IGF-1R and ErbB3/HER3 contribute to enhanced proliferation and carcinogenesis in trastuzumab-resistant ovarian cancer model

Yanhan Jia [a,b,1], Yan Zhang [c,1], Chunxia Qiao [b,1], Guijun Liu [b], Qing Zhao [b,c], Tingting Zhou [b], Guojiang Chen [b], Yali Li [c], Jiannan Feng [b], Yan Li [b], Qiuping Zhang [a,*], Hui Peng [b,d,*]

[a] Department of Immunology, School of Basic Medical Sciences, Wuhan University, Wuhan, Hubei 430071, China
[b] Department of Immunology, Institute of Basic Medical Sciences, Beijing 100850, China
[c] Department of Gynaecology and Obstetrics, PLA General Hospital, Beijing 100853, China
[d] Cardiovascular Drug Research Center, Institute of Health and Environmental Medicine, Beijing 100850, China

ARTICLE INFO

ABSTRACT

Trastuzumab (Herceptin®) has demonstrated clinical potential in several types of HER2-overexpressing human cancers. However, primary and acquired resistance occurs in many HER2-positive patients with regimens. To investigate the possible mechanism of acquired therapeutic resistance to trastuzumab, we have developed a preclinical model of human ovarian cancer cells, SKOV3/T, with the distinctive feature of stronger carcinogenesis. The differences in gene expression between parental and the resistant cells were explored by microarray analysis, of which IGF-1R and HER3 were detected to be key molecules in action. Their correctness was validated by follow-up experiments of RT-PCR, shRNA-mediated knockdown, downstream signal activation, cell cycle distribution and survival. These results suggest that IGF-1R and HER3 differentially regulate trastuzumab resistance and could be promising targets for trastuzumab therapy in ovarian cancer.

1. Introduction

The family of human epidermal growth factor receptor (EGFR) consists of EGFR, HER2, HER3 and HER4. The deregulated or dysfunctional expression of EGFR family has been implicated in the development and malignancy of several types of human cancers [1]. Trastuzumab (Herceptin®) was the first anti-HER2 humanized monoclonal therapeutic antibody and has shown considerable clinical efficacy and improvement of survival quality in patients with HER2-amplified breast cancer [2,3]. In metastatic breast cancer, a series of phase II and phase III clinical trials showed that trastuzumab plus chemotherapy increase time to progression, response rates and overall survival compared with chemotherapy alone [4]. However, many patients do not respond to initial trastuzumab treatment (primary resistance), and many trastuzumab-responsive patients develop resistance after continuous treatment within one year (acquired resistance) [5].

Overexpression of HER2 has also been found in patients with other types of cancers such as ovarian cancer [6], though correlation between HER2-overexpression and the prognostic significance or incidence remain unclear. In ovarian cancer patients, intrinsic resistance to trastuzumab was still under close scrutiny in clinical studies. A clinical Phase II trial exhibited a limited clinical value of trastuzumab in the treatment of HER2-positive ovarian cancer due to a low rate of objective responses [7]. These disappointing results have been confusing many researchers because the overexpression of HER2 in ovarian patients is at the approximate frequency as breast cancer patients do [8,9]. Indeed, several studies have clearly shown that the causes for low response rate of single-agent trastuzumab in ovarian cancer possibly correlate with multi cancer-related markers [10,11], gene mutations [12] or marker shifting during disease developing courses [13,14]. Identifying the needs and opportunities to develop predictive biomarker of trastuzumab response in ovarian cancer have also been suggested by a recent study [15].

To identify possible effective and clinically practical resistant mechanisms in ovarian cancer, we developed a preclinical model of trastuzumab-resistant cell line SKOV3/T by means of chronic exposure to trastuzumab. Surprisingly, SKOV3/T cells exhibit enhanced proliferation and carcinogenesis *in vivo* without the amplification of EGFR and HER2. Therefore, microarray analysis was subsequently used to clarify differential-expressed genes

* Corresponding authors. Address: Department of Immunology, School of Basic Medical Sciences, Wuhan University, 185 Dong Hu Road, Wuhan, Hubei 430071, China. Fax: +86 27 68759827 (Q. Zhang), Department of Immunology, Institute of Basic Medical Sciences, 27 Taiping Road, Beijing 100850, China. Fax: +86 10 68159436 (H. Peng).
    E-mail addresses: qpzhang@whu.edu.cn (Q. Zhang), p_h2002@hotmail.com (H. Peng).
1 These authors contributed equally to this work.

between resistant and parental cell lines, confirming that the up-regulation of IGF-1R and HER3 genes was involved in resistant cells. Follow-up experiments verified their contribution to the enhanced proliferation in the resistant cells, suggesting that they could be suitable for prediction of the clinical response and potential therapeutic targets in ovarian cancer patients on trastuzumab-based therapy.

