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Research Article

HER2 decreases drug sensitivity of ovarian cancer cells via inducing stem cell-like property in an NFκB-dependent way

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Increasing evidence shows that cancer stem cells are responsible for drug resistance and relapse of tumors. In breast cancer, human epidermal growth factor receptor 2 (HER2) induces Herceptin resistance by inducing cancer stem cells. In the present study, we explored the effect of HER2 on cancer stem cells induction and drug sensitivity of ovarian cancer cell lines. First, we found that HER2 overexpression (HER2 OE) induced, while HER2 knockdown (HER2 KD) decreased CD44+/CD24− population. Consistently, HER2 expression was closely correlated with the sphere formation efficiency (SFE) of ovarian cancer cells. Second, we found that NFκB inhibition by specific inhibitor JSH23 or siRNA targetting subunit p65 dramatically impaired the induction of ovarian cancer stem cells by HER2, indicating that NFκB mediated HER2-induced ovarian cancer stem cells. Third, we found that HER2 KD significantly attenuated the tumorigenicity of ovarian cancer cells. Further, we found that HER2 inhibition increased drastically the sensitivity of ovarian cancer cells to doxorubicin (DOX) or paclitaxel (PTX). Finally, we examined the correlation between HER2 status and stem cell-related genes expression in human ovarian tumor tissues, and found that expressions of OCT4, COX2, and Nanog were higher in HER2 positive tumors than in HER2 negative tumors. Consistently, the 5-year tumor-free survival rate of HER2 positive patients was dramatically lower than HER2 negative patients. Taken together, our data indicate that HER2 decreases drug sensitivity of ovarian cancer cells via inducing stem cell-like property.

Introduction

Ovarian cancer is the fifth leading cause of cancer-related deaths in women worldwide. Ovarian cancer has the highest rate of cancer-related mortality amongst all the gynecological cancers in the western world. Epithelial ovarian cancer is the most lethal of the gynecologic cancers, with a 5-year survival rate less than 50%. Each year, an estimated 22000 women are diagnosed with and 14000 women die from the disease [1]. However, the signaling pathways regulating development of ovarian cancer are largely unknown.

Human epidermal growth factor receptor 2 (HER2) is a well-known oncogene inducing breast cancer. Recently, several studies have shown HER2 overexpression (HER2 OE) is correlated with prognosis in ovarian cancer patients [2–6]. However, the underlying mechanism is not clear. Increasing evidence shows that cancer stem cells contribute to HER2-induced breast tumorigenesis, and they are responsible for drug resistance and relapse of tumors [7–9]. Therefore, we hypothesized that HER2 facilitated the development and poor prognosis of ovarian cancer via inducing cancer stem cells.

It is well-established that CD44+/CD24− population has the property of cancer stem cells in breast cancer, including (i) ability to form mammary sphere; (ii) ability to initiate tumor more efficiently *in vivo*; and (iii) ability to regenerate the whole population (including CD44+/CD24− and CD44+/CD24+ and CD44−/CD24− and CD44−/CD24+) [10]. HER2 has been shown to induce CD44+/CD24− population

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in breast cancer [7]. Therefore, we explored the role of HER2 on induction of ovarian cancer stem cells by checking CD44+/CD24− population and sphere formation efficiency (SFE) in the present study. We found that HER2 up-regulated the percentage of CD44+/CD24− population, promoted sphere formation of ovarian cancer cells, and facilitated tumor initiation *in vivo*. Mechanistically, NFκB mediated the regulation of HER2 on cancer stem cells. Further, HER2 decreased the sensitivity of ovarian cancer cells to chemotherapy drugs. More importantly, there was close correlation between HER2 status and expression of cancer stem cells related genes in human ovarian cancer tissues. Our data indicate that HER2 may be a promising target for treatment of HER2-positive ovarian cancer patients.

