Suppression of Breast Cancer Cell Growth by Her2-Reduced AR Serine 81 Phosphorylation

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Abstract

Breast cancer is a hormone-related carcinoma and the most commonly diagnosed malignancy in women. Although Her-2, estrogen receptor (ER), and progesterone receptor (PR) are the major diagnostic markers and therapeutic targets to breast cancer, searching for additional molecular targets remains an important issue and one of the candidates is androgen receptor (AR). AR has been shown expressed in 70% breast cancer patients and connects to low recurrence and high survival rate. Our previous study demonstrates that Ser81 phosphorylation of AR in prostate cancer cells is critical for its protein stability modulated by human epidermal growth factor receptor-2 (Her2). The aim of this study is to investigate the influence of Her2 and AR in proliferation of breast cancer cell line, MDA-MB-453. The data show that AR which was activated by synthetic androgen R1881 suppressed the proliferation of MDA-MB-453 cells. Notably, AR activation decreased the protein levels of cell growth-related proteins, including cyclin A, cyclin B, and early growth response protein 1 (Egr1), while cell-cycle inhibitor protein p27 was increased. Besides, Heregulin (HRG)-induced Her2 activation decreased the AR protein levels and its Ser81 phosphorylation. Her2 small molecular inhibitor, Lapatinib, dose-dependently suppressed cell proliferation while the levels of phospho-Ser81 AR and p27 protein were increased. Phospho-Ser81 AR was also increased after Her2 knockdown. Specifically, the influence of phospho-Ser81 AR by Lapatinib was primarily found in the nucleus of MDA-MB-453 cells, where the cell proliferation might directly be interfered. In conclusion, our findings indicate that Her2 might negatively regulate AR phosphorylation/activation and contribute to regulate the proliferation of MDA-MB 453 cells.

Key Words: androgen receptor, breast cancer, Her2, Lapatinib, MDA-MB-453 cell line

Introduction

Breast cancer is a hormone-related malignancy in women (31). The expression levels of estrogen receptor (ER) and progesterone receptor (PR) are important for diagnosis and as biomarkers of hormone
replacement therapy in breast cancer (6, 8, 27). In addition to ER and PR, human epidermal growth factor receptor-2 (Her2) possesses critical roles in the activation of subcellular signaling transduction and contributes to breast cancer development (16). Her2 overexpression occurs in approximately 30% breast cancer patients who have both poor prognosis and lower survival rate (24). Therapies targeting Her2 by anticancer agents such as trastuzumab (Herceptin), Lapatinib, and bevacizumab have been proven beneficial to Her2-overexpressing breast cancer patients (18, 20). Although these three biomarkers are valuable as predictive and prognostic factors to breast cancer, it remains difficult to predict and survey the therapeutic efficiency via such few biomarkers due to the heterogeneous features of breast cancer (6). Therefore, searching for additional molecular biomarkers is still an important issue.

In addition to ER and PR, breast cancer cells express other nuclear hormone receptors. In which, androgen receptor (AR) occurs in 60-80% of breast cancer patients regardless of ER status (1, 14). Interestingly, in the ER-negative breast cancer, AR expression is correlated with the status of Her2 overexpression (23). In prostate cancer, Her2 regulates AR protein stability and activation via modulating Ser81 phosphorylation (12, 17). Several lines of evidence have indicated that AR phosphorylation at Ser81 site mediate AR function and are regulated by different kinases (3, 9). Notably, the functional connection between AR and Her2 has been reported in ER-negative breast cancer cells (21), however, the physiological relevance and its detailed mechanisms remain unclear.

In this study, we identified that AR might be important in Her2-regulated breast cancer cell proliferation and therefore provides a potential diagnostic and therapeutic target to breast cancer in the future.

