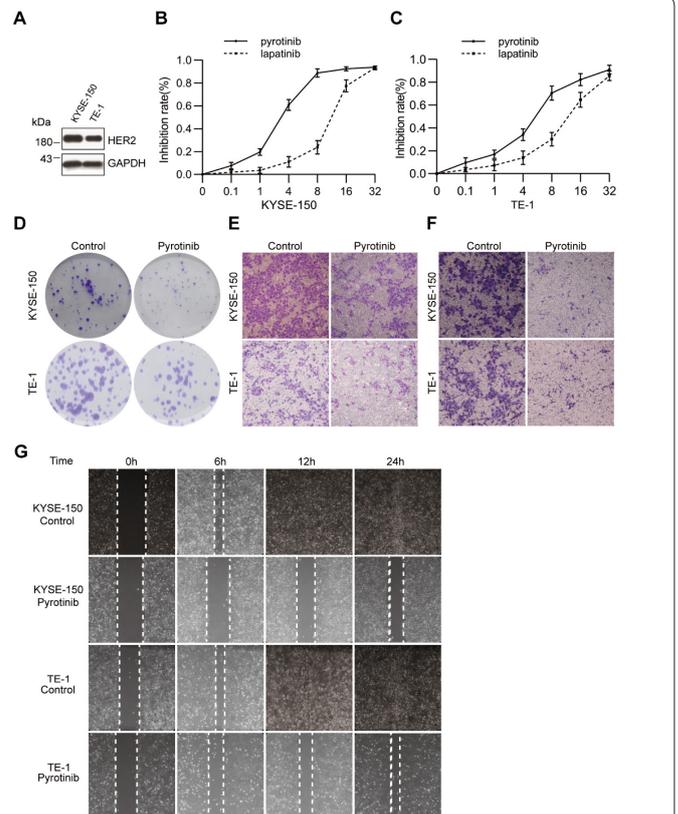
## Results

### Pyrotinib more potently inhibits cell proliferation than lapatinib in HER2-positive esophageal cancer cells

Two esophageal cancer cell lines, KYSE-150 and TE-1 cells expressing a high level of HER2 were used in this study (Figure 1A). We first tested the growth inhibition effect of pyrotinib relative to lapatinib by a CCK8 assay, and the results showed that pyrotinib treatment could inhibit the growth of both KYSE-150 and TE-1 cells in a dose-dependent manner with a significant effect starting at 0.1  $\mu\text{M}$  in relative to lapatinib at 1  $\mu\text{M}$ , suggesting that pyrotinib is more potent than lapatinib for anti-tumor activity (Figure 1B and C).

We further performed colony formation assay as well as transwell migration and invasion assay to test the anti-tumor efficacy of pyrotinib, and found that compared with the control group, pyrotinib significantly inhibits the colony forming efficiency ( $P < 0.001$ ) (Figure 1D) and invasive and metastatic potentials ( $P < 0.001$ ) (Figure 1E and F). The scratch assay results also support the above findings showing that the scratch healing time of the two cells treated with pyrotinib is significantly longer than that of the vehicle control group ( $P < 0.001$ ) (Figure 1G).

In summary, pyrotinib is superb over lapatinib in inhibiting the proliferation of HER2-positive esophageal cancer cells, and meanwhile, pyrotinib also showed potent activity in inhibiting cancer cell migration and invasion.