Materials and methods

Cell lines

Ovarian cancer cell lines SKOV3 and A2780 were purchased from the European Collection of Cell Cultures (ECACC®) via Sigma. OVCAR-5 and IGVOA-1 were obtained from Dr W. Cliby (William Cliby, MD, Chair, Division of surgery, The Mayo Clinic, Rochester, Minnesota). SKOV3 was maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 1% sodium pyruvate (Invitrogen), 0.2% non-essential amino acids (Invitrogen), and 5% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. A2780 was cultured in RPMI1640 with 2 mM glutamine and 10% FBS. OVCAR-5 was cultured in DMEM, 10% FBS, 1% PSG, and 0.1 mM non-essential amino acids. IGROV-1 was cultured with DMEM with 10% FBS. For generation of HER or p65 knockdown cell line, SKOV3 transfected with pSIH-H1-p65 plasmid or pSIH-H1-HER2 plasmid was screened using puromycin for 15 days. The survived single clones were picked up and expanded. Expression of HER2 or p65 was examined by Western. The clones with high efficiency of HER2 or p65 knockdown were used for experiments in the present study.

Chemicals

LY294002 (Cat. #440202) and JSH23 (Cat. #481408) were purchased from Calbiochem (CA, U.S.A.). PD0325901 (Cat. #13034) and PP2 (Cat. #13198) were purchased from Cayman Chemical Company (MI, U.S.A.). Lapatinib (Cat. #S1028) was purchased from Selleckchem.com (TX, U.S.A.). Paclitaxel (PTX) (Cat. #RS036) was purchased from TSZCHEM (MA, U.S.A.). Doxorubicin (DOX) was a gift from Dr Marj Pena (Associate Professor, Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, SC, USA).

Human ovarian tumor tissues

Human Ethics approval was received from Central Hospital of Xinxiang. In brief, all tumor tissues obtained from the Tissue Bank of Central Hospital of Xinxiang were de-identified, granted an exemption from requiring ethics approval by the Institutional Review Board of Central Hospital of Xinxiang. The permit was obtained from the patients before the tumor tissues were collected by the Tissue Bank of Central Hospital of Xinxiang for research purpose and possible publication. Their clinicopathological characteristics are summarized in Table 1.

Plasmids

pcDNA-HER2 was purchased from Addgene (Plasmid #16257, Cambridge, MA, U.S.A.). pSIH-H1-HER2 and pSIH-H1-p65 were generated by inserting specific siRNA sequence into vector pSIH-H1. The specific siRNA sequences for HER2 and p65 are GGACGAATTCTGCACAATG and GCCCTATCCCTTTACGTCA, respectively.

Sphere assay

Cells were cultured in serum-free corresponding medium supplemented with 5 μg/ml insulin (Sigma, St. Louis, MO, U.S.A.), 20 ng/ml human recombinant epidermal growth factor (EGF, Invitrogen), 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen), and 0.4% BSA (Invitrogen) in six-well Ultra-Low Attachment plates (Corning, Corning, NY, U.S.A.) at a density of 10^4/well for 10 days. Spheres with a size of 20 μm or above were counted.

FACS

Cells were suspended in PBS and labeled with anti-human FITC–conjugated CD44 and PE-conjugated CD24 antibodies (BD Pharmingen™, San Jose, CA, U.S.A.) per one million cells in a final volume of 100 μl for 1 h at 4°C in the dark. Unbound antibodies were washed off and cells were analyzed on a BD FACSCalibur.