2. Materials and methods

2.1. Regents

Antibodies were purchased from companies as follows: anti-EGFR, anti-phospho-EGFR, anti-HER2, anti-phospho-HER2, anti-HER3, anti-phospho-HER3, anti-Akt, anti-phospho- Akt, anti-ERK$_{1/2}$, anti-phospho-ERK$_{1/2}$, anti-IGF-1R, anti-phospho-IGF-1R, anti-GAPDH and corresponding secondary antibodies (Cell Signaling Technology); PE conjugated anti-EGFR, anti-HER2 and anti-HER3 (eBiosciences). Regents were purchased from companies as follows: Trastuzumab (F. Hoffmann-La Roche Ltd.); SYBR® Green Real-time PCR Master Mix (TOYOBO); Electrophoresis reagents and polyvinylidene difluoride (PVDF) membranes (Bio-Rad); BCA protein assay and enhanced chemiluminescent (ECL) reagents (Pierce); The IGF-1R inhibitor NVP-ADW742 (Selleck Chemicals); shRNA (short hairpin RNA) to knock down HER3 (Biomics Biotechnologies); DMSO, TRIzol, G418 and Lipofectamine 2000 (Invitrogen); Human NRG1-β1/HRG1-β1 (RD); DMEM and FBS (HyClone); Human IGF-1 and sulforhodamine B (SRB) (Sigma–Aldrich). All other chemicals were obtained from commercial source of analytical grade.

2.2. Cell culture and shRNA transfection

Human ovarian cancer cell line SKOV3 from ATCC (No. HTB-77) was conserved in our lab. The cells were cultivated in DMEM supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 10% FBS and 4 mM L-glutamine. All the cells were incubated in a humidified incubator (Thermo, USA) at 37 °C with 5% CO$_2$. Acquired trastuzumab resistant cell line SKOV3/T by eight-month persistent treatment of trastuzumab was prepared in our lab. For shRNA transfection, the shRNA-lipo complexes were prepared using Lipofectamine 2000 according to the manufacturer's instruction and then the mixtures were added into SKOV3/T cells. Stable knockdown clones were established by selection with a medium containing 600 μg/ml G418 for 3 weeks. G418 resistant clones were isolated and amplified in complete medium containing 400 μg/ml G418.

2.3. SKOV3/T model generation

Trastuzumab-resistant ovarian cell line SKOV3/T was developed through continuously culturing SKOV3 cells in the presence of gradually increasing concentrations of trastuzumab (from 20 μg/ml to 50 μg/ml) for 8 months, during which the medium was replaced every 3 days [5]. SKOV3/T cells are now maintained in the presence of 20 μg/ml trastuzumab.

2.4. Cell survival assay

Cells were seeded in triplicate in 96-well plate with 5×10^3 cells/well. After 24 h, the cells were treated with NVP-ADW742 (2 μM) or trastuzumab (20 μg/ml) for defined time and then cell viability was determined by SRB assay as previously described [16].

2.5. Flow cytometry

Flow cytometry and data analysis (the percentage and intensity of stained cells, etc.) was performed exactly as previously described [16].

2.6. Cell cycle distribution analysis

Cells were treated, digested and prepared by fixation in 75% ethanol overnight at −20 °C. In the second day, cells were stained with 50 μg/ml PI in the presence of 100 μg/ml RNase A for 30 min at 37 °C in the dark. Quantification of the cell cycle distribution was done by flow cytometric analysis.

2.7. In vivo carcinogenic and trastuzumab treatment assays

Groups of 5-wk-old female BALB/c athymic, nu/nu (nude) mice were inoculated on fat pad with 1×10^6/0.1 ml SKOV3 or SKOV3/T cells on day 0. Since day 7, mice bearing palpable tumors were randomized into four groups with 6 mice per group ($n = 6$) and treated once weekly with trastuzumab i.v. for four times at the dose of 5 mg/kg. Then mice were observed twice a week about body weight, survival rates and tumor volumes according to the following equation: Tumor volume $(\text{mm}^3) = 1/2 \times (\text{length}) \times (\text{width})^2$. Data are presented as mean ± S.D. of relative tumor volume compared with day 7. Pairwise differences between groups were compared.

Care, use and treatment of mice in this study were in strict agreement with international guidelines for the care and use of laboratory animals and approved by Animal Ethics Committee of Institute of Basic Medical Sciences.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells using TRIzol and reverse-transcribed into cDNA using the SuperScript III First Strand cDNA synthesis system according to the manufacturer's instruction (Invitrogen). qRT-PCR was performed on a Bio-Rad iCycler using SYBR® Green Real-time PCR Master Mix. All reactions were carried out on Bio-Rad iQ5 Optical Module Real-Time PCR instrument. Each sample was run in triplicate. GAPDH as the internal control was also amplified under the same conditions. The relative fold change of each sample was normalized to that of GAPDH. The standard curve and data analysis were produced using Bio-Rad iQ5 software.