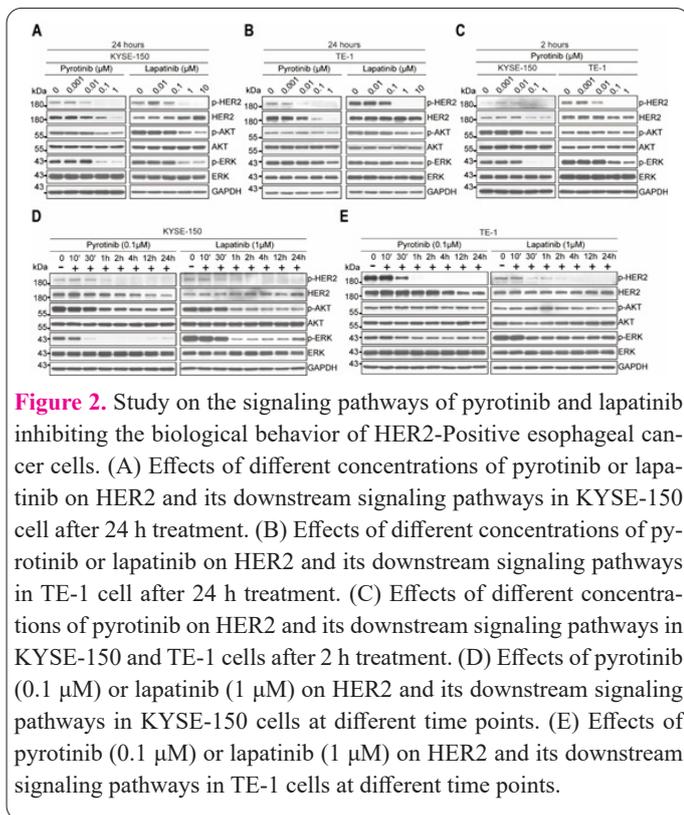


**Figure 1.** Effect of pyrotinib on the biological behavior of HER2-positive esophageal cancer cells. (A) HER2 expression of KYSE-150 and TE-1 cells was detected by Western blot. (B, C) Inhibitory effect on cell proliferation of KYSE-150 and TE-1 cells treated with different concentrations of pyrotinib and lapatinib. (D) Detect the effect of pyrotinib (0.1  $\mu\text{M}$ ) on the proliferation of KYSE-150 and TE-1 cells by colony formation assay. (E) Transwell migration assays showed that pyrotinib (0.1  $\mu\text{M}$ ) inhibited the migration ability of KYSE-150 and TE-1 cells. (200 $\times$ ), scale bars=100  $\mu\text{m}$ . (F) Transwell invasion assays showed that pyrotinib (0.1  $\mu\text{M}$ ) inhibited the invasion ability of KYSE-150 and TE-1 cells. (200 $\times$ ), scale bars=100  $\mu\text{m}$ . (G) Pyrotinib (0.1  $\mu\text{M}$ ) inhibited the migration of KYSE-150 and TE-1 cells by wound healing assays. (40 $\times$ ), scale bars=500  $\mu\text{m}$ .

### Pyrotinib treatment decreased HER2 while lapatinib increased the HER2 protein level in HER2-Positive esophageal cancer cells

We then examined the inhibitory effect of pyrotinib vs. lapatinib on HER2 and its downstream signals. As expected, treatment with different concentrations of pyrotinib or lapatinib for 24 h could markedly decrease the levels of p-HER2, and its downstream p-ERK and p-AKT in KYSE-150 and TE-1 cells with an effective concentration of 0.1  $\mu\text{M}$  for pyrotinib and 1  $\mu\text{M}$  for lapatinib, which is consistent with their behaviors in a cell proliferation assay. It should be noted that the p-AKT level in TE-1 cells upon pyrotinib treatment only changed slightly. Intriguingly, pyrotinib treatment decreases the expression of HER2 protein, while lapatinib increases HER2 protein expression (Figure 2A-B). To exclude the possibility that the decreased HER2 protein level might result from the longer inhibition of p-HER2, we further treated the cells with pyrotinib for 2 h and observed a similar trend in HER2 protein expression (Figure 2C), suggesting that pyrotinib can simultaneously inhibit HER2 activation and expression.