Table 1 The clinicopathological characteristics of clinical samples

Patient Id#	Histology	Lymph node status	FIGO stage*	Differentiation
30225	Mucinous	Negative	I	High
45871	Serous	Negative	II	Middle
24780	Serous	Negative	I	High
40568	Mucinous	Negative	III	High
41523	*Clear cell carcinoma*	Negative	II	Middle
36987	Endometrioid	Negative	I	High
40254	Endometrioid	Negative	IV	Middle
41254	Mucinous	Negative	II	Low
40263	Endometrioid	Negative	II	High
41078	Serous	Negative	IV	Low
40482	Mucinous	Negative	II	Middle
41287	Mucinous	Negative	I	Middle
40965	*Clear cell carcinoma*	Positive	I	Low
40676	*Clear cell carcinoma*	Positive	III	Middle
23587	Mucinous	Positive	II	Middle
31958	Mucinous	Positive	II	Low
26972	Endometrioid	Positive	II	High
41109	Serous	Positive	III	Low
41163	Serous	Positive	I	Middle
40621	Endometrioid	Positive	I	Low
41452	Mucinous	Positive	III	Low
41079	*Clear cell carcinoma*	Positive	I	Low
41296	Serous	Positive	IV	High
41361	Endometrioid	Positive	II	Middle
29875	*Clear cell carcinoma*	Positive	II	High
36459	Serous	Positive	III	Low
40259	Serous	Positive	I	High
41344	Mucinous	Positive	II	Low
32956	*Clear cell carcinoma*	Positive	III	Low
41638	Serous	Positive	IV	Middle
10106	Mucinous	Positive	I	Middle
42968	Serous	Positive	II	Low
41895	Endometrioid	Positive	I	High
42541	Endometrioid	Positive	II	Low
33682	Serous	Positive	II	Low
35678	Endometrioid	Positive	IV	Middle
36985	Endometrioid	Positive	II	High

*FIGO stage, International Federation of Gynecology and Obstetrics.

Cell proliferation assay

Cells were seeded at a density of 5000 cells per well in 96-well tissue culture plates and treated with a series of concentrations of DOX and PTX for 3 days. The cells were then incubated with fresh medium containing MTT at 37°C for 4 h. After incubation, the formazan produced was then solubilized by the addition of 100 μl DMSO. The suspension was placed on a micro-vibrator for 5 min and then the absorbance was recorded at 570 nm by an ELISA reader.

Western blotting

Protein was extracted with lysis buffer (150 mM NaCl, 1% v/v NP-40, 0.1% v/v SDS, 2 μg/ml aprotinin, 1 mM PMSF). Fifty micrograms of proteins were loaded on 10% SDS/PAGE, and then transferred to nitrocellulose membrane. After blocking with 5% non-fat dry milk for 1 h at room temperature, the membrane was incubated with the primary antibodies overnight at 4°C. After washing with PBST, the membrane was incubated with second antibody for 2 h at room temperature. The signaling was detected with an enhanced chemiluminescence HRP substrate for Western blotting (Pierce, Rockford, IL, U.S.A.) using the Molecular Imager system GDS8000b (UVP Inc., Upland, CA, U.S.A.). All primary and second antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). HER2

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(#4290S, 1:2000 dilution), p-HER2 (#2243, 1:1000 dilution), p65 (#8242S, 1:2000 dilution), p-p65 (#3033S, 1:1000 dilution), p-AKT (#4060S, 1:1000 dilution), p-ERK (#4370, 1:1000 dilution), p-SRC (#2101, 1:2000 dilution), β-actin (#3700S, 1:5000 dilution), and HRP-linked anti-rabbit second antibody (#7074, 1:5000 dilution).

Real-time PCR

Total RNA was extracted from human ovarian tumor tissues using TRIzol reagents (Pufei Biotechnology, Shanghai, China). Reverse transcription was performed by using M-MLV reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Two micrograms of total RNA was used for reverse transcription reaction. Quantitative PCR was performed using SYBR-Green Real Time PCR Master Mix (Toyobo, Osaka, Japan). All sample values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression value (as the internal reference control). The PCRs consisted of 3 min at 95°C, followed by 45 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 40 s. A $\Delta\Delta$Cq method was then used to process these data to calculate relative gene expression. Human primer sequences are as follows:

Oct4 forward: 5′-actgcagcagatcagccaca-3′ and reverse: 5′-tgagaaaggagacccagcag-3′;
Sox2 forward: 5′-tgaatgccttcatggtgtgg-3′ and reverse: 5′-tctccgacaaaagtttccac-3′;
Nanog forward: 5′-tgccttgctttgaagcatcc-3′ and reverse: 5′-gctgtcctgaataagcagat-3′.