2.9. Western blot

Cells were cultivated in 24-well plate and incubated with 10 ng/ml HRG-β1 or 20 ng/ml IGF-1 for 15 min. Then cell monolayers were washed with cold PBS before they were lysed in cell lysis buffer. Cell lysate supernatants were collected by 13,000g centrifugation for 15 min at 4 °C. Total protein concentrations in these fractions were determined by BCA protein assay. Western blot analysis was performed exactly as previously described [16].

2.10. Microarray analysis

Human 12×135 K Gene Expression Array manufactured by Roche NimbleGen was used to estimate the changes in gene expression between SKOV3 parental and drug resistant cell lines. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. Differentially expressed genes between two samples were identified through fold change filtering. Hierarchical clustering was performed using the Agilent GeneSpring GX software (version 11.5.1).

2.11. Statistical analysis

Student's *t* test was performed to analyze differences between groups. Changes of tumor size between groups were analyzed by ANOVA (two-tailed). For all tests, *P* < 0.05 was regarded as statistically significant. All experiments were replicated at least three times.

3. Results

3.1. Trastuzumab-resistant cell line SKOV3/T loses the sensitivity to trastuzumab

Through continuously culturing SKOV3 cells under trastuzumab for 8 months, we obtained an acquired trastuzumab-resistant cell line SKOV3/T. Cells were pooled and tested for their responses to trastuzumab. As shown in Fig. 1A, cell survival and proliferation of SKOV3 were inhibited by 20 μg/ml trastuzumab, while SKOV3/T lost the response to antibody treatment. These results were further confirmed by *in vivo* trastuzumab therapy assays (Fig. 1B), for there were significant difference in the therapeutic effect between SKOV3 and SKOV3/T xenograft mice treated with trastuzumab (*P* < 0.01), offering the evidence of SKOV3/T lacking the normal sensitivity to trastuzumab.

3.2. SKOV3/T enhances proliferation and carcinogenesis in vivo without up-regulation of EGFR or HER2

Surprisingly, compared with parental line, SKOV3/T enhanced profound cell proliferation and carcinogenesis *in vivo* (Fig. 1B). Further cell cycle assay analyzed by flow cytometry offered a possible explanation that long-term trastuzumab treatment in SKOV3/T cells aroused increment of G2/M and S phase cell population and a concomitant decrement of cell number at G0/G1 phase. Fig. 1C showed the percentage of S-phase cells in SKOV3/T was 28.2%, significantly higher than that in SKOV3 cells (15.61%).

EGFR and HER2 have been confirmed pivotal role to cell survival, proliferation, transcription, apoptosis and prognosis of cancer patients. As shown in Fig. 1D–F, flow cytometry, qRT-PCR and western blot analyses showed the down-regulation of EGFR and HER2 in SKOV3/T cells. Moreover, HER2, EGFR expression and intrinsic phosphorylative activation were also remarkably down-regulated. Src was detected as a positive control of resistance [5]. Therefore, we concluded that there should be other reasons lead to SKOV3/T enhanced proliferation and carcinogenesis *in vivo*.

3.3. IGF-1R and HER3 genes were significantly up-regulated in SKOV3/T based on microarray analysis

In order to find out the mechanisms of how trastuzumab resistance trickles the enhanced proliferation and *in vivo* carcinogenesis, Human 12 × 135 K Gene Expression Array was used to detect the changes in gene expression between SKOV3/T and SKOV3 cells. As prolonged trastuzumab treatment has been reported to reprogramming of various RTKs [5,17], therefore we focus on all the RTKs in the two cell lines. We performed the hierarchical clustering to analysis the differential expressed RTKs genes. Only 10 RTKs genes reaching a *P*-value cut-off of ⩽0.05 and a fold change of ⩾2.0 in both directions (marker with * in Fig. 2), among which IGF-1R and HER3 were within the top 5 genes in positive direction and thus chosen for further evaluation.