Materials and Methods

Cell Culture

Human breast cancer cell line MDA-MB-453 was obtained from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Taiwan. MDA-MB-453 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 1.5 g/L sodium bicarbonate (NaHCO₃), and penicillin/streptomycin (P/S) (100 unit/ml and 100 µg/ml, respectively) (Sigma, St. Louis, MO, USA). All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT Assay)

Cells were seeded in 96-well plate, and incubated 24 h after attachment. Cells were treated following the experimental designs and then MTT Assay was manipulated to quantify the proliferation of cells. The MTT stock solution (5 mg/ml, Sigma, St. Louis, MO, USA) was diluted to 0.5 mg/ml with complete cultured medium and added 0.1 ml into each well. The yellow MTT would be converted to blue formazan by living cells, which depends on mitochondrial enzyme activity. After using Dimethyl sulfoxide (DMSO) to dissolve blue formazan, the absorbance of converted MTT could be measured by equipment (Thermo, Multiskan FC) at the wavelength 570 nm.

Transfection

Cells were plated for at least 24 h and had reached 80% confluency prior to transfection. The siRNA was premixed within Lipofectamine 2000 transfection reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions, and then the liposome/nucleic acids complex was transfected into Opti-MEM (Gibco, Carlsbad, CA, USA)-incubated cells for 6 h, and then culture medium was added for exogenous protein expression. Commercial products of siRNA-Her2 (siHer2) and non-specific control siRNA (siCon) were purchased from Dharmacon (Lafayette, CO, USA).

Cellular Protein Lysates Preparation

Cells were collected by rubber scrapper and homogenized with Na₃VO₄/PBS (1:100). To extract whole-cell lysates, cells were homogenized with lysis buffer containing protease inhibitors cocktail for 45 min on ice. The supernatant was collected as total protein extract after centrifugation (15,400 × g). To isolate subcellular proteins, cells were collected and re-suspended in hypotonic buffer containing protease inhibitors cocktail for 10 min. After centrifugation (15,000 × g), the supernatant was collected as a fraction of cytoplasmic proteins. The pellet was washed three times with hypotonic buffer before lysis in a nuclear extraction buffer for 45 min on ice. Following centrifugation (15,000 × g), the supernatant was collected as a fraction of cytoplasmic proteins. The formulas of lysis, hypotonic, and nuclear extraction buffer were described (12).

Western Blot Analysis

The protein extraction was mixed with sampling buffer and boiled for 10 min, and then sodium dodecyl sulfate (SDS)-polyacrylamide gel electropho-
resis (SDS-PAGE) was processed. Proteins were transferred from SDS-PAGE onto polyvinylidene fluoride (PVDF) membrane. Primary antibodies was incubated with the membrane for overnight, and the horseradish peroxidase (HRP)-conjugated secondary antibodies (26) compared ECL (western lighting chemi-luminescence reagent plus, PerkinElmer, Waltham, MA, USA) reaction to show the protein expression levels through exposing X-ray films. Primary antibodies used in this study were listed: phospho-Her2 (1:1,000, 2064, Cell Signaling, Danvers, MA, USA), Her2 (1:1,000, sc-284, Santa Cruz, Dallas, TX, USA), phospho-AR (1:2,000, 07-1375, Merck Millipore, Darmstadt, Germany), AR (1:1,000, sc-7305, Santa Cruz), ERα (1:1,000, sc-543, Santa Cruz), p27 (1:2,500, 610241, BD Biosciences, San Jose, CA, USA), early growth response protein 1 (Egr1) (1:1,000, sc-110, Santa Cruz), cyclin A (1:1,000, sc-751, Santa Cruz), cyclin B (1:1,000, sc-752, Santa Cruz), PARP (1:2,000, 06-557, Upstate, Lake Placid, NY, USA), α-tubulin (1:10,000, 05-829, Upstate), and β-actin (1:10,000, MAB1501, Merck Millipore, Darmstadt, Germany).

Statistics

All values are given as the mean ± standard error of the mean (S.E.M). In all cases, Student’s t test was used to assess in the results of cell proliferation assay. A difference between two means was considered statistically significant with \( P < 0.05 \).