IHC staining

The core tissue biopsies exhibiting carcinoma with a diameter of 2.5 mm were punched from individual donor paraffin-embedded tissue blocks and precisely arrayed into a new recipient block. Four micron sections were cut and used for IHC analysis. HER2 expression was detected via immunohistochemical strepavidin peroxidase staining. Briefly, the sections were dewaxed with xylene and rehydrated by graded ethanol washing. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 min. After antigen retrieval in 10 mmol/l citrate phosphate buffer for 5 min, the sections were blocked in 10% normal goat serum for 30 min. The sections were then incubated with HER2 antibody (#4290S, Cell Signaling Technology, Danvers, MA, U.S.A.) at 4°C overnight. VECTASTAIN® Elite ABC Kits (Peroxidase) (Vector Laboratories, Burlingame, CA, U.S.A.) was used for detection of HER2 signaling.

HER2 staining was confirmed under high power fields (magnification ×400). Each slide was then scored by evaluating staining intensity and percentage of positive tumor cells. The degree of staining was defined as follows: 0 (no staining); 1 (faint yellow); 2 (yellowish brown); and 3 (brown). Percentage of HER2-positive cells was scored as follows, 0 (<5%); 1 (5–25%); 2 (26–50%); 3 (51–75%); and 4 (>75%). The final score was calculated by multiplying these two scores for each slide. The final score 0–2 was defined as negative (−); 3–4 as weakly positive (+); 5–8 as moderately positive (++); and 9–12 as strongly positive (+++). Thirty-seven human ovarian tumor tissues were classified into two groups: HER– (the final score ranged from 0 to 4) and HER2+ (the final score ranged from 5–12).

Animal experiment

The *in vivo* experiment was conducted in accordance with regulations of Central Hospital of Xinxiang, and approved by the Institutional Animal Care and Use Committee. Briefly, SKOV3 cells with a series of dilutions were inoculated subcutaneously into 6–8 weeks old nude mice (one injection for each mouse). Six mice were used for each group. Tumor initiation and growth were examined weekly. The volume of tumor was calculated by using the formula: $V = (\text{Width}^2 \times \text{Length})/2$ (whatever direction that had the largest diameter was viewed as the Length, and the corresponding vertical direction was viewed as the Width.)

Statistical analysis

All results were confirmed in at least three independent experiments, and all quantitative data were presented as mean ± S.D. Student's *t* test or one-way ANOVA test was employed for analyzing quantitative variables. Survival curves were evaluated using Kaplan–Meier method and the differences between these survival curves were tested by log-rank test. It was considered statistically significant when a two-sided *P*-value was less than 0.05.

Results

To maximize the possible effect of HER2 on induction of ovarian cancer stem cells, ovarian cancer cell lines A2780 (with low endogenous HER2 expression) and SKOV3 (with high endogenous HER2 expression) were chosen as cell models in the present study (Figure 1A). To overexpress HER2, A2780 was transfected with pcDNA-HER2. As shown in Figure 1B, HER2 was overexpressed after transfection for 4 days. As the classic downstreams of HER2 signaling,