3.4. IGF-1R and HER3 overexpression in SKOV3/T cells

The role of IGF-1R in cancer pathogenesis has been extensively studied. The IGF-1R gene was shown up-regulated in SKOV3/T cells
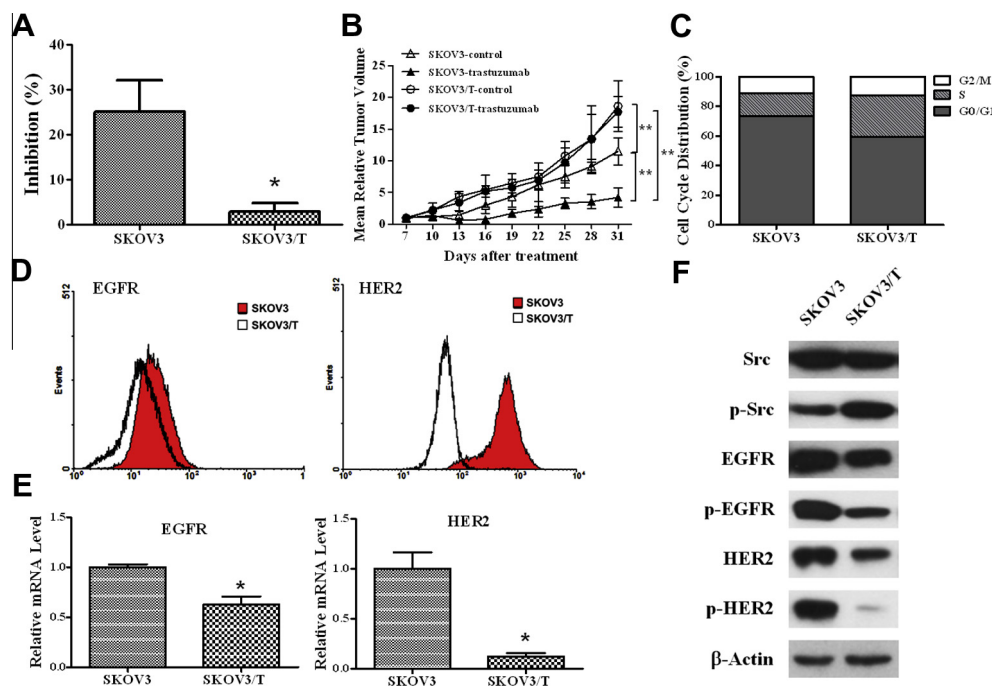

Fig. 1. Enhanced proliferation and *in vivo* carcinogenesis of acquired trastuzumab-resistant cell line SKOV3/T. (A) SKOV3 or SKOV3/T cells were treated with trastuzumab (20 μg/ml) followed by SRB assay. Values are mean ± SD from 3 independent experiments. *P* < 0.05, SKOV3/T group *vs*. SKOV3. (B) Nude mice bearing SKOV3 or SKOV3/T xenografts were treated with PBS (control) or trastuzumab. Values are the mean ± SD of relative tumor volume compared with day 7 (6 mice per group). **P* < 0.01; (C) cell cycle distribution of SKOV3 and SKOV3/T cell lines were determined by flow cytometry. The data are representative of 3 independent experiments. (D) Membrane protein expression of EGFR and HER2 in SKOV3 or SKOV3/T cells were detected by flow cytometry. The data are representative of 3 independent experiments. (E) mRNA transcription level of EGFR and HER2 in SKOV3 or SKOV3/T cells were determined by qRT-PCR. Values are mean ± SD from 3 independent experiments. *P* < 0.05, SKOV3/T group *vs*. SKOV3. (F) Western blot analyses for expression level of p-EGFR, EGFR, p-HER2 and HER2 in SKOV3 or SKOV3/T cells.
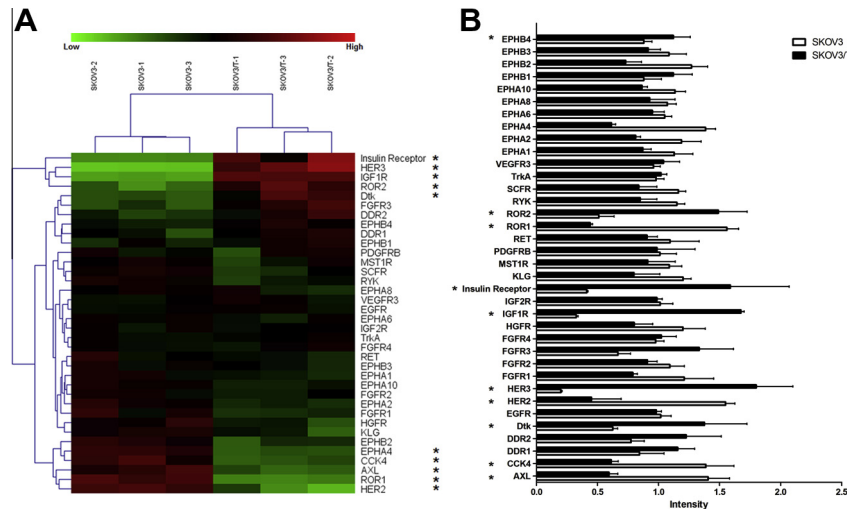
Fig. 2. Differential gene expression of RTKs between SKOV3 and SKOV3/T cell line. (A) Unsupervised hierarchical clustering based on the set of all RTK genes in SKOV3 and SKOV3/T. Rows represent gene, columns represent experimental cells. (B) Fold-change map of all RTKs. *Represent genes reaching a *P*-value cut-off of ⩽0.05 and a fold change of ⩾2.0 in both directions.

based on microarray analysis and its expression level was further confirmed by qRT-PCR (Fig. 3A) and western blot (Fig. 3B). With the treatment of IGF-1, a ligand for IGF-1R, phosphorylation of IGF-1R and downstream signal p-ERK$_{1/2}$ was remarkably stronger in SKOV3/T cells than in parental SKOV3 cells, suggesting the bio-functional significance of IGF-1R up-regulation associated with activation of downstream signals in SKOV3/T cells. The specificity of IGF-1R was further confirmed based on the offset of the above activation effect through co-incubation with the IGF-1R antagonist NVP-ADW742 (Fig. 3B).