Results

Suppressive Effect of AR Activation on MDA-MB-453 Cell Proliferation

We monitored the basal protein levels of Her2, AR, and related proteins in six breast cancer cell lines including MCF-7, T47D, BT474, HS578T, MDA-MB-231, and MDA-MB-453 (Fig. 1A). Among these cell lines, MDA-MB-453 expressed high AR and Her2 proteins and is suitable for our following experiments. To figure out the effect of AR activation on cell
proliferation, the synthetic androgen R1881 at different concentrations 0.1, 1, and 10 nM was used to activate AR in MDA-MB-453 cells and MTT assay was used to measure the cell proliferation. The result showed that R1881 significantly decreased cell proliferation after 96 h treatment (Fig. 1B). Furthermore, cell growth curves indicated similar growth inhibition caused by R1881 treatment (Fig. 1C). These results showed that AR activation might decrease the proliferation of MDA-MB-453 cells.

**Effects of AR Activation on Protein Levels of Cell Growth-Related Proteins**

Since AR might decrease MDA-MB-453 cell growth, we next surveyed the levels of cell growth-related proteins in MDA-MB-453 cells after treating with 10 nM R1881 for different time intervals. We found that the protein levels of cyclin A and cyclin B were decreased while AR phosphorylation at Serine 81 and cell cycle inhibitor p27 were elevated by R1881 treatment (Fig. 2). The Ser81 phosphorylation of AR has been proven to contribute to AR protein stability and activation in prostate cancer cells (11, 12). The Egr1 was also decreased in a time-dependent manner upon R1881 treatment (Fig. 2). Egr1 plays an important role in promoting cancer cell growth, invasion, migration, and tumor development partially through increasing expressions of cyclin D2 and cyclin-dependent kinase 4 (Cdk4) (2, 15, 25). According to these data, we demonstrate that the suppression of MDA-MB-453 cell proliferation by AR activation might be correlated with the decreases of cyclin A, cyclin B, and Egr1 as well as the upregulation of p27.

**Inhibitory Effect of Her2 on AR and Its Ser81 Phosphorylation**

Our previous study shows that Ser81 phosphorylation is critical for AR protein stability and modulated by Her2 in prostate cancer cells (11, 12). Therefore, the Her2 regulation on AR is the next to investigate. MDA-MB-453 cells were starved by incubating serum-free medium for 24 h prior to co-treatment of Her2 ligand, Heregulin (HRG), and R1881. The protein levels of AR and phospho-Ser81-AR were analyzed by western blotting and β-Actin served as an internal control.
phosphorylation might be the key of this regulation.

**Effect of Her2 Inhibition on AR Ser81 Phosphorylation**

Comparing to Her2 activated by HRG treatment, the Her2 inhibitor, Lapatinib, for breast cancer therapy (24) was used in our experimental design. The effect of Lapatinib (0, 0.1, 1, or 10 \( \mu \text{M} \)) on the growth of MDA-MB-453 cells was first determined by MTT assays. The data showed that Lapatinib significantly reduced cell proliferation in a time- and dose-dependent manner (Fig. 4A). The levels of phospho-Ser81-AR and AR protein were significantly increased by Lapatinib treatment (10 \( \mu \text{M} \)) in a time-dependent manner while Her2 phosphorylation was indeed inhibited (Fig. 4B). Notably, p27 protein expressions were increased after Lapatinib treatment (at least 24 h), which corresponded to the results of AR activation in Fig. 2. In addition to the treatment of Her2 inhibitor, Her2 knockdown by specific siRNA (siHer2) significantly elevated the levels of phospho-Ser81-AR and AR protein (Fig. 4C). Taken together, Her2 inhibition might increase AR activation and cause growth inhibition of MDA-MB-453 cells.

**Effects of Her2 Inhibition on AR Translocation**

Since the influence of AR protein levels by Lapatinib was not obvious with significant increase of phospho-Ser81-AR (Fig. 4B), it’s of interest to in-
Investigate the effect of Her2 on AR activation status.
As a transcriptional factor, activated AR translocates into nucleus and binds with specific promoter region to initiate downstream expression. To investigate the AR activation status, the analysis of cellular protein after nuclei/cytosol fractionation was performed as described in the Materials and Methods. The proteins including phospho-Her2, Her2, and phospho-Ser81-AR were detected by western blotting. PARP and α-tubulin served as markers for the nuclear and cytoplasmic fractions, respectively. The results of Input represented the proteins in total cell lysates and β-Actin served as an internal control.