We further monitored the HER2 and its downstream signaling levels at different time points post 0.1  $\mu\text{M}$  pyrotinib



**Figure 2.** Study on the signaling pathways of pyrotinib and lapatinib inhibiting the biological behavior of HER2-Positive esophageal cancer cells. (A) Effects of different concentrations of pyrotinib or lapatinib on HER2 and its downstream signaling pathways in KYSE-150 cell after 24 h treatment. (B) Effects of different concentrations of pyrotinib or lapatinib on HER2 and its downstream signaling pathways in TE-1 cell after 24 h treatment. (C) Effects of different concentrations of pyrotinib on HER2 and its downstream signaling pathways in KYSE-150 and TE-1 cells after 2 h treatment. (D) Effects of pyrotinib (0.1  $\mu$ M) or lapatinib (1  $\mu$ M) on HER2 and its downstream signaling pathways in KYSE-150 cells at different time points. (E) Effects of pyrotinib (0.1  $\mu$ M) or lapatinib (1  $\mu$ M) on HER2 and its downstream signaling pathways in TE-1 cells at different time points.

or 1  $\mu$ M lapatinib treatment. In KYSE-150 cells, a marked decrease in p-HER2, p-AKT and p-ERK levels emerged at 1 h post pyrotinib or lapatinib treatment. Meanwhile, the HER2 protein level in the pyrotinib group showed a progressive decrease along treatment time, in contrast to the lapatinib group where the HER2 protein level was kept constant during treatment with a slight increase at 24 h. A similar trend was also found in TE-1 cells except p-AKT showing a slight decrease post-treatment. (Figure 2D).

Overall, this result demonstrates that pyrotinib is more potent in inhibiting the activation of HER2 and its downstream signals than lapatinib. More importantly, different from lapatinib, pyrotinib could also decrease the HER2 protein level.

**Pyrotinib enhances HER2 ubiquitination and proteosomal degradation**

To determine whether HER2 suppression induced by pyrotinib or lapatinib treatment is at the transcriptional level, we employed RT-qPCR to quantify the expression level of HER2 mRNA before or after pyrotinib and lapatinib treatment, and the expression level of HER2 mRNA slightly increased with time (Figure 3A). As the proteosomal mechanism has been implicated in HER2 protein regulation (19), we then treat the cells with pyrotinib combined with or without proteasome inhibitor Velcade (MG-132). The result showed that Velcade (MG-132) substantially slows HER2 protein degradation (Figures 3B and 3C), whereas concomitant treatment with lysosomal inhibitor Baf-A1 didn't show this effect (Figure 3D), suggesting that HER2 protein expression is regulated through a proteasomal mechanism.

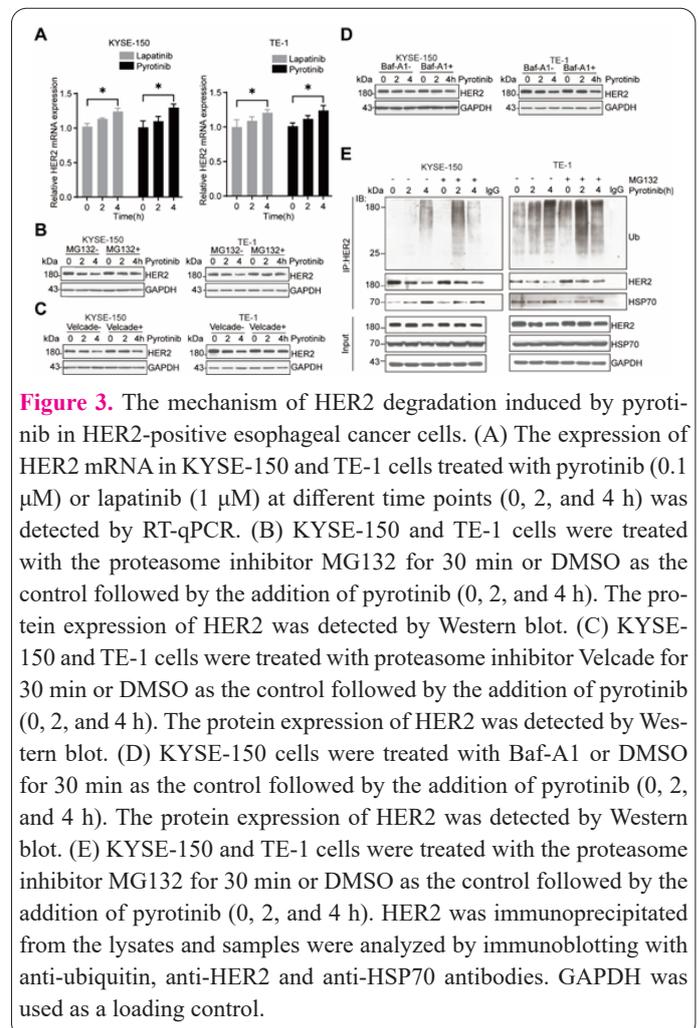
We next tested HER2 ubiquitination levels in both esophageal cells. Cells treated with pyrotinib in the presence or absence of MG-132 were lysed followed by immunoprecipitation with anti-HER2 antibody and Western blotting analysis with anti-Ub antibody. The result showed that the