Besides IGF-1R, HER3 was also identified as over-expression by qRT-PCR (Fig. 3C) and western blot (Fig. 3D). To investigate the effect of HER3-amplification on the downstream signaling in SKOV3/T, cells were stimulated with heregulin-β1 (HRG-β1), a well-known ligand for HER3. As shown in Fig. 3E, HRG-β1 induced phosphorylation and downstream p-AKT and p-ERK$_{1/2}$ activation only existed in SKOV3/T cells, indicating the presence of membrane HER3 in stark contrast to SKOV3 cells. Meanwhile, as shown in Fig. 3F, knockdown of HER3 in SKOV3/T by shRNA attenuated the specific activation (especially p-ERK$_{1/2}$), thus offering a direct evidence of HER3 over-expression in resistant cell line.
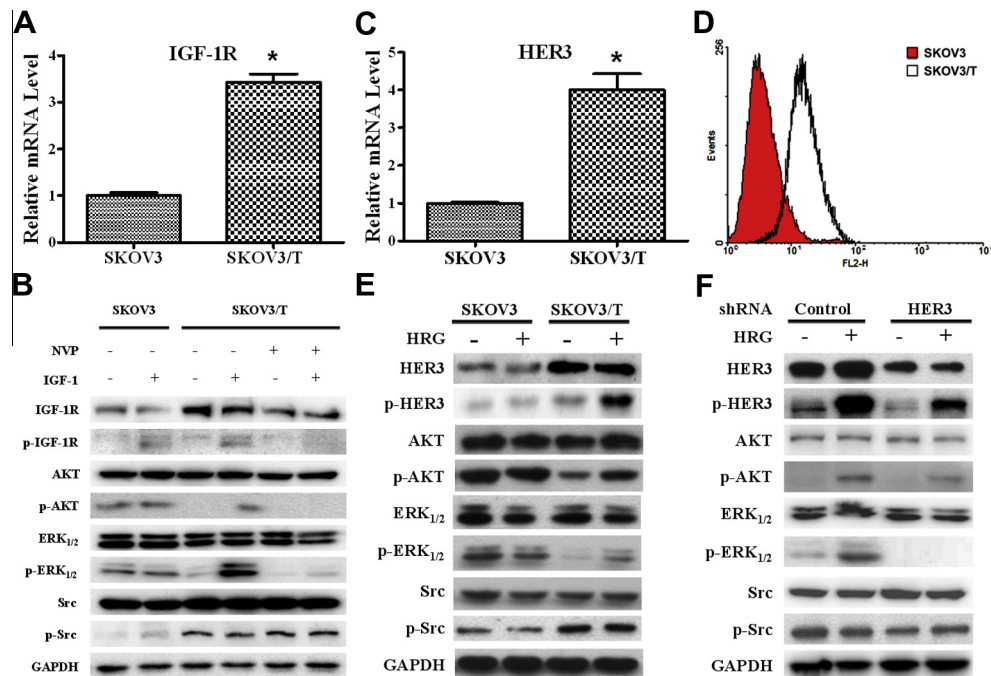
Fig. 3. IGF-1R or HER3 over-expression induces the activation of downstream signals in SKOV3/T cells. (A) IGF-1R transcription level was determined by qRT-PCR. Values are mean ± SD from 3 independent experiments. *$P < 0.05$, SKOV3/T group *vs.* SKOV3. (B) SKOV3 and SKOV3/T cells were treated with or without 1 μM NVP-ADW742 (NVP) in the absence or presence of IGF-1 (20 ng/ml) and lysed for western blot analysis. (C and D) HER3 expression level was identified by qRT-PCR (C) and flow cytometry (D). Values are mean ± SD from 3 independent experiments. *$P < 0.05$, SKOV3/T group vs. SKOV3. (E) SKOV3 or SKOV3/T cells were treated with 10 ng/ml HRG-β1 (HRG) for 15 min and lysed for western blot analysis. (F) SKOV3/T cells transfected HER3 or control shRNA were treated with 10 ng/ml HRG-β1 (HRG) for 15 min followed by western blot analysis. The data are representative of 3 independent experiments.

3.5. Targeting IGF-1R and/or HER3 inhibited the proliferation of SKOV3/T

To further identify the bio-functional association of HER3 and IGF-1R, SKOV3/T cells were treated with control or HER3 shRNA before cells were incubated with NVP-ADW742. Cell survival was subsequently calculated by SRB assay. As shown in Fig. 4A, both NVP-ADW742 and shRNA-HER3 transfection could dramatically inhibit SKOV3/T cell proliferation, suggesting that both IGF-1R and HER3 contributed to cell proliferation in SKOV3/T cells and IGF-1R seems to show greater importance than HER3. The combination of NVP-ADW742 and HER3 knockdown caused the greatest inhibition rate in SKOV3/T cells. Besides, NVP-ADW742 also showed enhanced inhibition of cell cycle in HER3 knockdown SKOV3/T cells. As shown in Fig. 4B, the S-phase was about 9% (HER3 knockdown and IGF-1R inhibition) contrasting to the value of 16% in SKOV3/T treated with NVP-ADW742 (IGF-1R inhibition), and that of 21% in HER3 knockdown cells and 29% (control, untreated) in SKOV3/T.