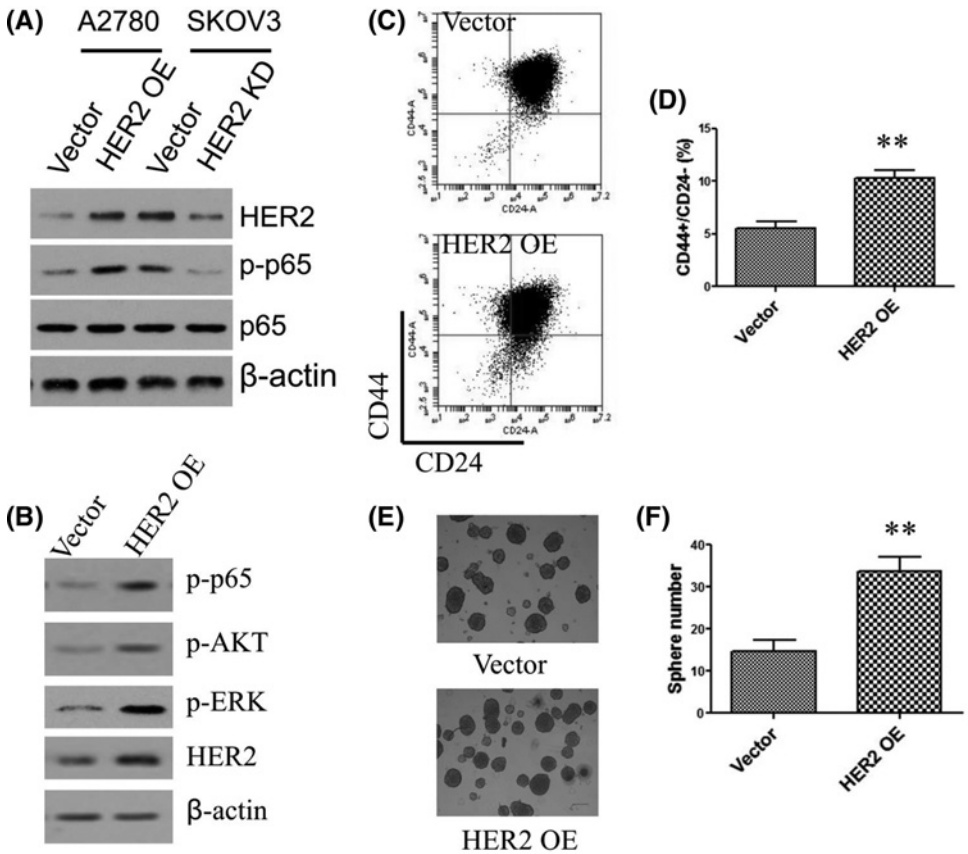

Figure 1. HER2 OE induces cancer stem cell-like population in ovarian cancer cells

(**A**) Endogenous HER2 expression level in A2780 and SKOV3 cells. (**B**) Overexpression of HER2. A2780 cells were transfected with pcDNA3 or pcDNA3-HER2 plasmid for 4 days. Cells were collected for Western blotting. (**C**,**D**) HER2 OE up-regulates CD44+CD24− population. A2780 cells were transfected with plasmids as in (B). Cells were then collected for staining of CD44 and CD24 and FACS analysis. Representative dot plot (C) and quantitative data (D). (**E**,**F**) HER2 OE facilitates sphere formation. A2780 cells were transfected with plasmids as in (B). SFE of cells was examined by seeding cells in a density of 10^4 for 2 weeks. Spheres were photographed (E) and counted (F). Scale bar, 50 μm. Data represent as mean ± S.D of three independent experiments. $^{**}P<0.01$.

p-AKT, p-ERK, and p-p65 were up-regulated significantly (Figure 1B), indicating that HER2 signaling was functional. The percentage of CD44+/CD24− population was then examined by FACS. As shown in Figure 1C,D, cancer stem cells population increased by 50% (5.5% compared with 10.3%) after overexpressing HER2. Another property of cancer stem cells is formation of sphere. Sphere formation ability of HER2-overexpressing and control A2780 was evaluated. As expected, SFE of A2780 overexpressing HER2 was much higher than control cells (Figure 1E). The number of spheres per 10^4 cells in HER2-overexpressing A2780 and control cells was 33.7 and 14.7, respectively (Figure 1F).

SKOV3 is an ovarian cancer cell line with high level of endogenous HER2 (Figure 1A). To confirm the role of HER2 on induction of cancer stem cells population, CD44+/CD24− percentage and SFE were measured in SKOV3 with endogenous HER2 knockdown (HER2 KD). Down-regulation of p-AKT, p-ERK, and p-p65 confirmed the inhibition of HER2 signaling (Figure 2A). As shown in Figure 2B,C, HER2 KD significantly decreased CD44+/CD24− population by more than 60% (5.6 compared with 2.0%). Consistently, SFE was impaired dramatically in HER2 KD SKOV3 cells (Figure 2D,E, 12.75 compared with 6.25 per 10^4 cells).

HER2-positive IGVOA-1 and HER2-negative OVCAR-5 (Supplementary Figure S1A), further confirmed the role of HER2 on cancer stem cells induction. As shown in Supplementary Figure S1B, HER2 KD decreased, while overexpression increased CD44+/CD24− population (Supplementary Figure S1B, 10.3 compared with 5.9% in IGVOA-1 and 4.3 compared with 8.6% in OVCAR-5). In-line with it, the SFE was closely associated with HER2 expression in IGVOA-1 and OVCAR-5 (Supplementary Figure S1C, 70.7 compared with 26.7 in IGVOA-1 and 40.2 compared with 112.0 in OVCAR-5).