HER2 ubiquitination level was increased upon pyrotinib treatment, and even pronounced at 2 h of pyrotinib plus MG-132 treatment (Figure 3E), indicating that pyrotinib enhances HER2 ubiquitination and the following proteasomal degradation.

HSP70 is a multifunctional chaperon participating in the regulation of protein stability (20). Our result showed that HSP70 protein levels are increased in the anti-HER2 immunoprecipitated complex, suggesting that pyrotinib can promote the association between HER2 and HSP70. But how this enhanced binding facilitates HER2 protein degradation needs further investigation.

**Pyrotinib treatment significantly suppresses the tumor growth of HER2-positive esophageal cancer in nude mice**

The therapeutic effect of pyrotinib on HER2-positive esophageal cancer was tested in nude mice. The in vivo Xenograft models of esophageal cancer were established and divided into four groups: control and three pyrotinib treatment groups (low: 5 mg/kg/day, medium: 20 mg/kg/day and high: 40 mg/kg/day). The results of antitumor experiments of different doses of pyrotinib on transplanted tumors in nude mice showed that the weights of implanted tumors in the control group are significantly greater than those in the pyrotinib treatment groups ( $P < 0.001$ ) (Figure 4A and B); The body weights of the nude mice in the four groups are not statistically different ( $P > 0.05$ ) (Figure 4C); The protein expression levels in the tumor tissues of KYSE-150 cell xenograft are detected by Western blot, and the results showed that the expression levels of p-



**Figure 3.** The mechanism of HER2 degradation induced by pyrotinib in HER2-positive esophageal cancer cells. (A) The expression of HER2 mRNA in KYSE-150 and TE-1 cells treated with pyrotinib (0.1  $\mu$ M) or lapatinib (1  $\mu$ M) at different time points (0, 2, and 4 h) was detected by RT-qPCR. (B) KYSE-150 and TE-1 cells were treated with the proteasome inhibitor MG132 for 30 min or DMSO as the control followed by the addition of pyrotinib (0, 2, and 4 h). The protein expression of HER2 was detected by Western blot. (C) KYSE-150 and TE-1 cells were treated with proteasome inhibitor Velcade for 30 min or DMSO as the control followed by the addition of pyrotinib (0, 2, and 4 h). The protein expression of HER2 was detected by Western blot. (D) KYSE-150 cells were treated with Baf-A1 or DMSO for 30 min as the control followed by the addition of pyrotinib (0, 2, and 4 h). The protein expression of HER2 was detected by Western blot. (E) KYSE-150 and TE-1 cells were treated with the proteasome inhibitor MG132 for 30 min or DMSO as the control followed by the addition of pyrotinib (0, 2, and 4 h). HER2 was immunoprecipitated from the lysates and samples were analyzed by immunoblotting with anti-ubiquitin, anti-HER2 and anti-HSP70 antibodies. GAPDH was used as a loading control.