4. Discussion

Approximately 20–30% of ovarian cancer patients have been shown HER2 overexpression [18]. Trastuzumab was evaluated to have limited clinical value in the treatment of HER2-positive ovarian cancer based on low response rate [7]. However, the reasons for low response rate remain unclear. Luistro et al. [13] were pioneers in the development of the first preclinical trastuzumab-resistant ovarian carcinoma model to investigate the mechanism of trastuzumab resistance in ovarian cancer. The model provided highly valuable clues for further research of the inefficiency of trastuzumab in patients with HER2-overexpressing ovarian cancer. They showed that overexpression of MUC1 might hinder trastuzumab binding to HER2 receptors, thereby abrogating the antitumor effects of trastuzumab and thus giving rise to resistance to trastuzumab therapy. In our study, the results of microarray analysis also showed up-regulation of MUC1 (data not shown), supporting their conclusion.

We also established a preclinical trastuzumab-resistant ovarian cancer model SKOV3/T to further investigate the trastuzumab resistance in ovarian cancer. Surprisingly, we found that SKOV3/T cells possessed enhanced proliferation and carcinogenesis characteristic in vivo without the amplification of EGFR and HER2. Furthermore, microarray analysis was performed to clarify differential-expressed genes between SKOV3 and SKOV3/T, from which we preliminarily singled out IGF-1R and HER3 to be the key molecules. Follow-up experiments were performed to characterize their bioactivities by RT-PCR, shRNA-mediated

knockdown, downstream signal activation, cell cycle distribution and survival, respectively. These results supported the fact that IGF-1R plus HER3 contribute to the enhanced proliferation and carcinogenesis in acquired trastuzumab-resistant SKOV3/T cells.

A positive HER3 expression was observed in 53.4% of the cancer patients and considered as a unique tumor target in ovarian cancer when anti-HER2 antibody pertuzumab plus gemcitabine treatment of platinum-resistant ovarian cancer benefited with low HER3 mRNA [19]. Bezler et al. [20] postulated that activation of the HER3-PI3K-AKT cascade represents a major mechanism of chemo-resistance in ovarian cancer as cells treated with doxorubicin or cisplatin resulted in activation of the HER3. In our study, HER3 up-regulation was also identified significantly in acquired trastuzumab resistant SKOV3 cells, indicating its important role in ovarian cancer.

IGF-1R is highly expressed in ovarian cancer tissues and associated with the occurrence, development and poor prognosis of ovarian cancers [21,22]. IGF-IR is reported as a potential new molecular target in ovarian cancer and blocking its action is becoming a new potential ovarian cancer therapy [23]. An anticancer activity of IGF-1R kinase inhibitor NVP-AEW541 in ovarian cancer has been documented [24]. A commercially available fully human neutralizing monoclonal antibody specific to IGF-IR, robatumumab, has shown potent antitumor effects in ovarian cancer in vitro and in vivo. Some reports have revealed that IGF-1R/HER2 heterodimerization [25] and HER2/HER3/IGF-1R heterotrimerization [26] played key roles in trastuzumab-resistant breast cancer and the levels of HER2, HER3, and IGF-IR were similar between the parental and resistant breast cancer cells. But our study showed down-regulation of HER2 as well as up-regulation of IGF-1R and HER3 in the trastuzumab-resistant ovarian carcinoma cells, possibly attributing to the different type of cancer. Nevertheless, our data confirmed that jointly targeting IGF-1R and HER3 could overcome the trastuzumab resistance in ovarian cancer cells, supporting their assumptions.

