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YAP/TAZ Pathway Promoted the Trastuzumab Resistance in HER2-Positive Breast Cancer

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WW and MZ conceived and designed experiments, performed experiments and data analysis, and wrote the manuscript. YL, FZ, ZW, SM and YQW provided technical support, data collection and analysis. All authors provided final approval for submitted and published version.

Key words
HER2-positive breast cancer, Trastuzumab resistance, YAP/TAZ

ABSTRACT

The developed resistance of trastuzumab remained a problem for clinical therapy of HER2-positive breast cancer. However, effects of YAP/TAZ pathway on resistance of trastuzumab have not been explored. Tumor tissues were collected from 40 breast cancer patients for clinical studies. For *in vitro* studies, human breast cancer cell lines SK-BR-3-TS was obtained, and trastuzumab resistant model SK-BR-3-TR was constructed. Cell viability was determined using MTT assay. Cell apoptosis was analyzed by flow cytometry. Protein and mRNA expression was measured using western blotting and RT-qPCR, respectively. The mRNA and protein level of YAP was significantly increased in the tumor tissues of HER2-positive breast cancer patients. Consistently, the expression of YAP and TAZ were both dramatically upregulated in SK-BR-3-TR cells. The cell viability was increased, while cell apoptosis was inhibited in SK-BR-3-TS cells compared with SK-BR-3-TS. The depletion of YAP by si-YAP reversed the YAP/TAZ expression, cell viability and cell apoptosis in SK-BR-3-TR cells. YAP/TAZ pathway might induce the trastuzumab resistance in HER2-positive breast cancer and targeting YAP would be an alternative way for the clinical therapeutic methods of HER2 positive breast cancer.

INTRODUCTION

Breast cancer has been regarded as one of the cancers that caused significant morbidity and mortality in female (Akram *et al*., 2017). It was estimated that the 5-year survival rate was 80% in developed countries, whereas that was only 40% for developing countries (Coleman *et al*., 2008). Human epidermal growth factor receptor 2 (HER2) belonged to HER family, whose expression was demonstrated to be increased in around 20% breast cancer and associated with aggressive cancers and disease recurrence (Kunte *et al*., 2020). Trastuzumab has been identified as a monoclonal antibody, which could target against the extracellular domain of the HER2 receptor, and chemotherapy combined with trastuzumab was the standard therapeutic methods for the early-stage therapy of breast cancer in the past few decades (Zhang, 2021). However, it was reported that only a small part of metastatic patients was responded to trastuzumab, and around 60% of them developed resistance (Han *et al*., 2020). Therefore, investigations on the mechanisms regarding resistance occurrence and approaches to reducing resistance are of vital importance.

YAP was identified as a 65 kDa protein that contained transcriptional activation domain and TEAD binding domain, and the sequence identity between TAZ and YAP was 50% (Reuven *et al*., 2019). The domains of YAP/TAZ played various biological roles in the cellular processes of tumorigenesis. The transcriptional co-activators YAP/

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TAZ could be regulated by multiple upstream signals, of which Hippo pathway accounted for a main part (Zhao *et al*., 2010). Previous studies also revealed that YAP/TAZ pathway participated in the proliferation and adhesion of breast cancer (Zhao *et al*., 2021). Recent studies indicated that YAP was able to induce cisplatin resistance through cellular autophagy and could mediate paclitaxel resistance in ovarian cancer cells (He *et al*., 2019; Xiao *et al*., 2016). However, the relations of YAP and trastuzumab resistance has not been comprehensively explored in breast cancer.

In the present study, we hypothesized that the YAP/TAZ pathway could induce the trastuzumab resistance in HER2-positive breast cancer. The clinical tissues were collected, and the expression of YAP were detected. Moreover, the drug-resistant cell model was established for *in vitro* verifications. With the deepening understanding of the biological behavior of breast cancer and the transformation and updating of the treatment concept, this study is expected to provide new ideas for the mechanism of acquired drug resistance in breast cancer.