Discussion

Breast cancer is a common malignancy in women and sex hormones play important roles in cancer development (28). Estrogen binds to its primary receptor ERs which has widely been recognized to promote breast cancer progression (10, 13, 19), whereas the roles of androgen and the receptor, AR, in breast cancer remain uncertain and controversial. Breast cancer cell line MDA-MB-453 is seen as a model for molecular apocrine breast carcinoma, which is characterized by the profiles of AR overexpressing with ER/PR negative (4, 7, 21). In addition to steroid hormone receptor, Her2 is considered as a diagnostic and therapeutic target and often overexpresses in the molecular apocrine breast cancer subtype (7). Thus, MDA-MB-453 is suitable for investigating the role of AR in breast cancer and tracing the regulatory mechanism between AR and Her2.

The characteristics of MDA-MB-453 cells with AR-positive, ER-negative, and Her2 overexpression were confirmed in our data (Fig. 1A). Further results exhibited that synthetic androgen R1881 suppressed cell proliferation after 4 days treatment analyzed by MTT assay (Fig. 1B) and at least 3-days-treatment detected by cell counting (Fig. 1C). Although some studies suggest the positive regulation of androgen on breast cancer cell proliferation (5, 21, 22), there are several lines of evidence supporting of our findings that AR activation suppresses the growth of breast cancer cells (29, 30). Notably, Wang et al. demonstrate the opposite effects of AR activation on regulating the proliferation of prostate cancer cells versus breast cancer cells (30). The mechanism of the opposite regulations in prostate cancer and breast cancer has been addressed on the distinct expression regulation of PTEN by AR (30). This report suggests that cancer types and signaling molecules may decide the roles AR plays. In our study, R1881 treatment activated AR with the elevation of Ser81 phosphorylation while p27 increase and cyclin A/cyclin B/Egr-1 decreases were observed in a time-dependent manner (Fig. 2). Therefore, we suggest that AR activation contributes to suppress the proliferation of MDA-MB-453 cells possibly through regulating cell cycle-related proteins and mitogen-responsive proliferation factor.

Our previous results show that AR phosphorylation at Serine 81 facilitates its protein stability and transcriptional activity during Her2 activation in prostate cancer (11, 12). The functional correlation between AR and Her2 has been reported in ER-negative breast cancer cells (21); however, AR phosphorylation was not discussed in the regulation. Interestingly, we explore that Her2 activated by HRG might reduce phospho-Ser81-AR after 4 h in the presence of R1881 (Fig. 3). In addition to Her2 activation, the small molecular inhibitor of Her2, Lapatinib was used to further investigate the regulatory mechanism. The growth of MDA-MB-453 cells was significantly suppressed by Lapatinib treatment in dose- and time-dependent manners (Fig. 4A). Interestingly, Her2 inhibited by Lapatinib elevated phospho-Ser81-AR level with p27 increase, which was similar with the data of AR activated by R1881 (Figs. 2 and 4B). Correspondingly, phospho-Ser81-AR increase was observed when Her2 was knocked down by siHer2 (Fig. 4C). Both results from Her2 activation or inhibition suggest that Her2 inversely regulated AR activation
and AR protein stability might be modulated via Ser81 phosphorylation.

In our previous findings, Cdk5 may phosphorylate AR at Ser81 and increase its nuclear accumulation and AR activity is therefore enhanced (11). In order to confirm if AR is transactivated after Ser81 phosphorylation triggered by Her2 inhibition, subcellular distribution of phospho-Ser81-AR was monitored. Corresponding to our hypothesis, the nuclear levels of phospho-Ser81-AR was increased after Lapatinib-dependent Her2 inhibition (Fig. 5). In this study, we shed light on the role of AR and its Ser81 phosphorylation under Her2 regulation and provide evidence answering an old riddle of Her2 and AR regulation in breast cancer.

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