Under the stress of drugs, e.g. antibody, cancer cells often chose "evolution" to adapt it, including surface marker shifting, mutation and/or cytokine release to change the microenvironment, etc. Our new findings complemented the existing knowledge of acquired resistance mechanisms that IGF-1R and HER3 up-regulation contributed to the enhanced proliferation and carcinogenesis of trastuzumab-resistant cancer cells. The proposed mechanism of trastuzumab resistance supports new therapies that target IGF-1R and/or HER3 with HER2 to achieve increased antitumor efficacy. We maintain that the new carcinogenic marker, such as HER3 or IGF-1R formerly considered as negative/low should also be screened during the course of trastuzumab treatment in the future. Moreover, our work has identified a gene signature that may be responsible for the onset of resistance to trastuzumab and
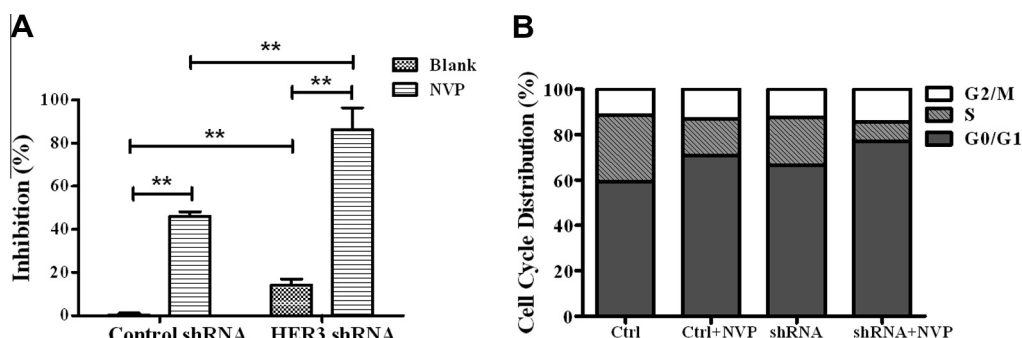
Fig. 4. HER3 plus IGF-1R up-regulation contribute to enhanced SKOV3/T cell proliferation. (A) SKOV3/T cells transfected with HER3 or control shRNA were treated with 2 μM NVP-ADW742 (NVP) followed by SRB assay. Values are mean ± SD from 3 independent experiments. **P < 0.01, NVP-ADW742 treated group or HER3 shRNA group vs. control. (B) SKOV3/T cells transfected with HER3 or control shRNA were treated with or without 2 μM NVP-ADW742 (NVP) and their cell cycle distribution determined by flow cytometry. The data are representative of 3 independent experiments.

provided the basis for elucidating the *de novo* lack of trastuzumab activity in patients with ovarian cancer, for the reason that other carcinogenic markers escape the targeting therapy of antibody. Although further investigation is certainly warranted, the inhibition of these genes and proteins may result in the reversal of resistance and lead to the discovery of more effective therapies for breast, ovarian and other cancers.

Acknowledgments

References

[1] M.A. Olayioye, R.M. Neve, H.A. Lane, N.E. Hynes, The ErbB signaling network: receptor heterodimerization in development and cancer, EMBO J. 19 (2000) 3159–3167.

[2] D. Slamon, W. Eiermann, N. Robert, T. Pienkowski, M. Martin, M. Press, J. Mackey, J. Glaspy, A. Chan, M. Pawlicki, T. Pinter, V. Valero, M.C. Liu, G. Sauter, G. von Minckwitz, F. Visco, V. Bee, M. Buyse, B. Bendahmane, I. Tabah-Fisch, M.A. Lindsay, A. Riva, J. Crown, Adjuvant trastuzumab in HER2-positive breast cancer, N. Engl. J. Med. 365 (2011) 1273–1283.

[3] C.L. Vogel, M.A. Cobleigh, D. Tripathy, J.C. Gutheil, L.N. Harris, L. Fehrenbacher, D.J. Slamon, M. Murphy, W.F. Novotny, M. Burchmore, S. Shak, S.J. Stewart, M. Press, Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer, J. Clin. Oncol. 20 (2002) 719–726.

[4] D.J. Slamon, B. Leyland-Jones, S. Shak, H. Fuchs, V. Paton, A. Bajamonde, T. Fleming, W. Eiermann, J. Wolter, M. Pegram, J. Baselga, L. Norton, Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2, N. Engl. J. Med. 344 (2001) 783–792.

[5] S. Zhang, W.C. Huang, P. Li, H. Guo, S.B. Poh, S.W. Brady, Y. Xiong, L.M. Tseng, S.H. Li, Z. Ding, A.A. Sahin, F.J. Esteva, G.N. Hortobagyi, D. Yu, Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways, Nat. Med. 17 (2011) 461–469.

[6] D.J. Slamon, W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove, A. Ullrich, et al., Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer, Science 244 (1989) 707–712.

[7] M.A. Bookman, K.M. Darcy, D. Clarke-Pearson, R.A. Boothby, I.R. Horowitz, Evaluation of monoclonal humanized anti-HER2 antibody, trastuzumab, in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2: a phase II trial of the gynecologic oncology group, J. Clin. Oncol. 21 (2003) 283–290.

[8] J.A. Wilken, K.T. Webster, N.J. Maihle, Trastuzumab sensitizes ovarian cancer cells to egfr-targeted therapeutics, J. Ovarian Res. 3 (2010) 7.

[9] J.N. McAlpine, K.C. Wiegand, R. Vang, B.M. Ronnett, A. Adamiak, M. Kobel, S.E. Kalloger, K.D. Swenerton, D.G. Huntsman, C.B. Gilks, D.M. Miller, HER2 overexpression and amplification is present in a subset of ovarian mucinous carcinomas and can be targeted with trastuzumab therapy, BMC Cancer 9 (2009) 433.