MATERIALS AND METHODS

Clinical studies

The frozen tumor tissues and the adjacent tissues were collected from 40 breast cancer patients. The experiments obtained the informed consent of all the patients. The study was performed as per the guidelines of the committee and the declaration of Helsinki. All the clinical experiments were approved by the Ethics Committee of the Hainan General Hospital, Hainan Medical University.

Cell culture

The human breast cancer cell lines SK-BR-3-TS was obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in cultured in Roswell Park Memorial Institute (RPMI, Gibco, USA) media containing 10% fetal bovine serum and 1% penicillin/streptomycin. For construction of trastuzumab resistant models, cells at logarithmic growth phase were collected, after which 0.5 μg/ml (10 times of 50% inhibition concentration) trastuzumab (Roche, Shanghai, China) was added into complete medium. Subsequently, the concentration of trastuzumab was added (0.5, 1, 2, 4, 6 to 8 μg/ml), and the SK-BR-3-TR cells were collected when cells stably grew in medium containing 8 μg/ml trastuzumab. For further experiments, cells were maintained in medium containing 4 μg/ml trastuzumab. All the cells were incubated in a 5% CO_2 humidified incubator at 37°C.

Cell transfection

For constructing the knockdown model of YAP, si-YAP was synthesized by GenePharma Co., Ltd. (Shanghai, China). Cell transfection was performed when the confluence of cells reached 90% using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as per the protocol of manufacturer.

Cell viability

MTT assay was employed for measurement of cell viability. Briefly, cells were seeded into 96-well plates at a density of 5×10^3 cells/well and then treated under various conditions for 48 h. After three PBS washes, the cells were treated with MTT solution (0.1 mg/ml, Solarbio, Beijing, China) as per the protocol of manufacturer and incubated for 4 h at 37°C. The absorbance was detected at 570 nm by a microplate reader (Bio-Tek, Norcross, GA, USA).

Cell apoptosis

The cell apoptosis was determined by flow cytometer analysis. Briefly, cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well and then treated under various conditions for 48 h. After three PBS washes, the cells were treated with Annexin V-FITC/PI apoptosis detection kit (BD Bioscience, USA) as per the manufacturer's instructions. Briefly, cells were suspended with 100 μL 1×Binding Buffer and 5 μL Annexin V solutions and incubated for 5 min at room temperature, after which cells were centrifugated at 1000 rpm for 5 min and resuspended with 10 μL propidium iodide and 190 μL buffer. The double-stained cells were analyzed by a flow cytometer (Thermo Scientific, Waltham, MA, USA).

RT-qPCR

Total RNA was extracted from cells by TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Then the cDNA was synthesized using Prime Script reverse transcription reagent kit (TaKaRa, Japan). RT-qPCR were performed with quantitative fluorescent PCR kit (Cwbiotech, Beijing, China) and performed on ABI Prism system (Applied Biosystems, USA). The relative expressions of mRNA were measured using $2^{-\Delta\Delta Ct}$ method *via* normalization to GAPDH. The primers were list as follows:

YAP-F: 5'- CCTTCTTCAAGCCGCCGGAG -3'
5'- CAGTGTCCCAGGAGAAACAGC -3'
TAZ-F: 5'- TATCCCAGCCAAATCTCGTG -3'
5'- TTCTGCTGGCTCAGGGTACT -3'
GAPDH-F: 5'- TCAAGAAGGTGGTGAAGCAGG - 3'
5'- TCAAAGGTGGAGGAGTGGGT -3'

Western blotting

Total protein was extracted from cells using using RIPA buffer (Sigma-Aldrich, USA) that supplemented with protease (1:100) and phosphatase (1:100) inhibitors.

The concentration was determined using the BCA Assay Kit (Beyotime, Shanghai, China). The samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Massachusetts, USA). The nonfat milk was obtained for blocking at room temperature for 1 h. Then the membrane was incubated with primary antibodies that included anti-YAP (Abcam, ab52771, 1:1000), anti-TAZ (Abcam, ab110239, 1:1000) and anti-GAPDH (Abcam, ab8245, 1:1000). Then the samples were incubated with horseradish peroxidase-conjugated secondary antibody (Abcam, ab6721 1:1000) for 2 h at room temperature.