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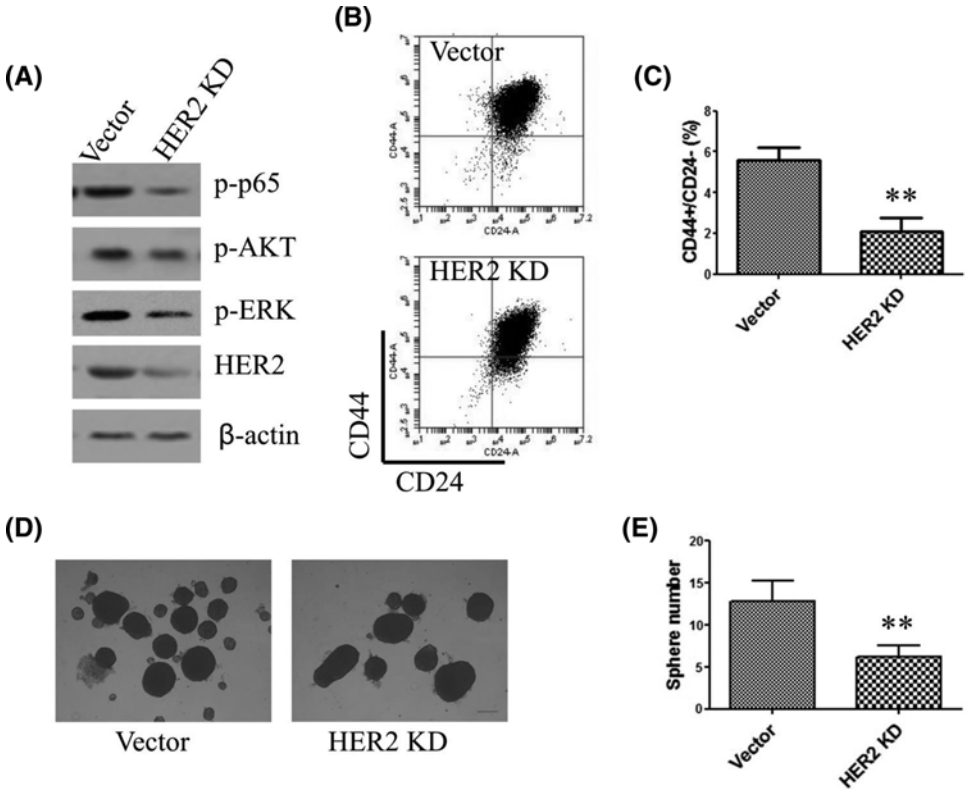

Figure 2. HER2 KD decreases cancer stem cell-like population in ovarian cancer cells

(**A**) Knockdown of HER2. SKOV-3 cells were transfected with pSIH-H1 or pSIH-H1-HER2 plasmid for 4 days. Cells were collected for Western blotting. (**B**,**C**) Knockdown of endogenous HER2 down-regulates CD44+CD24− population. SKOV-3 cells were transfected with plasmids as in (A). The cells were collected to examine the percentage of CD44+CD24− population by FACS. Representative dot plot (B) and quantitative data (C). (**D**,**E**) Knockdown of endogenous HER2 impairs SFE of ovarian cancer cells. SKOV-3 cells were transfected with plasmids as in (A). Cells were collected to examine SFE as in Figure 1F. Spheres were photographed (D) and counted (E). Scale bar, 50 μm. Data represent as mean ± S.D. of three independent experiments. $^{**}P<0.01$.