[10] K.D. Steffensen, M. Waldstrom, R.F. Andersen, D.A. Olsen, U. Jeppesen, H.J. Knudsen, I. Brandslund, A. Jakobsen, Protein levels and gene expressions of the epidermal growth factor receptors, HER1, HER2, HER3 and HER4 in benign and malignant ovarian tumors, Int. J. Oncol. 33 (2008) 195–204.

[11] V. Kulasingam, M.P. Pavlou, E.P. Diamandis, Integrating high-throughput technologies in the quest for effective biomarkers for ovarian cancer, Nat. Rev. Cancer 10 (2010) 371–378.

[12] D.D. Bowtell, The genesis and evolution of high-grade serous ovarian cancer, Nat. Rev. Cancer 10 (2010) 803–808.

[13] L.L. Luistro, J.A. Rosinski, H. Bian, S. Bishayee, P. Rameshwar, N.M. Ponzio, S.R. Ritland, Development and characterization of a preclinical ovarian carcinoma model to investigate the mechanism of acquired resistance to trastuzumab, Int. J. Oncol. 41 (2012) 639–651.

[14] B. Liu, Z. Fan, S.M. Edgerton, X. Yang, S.E. Lind, A.D. Thor, Potent anti-proliferative effects of metformin on trastuzumab-resistant breast cancer cells via inhibition of erbB2/IGF-1 receptor interactions, Cell Cycle 10 (2011) 2959–2966.

[15] A.H. Sims, A.J. Zweemer, Y. Nagumo, D. Faratian, M. Muir, M. Dodds, I. Um, C. Kay, M. Hasmann, D.J. Harrison, S.P. Langdon, Defining the molecular response to trastuzumab, pertuzumab and combination therapy in ovarian cancer, Br. J. Cancer 106 (2012) 1779–1789.

[16] H. Peng, Z. Dong, J. Qi, Y. Yang, Y. Liu, Z. Li, J. Xu, J.T. Zhang, A novel two mode-acting inhibitor of ABCG2-mediated multidrug transport and resistance in cancer chemotherapy, PLoS One 4 (2009) e5676.

[17] M. Narayan, J.A. Wilken, L.N. Harris, A.T. Baron, K.D. Kimbler, N.J. Maihle, Trastuzumab-induced HER reprogramming in "resistant" breast carcinoma cells, Cancer Res. 69 (2009) 2191–2194.

[18] I. Hellstrom, G. Goodman, J. Pullman, Y. Yang, K.E. Hellstrom, Overexpression of HER-2 in ovarian carcinomas, Cancer Res. 61 (2001) 2420–2423.

[19] S. Makhija, L.C. Amler, D. Glenn, F.R. Ueland, M.A. Gold, D.S. Dizon, V. Paton, C.Y. Lin, T. Januario, K. Ng, A. Strauss, S. Kelsey, M.X. Sliwkowski, U. Matulonis, Clinical activity of gemcitabine plus pertuzumab in platinum-resistant ovarian cancer, fallopian tube cancer, or primary peritoneal cancer, J. Clin. Oncol. 28 (2010) 1215–1223.

[20] M. Bezler, J.G. Hengstler, A. Ullrich, Inhibition of doxorubicin-induced HER3-PI3K-AKT signalling enhances apoptosis of ovarian cancer cells, Mol. Oncol. 6 (2012) 516–529.

[21] Y. An, L. Cai, Y. Wang, D. Zhu, Y. Guan, J. Zheng, Local expression of insulin-like growth factor-I, insulin-like growth factor-I receptor, and estrogen receptor alpha in ovarian cancer, Onkologie 32 (2009) 638–644.

[22] D. Liu, J. Liu, C. Wang, B. Lin, Q. Liu, Y. Hao, S. Zhang, M. Iwamori, The stimulation of IGF-1R expression by lewis(y) antigen provides a powerful development mechanism of epithelial ovarian carcinoma, Int. J. Mol. Sci. 12 (2011) 6781–6795.

[23] I. Bruchim, H. Werner, Targeting IGF-1 signaling pathways in gynecologic malignancies, Expert Opin. Ther. Targets 17 (2013) 307–320.

[24] W.H. Gotlieb, I. Bruchim, J. Gu, Y. Shi, A. Camirand, M.J. Blouin, Y. Zhao, M.N. Pollak, Insulin-like growth factor receptor I targeting in epithelial ovarian cancer, Gynecol Oncol. 100 (2006) 389–396.

[25] R. Nahta, L.X. Yuan, B. Zhang, R. Kobayashi, F.J. Esteva, Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells, Cancer Res. 65 (2005) 11118–11128.

[26] X. Huang, L. Gao, S. Wang, J.L. McManaman, A.D. Thor, X. Yang, F.J. Esteva, B. Liu, Heterotrimerization of the growth factor receptors erbB2, erbB3, and insulin-like growth factor-i receptor in breast cancer cells resistant to herceptin, Cancer Res. 70 (2010) 1204–1214.