Statistical analysis

All data were expressed as means ± standard derivation (SD). GraphPad Prism 8 was used for statistical analysis. Student's *t* tests were used for comparisons between two groups. One-way ANOVA analysis was performed for comparisons among multiple groups. *P*-values < 0.05 were considered to indicate statistically significant results.

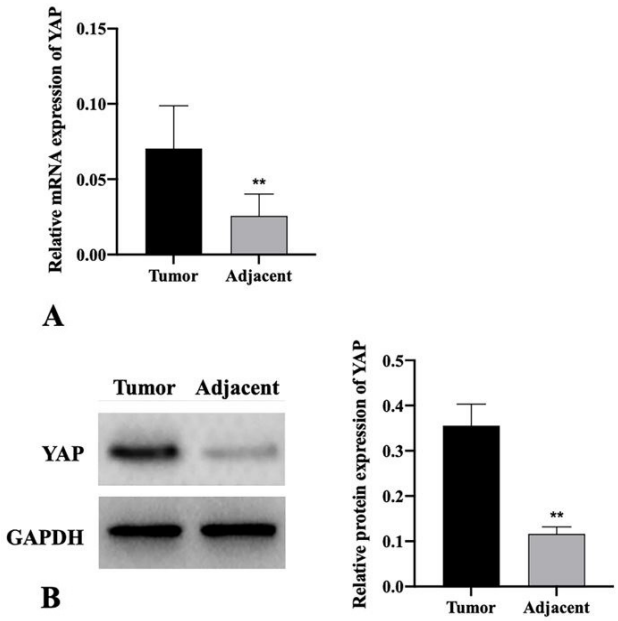

Fig. 1. Expression of YAP in clinical tissues of HER2 positive breast cancer patients. (A) Protein expression of YAP detected by western blotting. (B) Relative mRNA expression of YAP determined using RT-qPCR. ** P<0.01 vs tumor group (Student's *t* tests).

RESULTS

The analysis on clinical tissues revealed that the mRNA (Fig. 1A) and protein (Fig. 1B) level of YAP was dramatically upregulated in the tumor tissues compared with adjacent tissues in HER2-positive breast cancer patients. Consistently, the *in vitro* experiments showed that the mRNA (Fig. 2A) and protein (Fig. 2B) level of YAP and TAZ were dramatically upregulated in SK-BR-3-TR cells compared with SK-BR-3-TS. Moreover, MTT assay indicated that the cell viability was increased (Fig. 3A), while flow cytometry results (Fig. 3B) proved that cell apoptosis was inhibited in SK-BR-3-TR cells compared with SK-BR-3-TS. For elucidating the role of YAP, the depletion of YAP was achieved by transfection of si-YAP. The protein level of YAP and TAZ was downregulated after transfection of si-YAP (Fig. 4A), indicating the successful construction of YAP knockdown model. The cell apoptosis rate was enhanced after transfection of si-YAP (Fig. 4B), and the cell viability was decreased in si-YAP group (Fig. 4C). The above results suggested that inhibition of YAP might alleviate the tumor processes of trastuzumab resistant HER2-positive breast cancer cells.