HER2, HER2, p-AKT and p-ERK in the tumor tissues of pyrotinib treatment groups are markedly lower than those in the control group (Figure 4D). Immunohistochemistry staining result proved that the protein expression of HER2 in tumor tissues is significantly decreased in the groups of low, medium, and high pyrotinib doses compared with that in the control group (Figure 4E). Thus, pyrotinib exhibits potent antitumor activity against HER2-positive esophageal cancer in nude mouse xenograft models by promoting HER2 degradation and inhibiting its downstream MAPK/ERK and PI3K/AKT signaling pathways.

## Discussion

Both Lapatinib and pyrotinib are potent TKIs simultaneously inhibiting HER1 (EGFR), HER2 and HER4 and have been approved for the treatment of patients with metastatic HER2-positive breast cancer (13, 21). But only neoadjuvant pyrotinib, instead of lapatinib, showed promising efficacy (14, 18, 22). In a small number of non-breast solid cancer including lung and gastric, pyrotinib also exhibited promising benefits with well-manageable toxicities (23). However, the efficacy of pyrotinib and

its superiority over lapatinib in HER2-positive esophageal cancer remains to be elucidated. In this study, we employed HER2-positive esophageal cancer cell lines and demonstrated that the anti-tumor potency of pyrotinib was much higher than that of lapatinib, evidenced by the observation that a much lower concentration of pyrotinib is needed to achieve the goals of inactivating HER2 and its downstream signaling. Meanwhile, the in vitro cell invasiveness and in vivo tumorigenic potential can be significantly suppressed by pyrotinib, suggesting the potential value of pyrotinib as a neo-adjuvant agent in the treatment of HER2-positive esophageal cancer.

Ubiquitination is an essential post-translational modification embarking the target proteins for degradation by a proteasome pathway or initiating non-degradation biological functions (24). The current evidence supports that HER2 function was tightly regulated by the ubiquitination pathway. Both a bispecific tetravalent anti-HER2 antibody (Anti-HER2-Bs) and a pan-TKI (neratinib) have been shown to induce the rapid internalization and efficient degradation of HER2 receptor through enhancing its ubiquitination level in HER2-positive breast cancer or ovarian cancer (25, 26). In support, we also observed that pyrotinib, but not lapatinib, promotes HER2 protein ubiquitination and subsequent degradation through a proteasome-dependent pathway. This result mechanistically explains the underlying mechanism for the superior anti-tumor effect of pyrotinib relative to lapatinib.

We also observed an increased association of HER2 with heat shock protein 70 (HSP70) following pyrotinib-induced HER2 ubiquitination and degradation. HSP70 is the ubiquitous molecular chaperone regulating nearly all stages of the life of proteins from synthesis to degradation through cooperating with other cellular chaperone systems, such as HSP90 (27). HER2 has been shown to interact with HSP90 to avoid its internalization, whereas HSP90 inhibition leads to HER2 internalization and degradation (28, 29). But how HSP70 chaperone promotes HER2 ubiquitination and proteasomal degradation through an increased association is not clear and needs further investigation.