To dissect the signaling pathway that mediates HER2-induced cancer stem cells in ovarian cancer, SKOV3 cells were treated with inhibitors for classic downstream pathways of HER2, including LY294002 (PI3K/AKT inhibitor,10 μM), PD0325901 (MAPK inhibitor, 100 nM), JSH23 (NFκB inhibitor, 20 μM), and PP2 (SRC inhibitor, 10 μM). As shown in Supplementary Figure S2A, each inhibitor blocked efficiently its target signaling. CD44+/CD24− population was then examined after treating cells with these inhibitors for 4 days. LY294002, PD0325901, or PP2 treatment had no effect on CD44+/CD24− percentage (Supplementary Figure S2B). In contrast, NFκB inhibition by JSH23 treatment for 4 days (Figure 3A) decreased significantly the percentage of CD44+/CD24− population in SKOV3 cells (Figure 3B, 5.3 compared with 2.1%). In-line with it, JSH23 treatment significantly attenuated sphere formation ability of SKOV3 (Figure 3C, 12.5 compared with 6.1%). To exclude the non-specific effect of JSH23 on cancer stem cells induction, pSIH-H1-p65 plasmid (subunit of NFκB) was transfected into cells to knock down NFκB (Figure 3D). As shown in Figure 3E, NFκB knockdown decreased CD44+/CD24− percentage by 50% (Figure 3E, 6.5 compared with 3.1%). Further, SFE of SKOV3 with NFκB knockdown was much lower than control cells (Figure 3F, 13.2 compared with 8.7%). Taken together, NFκB knockdown significantly impaired the induction of ovarian cancer stem cells by HER2.

One of the most important properties of cancer stem cells is tumorigenesis. To further confirm the role of HER2 on ovarian cancer stem cells induction, cell lines with HER2 or NFκB knockdown were generated using SKOV3 (Figure 4A). The cells were inoculated orthotopically in a variety of amount into nude mice. As shown in Figure 4B, one out of six (17%) mice got tumor when inoculated with 500 cells, while 100% mice got tumors when inoculated with 10^4 cells in control group. However, in HER2 KD group, no mouse got tumor at the lowest dosage (500 cells) and only 67% (four out of six) mice got tumors at the higher dosage (10^4 cells). Similarly, NFκB knockdown also dramatically attenuated tumorigenicity of SKOV3 with no tumor formation at the lowest dosage (500 cells) and 50% tumor initiation efficiency at higher dosage (10^4 cells). The growth rate of tumors was also examined. As shown in