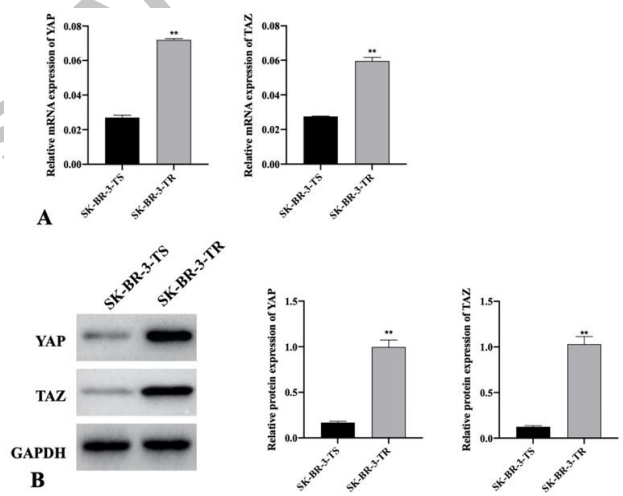

Fig. 2. Expression of YAP/TAZ *in vitro*. (A) Protein expression of YAP and TAZ detected by western blotting. (B) Relative mRNA expression of YAP and TAZ determined using RT-qPCR. ** P<0.01 vs SK-BR-3-TS group (Student's *t* tests).

DISCUSSION

HER2 was reported to be overexpressed in around 20% of breast cancers, which contributed to increased clinical behavior (Guarneri *et al*., 2010; Zimmer *et al*., 2022). The poor prognosis has always been an important problem for patients with breast cancer till the availability of HER2-directed monoclonal antibodies (Hudis, 2007). The diagnosis and treatment of HER2 positive breast cancer was considerably changed in the past few decades, and the

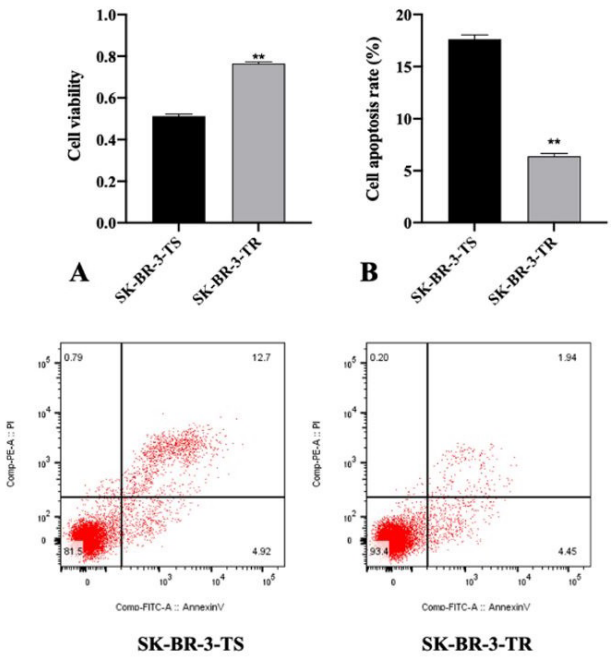

Fig. 3. Cell viability was increased while cell apoptosis was inhibited in SK-BR-3-TR cells. (A) Cell viability detected by MTT assay. (B) Cell apoptosis analyzed by flow cytometry. ** P<0.01 vs SK-BR-3-TS group (Student's *t* tests).

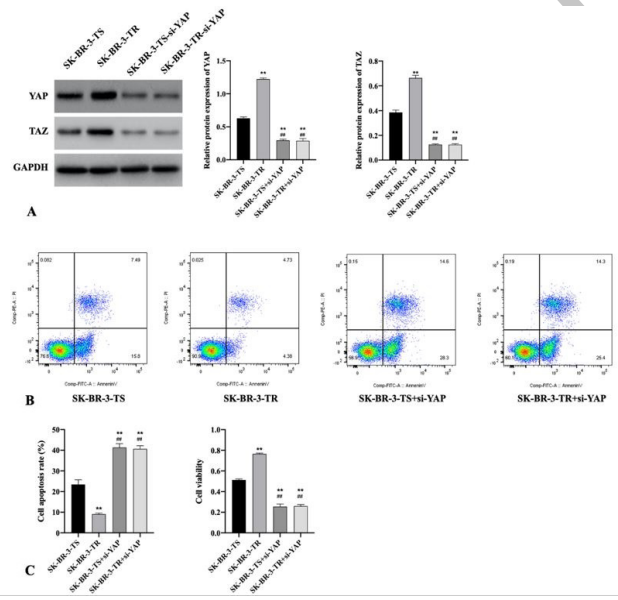

Fig. 4. Depletion of YAP reversed the induced trastuzumab resistance in SK-BR-3-TR cells. (A) Protein expression of YAP and TAZ detected by western blotting. (B) Cell apoptosis analyzed by flow cytometry. (C) Cell viability detected by MTT assay. ** P<0.01 vs SK-BR-3-TS group. ## P<0.01 vs SK-BR-3-TR group (One way ANOVA).