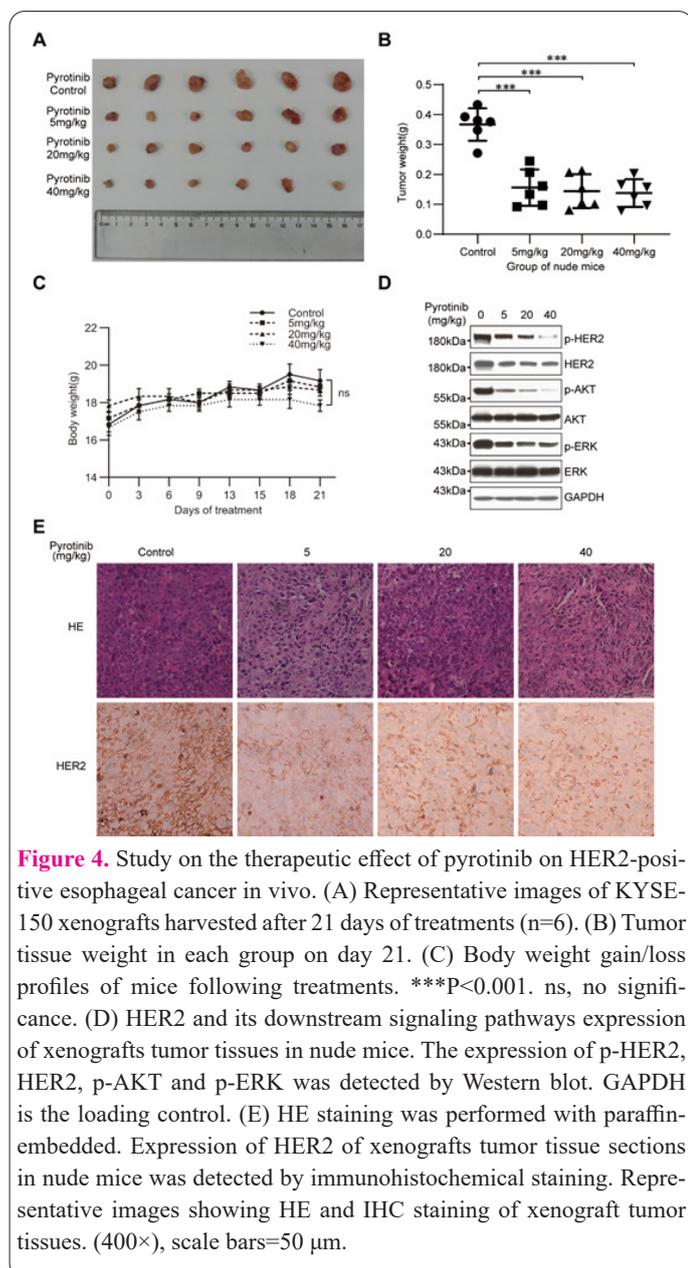
Esophageal cancer patients with overexpressed HER2 are considered as good candidates for the HER2-targeted therapy (2,3). Although combination of HER2 blockade with chemotherapy has been successful in HER2-positive breast cancer (7-9), the addition of trastuzumab (Heceptin) to neoadjuvant chemoradiotherapy for HER2-overexpressing esophageal cancer was not effective (3). The observation from our and others suggest that pan-HER TKI pyrotinib can serve as a better alternative for a neo-adjuvant strategy in inhibiting HER2 activation and treatment of HER2-positive esophageal cancer.

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## Author Contributions

Conceptualization, TN Z and F Z; Formal analysis, F Z and JK L; Experimental studies and Methodology, F Z and WJ R; Writing—original draft, F Z; Writing—review & editing, F Z and RJ Z All authors have read and agreed to the published version of the manuscript.



**Figure 4.** Study on the therapeutic effect of pyrotinib on HER2-positive esophageal cancer in vivo. (A) Representative images of KYSE-150 xenografts harvested after 21 days of treatments (n=6). (B) Tumor tissue weight in each group on day 21. (C) Body weight gain/loss profiles of mice following treatments. \*\*\*P<0.001. ns, no significance. (D) HER2 and its downstream signaling pathways expression of xenografts tumor tissues in nude mice. The expression of p-HER2, HER2, p-AKT and p-ERK was detected by Western blot. GAPDH is the loading control. (E) HE staining was performed with paraffin-embedded. Expression of HER2 of xenografts tumor tissue sections in nude mice was detected by immunohistochemical staining. Representative images showing HE and IHC staining of xenograft tumor tissues. (400×), scale bars=50 μm.

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## Conflicts of Interest

The authors declare no conflict of interest.

## Ethics approval and consent to participate

Not applicable.

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