standard for the HER2 positive breast cancer patients at the metastatic stage was dual antibody therapy that included pertuzumab and trastuzumab (Indini *et al*., 2021; Kunte *et al*., 2020). Although the HER2 positive targeted drugs achieved successful in extending the overall survival of patients, the developed drug resistance remained a major clinical problem. The multiple reagents that targeted HER2 truncation or PI3K pathway activation has been used clinically in combination with trastuzumab to decrease the effects of trastuzumab-resistance (Hunter *et al*., 2020). Moreover, it was reported that trastuzumab-resistance could be reversed by combination therapy with small-molecule inhibitors of multidrug-resistance transporters (Gottesman and Pastan, 2015). However, because of the high expression of efflux transporters in tumor tissues, the inhibitors were rarely applicated in clinical therapy. The present study investigated the molecular mechanisms for trastuzumab-resistance in HER2 positive breast cancer, aiming at providing new clinical therapeutic targets.

Transcriptional co-activators YAP and TAZ could interact with transcription factors and further regulated the cis-regulatory elements, which was essential for cancer progression and tumor metastasis (Cai *et al*., 2021; Zanconato *et al*., 2016). It was reported that the upregulated YAP/TAZ was positively correlated with the poor histological status and metastasis proclivity in tumor tissues of breast cancer patients (Zanconato *et al*., 2015). Chen *et al*. (2014) also demonstrated that transgenic expression of HER2 significantly activated YAP/TAZ signaling pathway in mouse mammary gland (Chen *et al*. 2014). Consistently, the present study indicated that the expression of YAP was dramatically increased in tumor tissues compared with adjacent tissues in HER2-positive breast cancer patients. Gao *et al*. reported that YAP/TAZ signaling positively regulated the expression of SLC7A11, which belonged to key transporters that maintained homeostasis of intracellular glutathione, and increased cell resistance to sorafenib (Gao *et al*., 2021). In lung cancer cells, the expression of YAP was upregulated, and the increased YAP level was positively correlated with the enhanced cisplatin resistance, while depletion of YAP effectively sensitized the cells to cisplatin treatment (Song *et al*., 2018). Moreover, a previous study found silencing of YAP inhibited tumor growth and increased responsiveness of parental cells to ALK tyrosine kinase inhibitors that used for clinical treatment of lung cancer (Yun *et al*., 2019). It was reported that nuclear YAP/TAZ was able to bind to the TEAD family of transcription factors, which further activated the down streaming pro-proliferative and survival enhancing gene programs (Cunningham and Hansen, 2022). In this study, we newly reported that YAP/TAZ might affect the resistance of HER2 positive

breast cancer cells to trastuzumab. The depletion of YAP significantly increased apoptosis rate and decreased cell viability of SK-BR-3-TR cells.

In conclusion, the present study elucidated that YAP/TAZ pathway might induce the trastuzumab resistance in HER2-positive breast cancer and targeting YAP would be an alternative way for the clinical therapeutic methods of HER2 positive breast cancer. However, further *in vivo* investigations and clinical studies are needed to comprehensively understand and verify the role of YAP/TAZ signaling in trastuzumab resistance in HER2-positive breast cancer. The down streaming pathways of YAP/TAZ signaling in trastuzumab resistance also needs further exploration.

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DECLARATIONS

Ethics approval and consent to participate

The ethic approval was obtained from the Ethic Committee of Hainan General Hospital, Hainan Medical University and written informed consent was obtained from all patients.

Consent to publish

All of the authors have Consented to publish this research.

Availability of data and materials

The data are free access to available upon request.

Competing interests

All authors declare no conflict of interest.

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