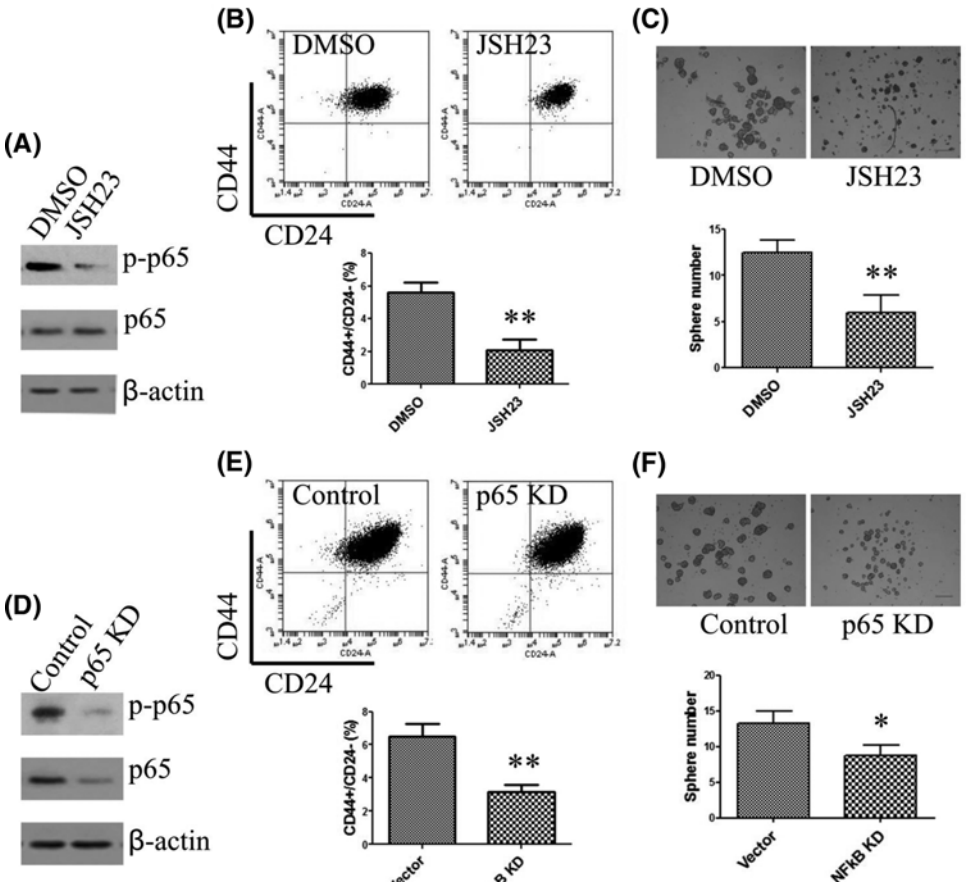

Figure 3. NFκB mediates HER2-induced cancer stem cell population

(**A**) Inhibition of NFκB signaling by JSH23. SKOV-3 cells were treated with JSH23 (20 μM) for 4 days. Cells were collected for Western blotting. (**B**) Inhibition of NFκB blocks the induction of CD44+CD24− population by HER2. SKOV-3 cells were treated with JSH23 as in (A). The cells were collected to examine the percentage of CD44+CD24− population by FACS. Representative dot plot (upper) and quantitative data (lower). (**C**) Inhibition of NFκB suppresses sphere formation of ovarian cancer cells. SKOV-3 cells were treated with JSH23 as in (A). The cells were collected to examine SFE by seeding cells at a density of 10^4 for 1 week. Spheres were photographed (upper) and counted (lower). Scale bar, 50 μm. (**D**) Inhibition of NFκB signaling by knocking down p65. SKOV-3 cells were transfected with pSIH-H1 or pSIH-H1-p65 plasmid for 4 days. Cells were collected for Western blotting. (**E**) Knockdown of NFκB significantly inhibits CD44+CD24− population induction by HER2. SKOV-3 cells were transfected with plasmids as in (D). The cells were collected to examine the percentage of CD44+CD24− population by FACS. Representative dot plot (upper) and quantitative data (lower). (**F**) Knockdown of NFκB decreases sphere number. SKOV-3 cells were transfected with plasmids as in (D). The cells were collected to examine SFE by seeding cells in a density of 10^4 for 1 week. Spheres were photographed (upper) and counted (lower). Scale bar, 50 μm. Data represent as mean ± S.D. of three independent experiments. *$P<0.05$, **$P<0.01$.

Figure 4C,D, knockdown of HER2 or NFκB drastically hampered tumor growth compared with control group. Briefly, HER2 enhanced tumorigenicity of SKOV3 via NFκB.

Increasing evidence shows that cancer stem cells are more resistant to chemotherapy. It is well known that PTX and DOX treatment dramatically increases cancer stem cells [9]. We thus mainly focussed on whether decrease in cancer stem cells via inhibiting HER2/NFκB signaling sensitized ovarian cancer cells to PTX or DOX treatment. As expected, SKOV3 cells with HER2 or NFκB knockdown were much more sensitive to DOX and PTX than parental SKOV3. The IC_{50} of DOX in HER2 KD, NFκB KD, and control SKOV3 was 2.6, 2.0, and 6.0 μM respectively (Figure 5A), and the IC_{50} of PTX was 3.5, 2.2, and 19.1 nM, respectively (Figure 5B). The effect of HER2 on drug sensitivity was confirmed in other ovarian cancer cell lines. As shown in Supplementary Figure S3A,B, HER2 KD decreased the IC_{50} of DOX by 60% (Supplementary Figure S3A, 1.75 compared with 0.72 μM), and PTX by 50% (Supplementary Figure S3B, 117 compared with 55 nM) in IGROV-1. In contrast, HER2 OE decreased the sensitivity of A2780 and OVCAR-5 to DOX (Supplementary Figure S3C, 0.04 compared with 0.09 μM and Supplementary Figure S3E, 0.4 compared

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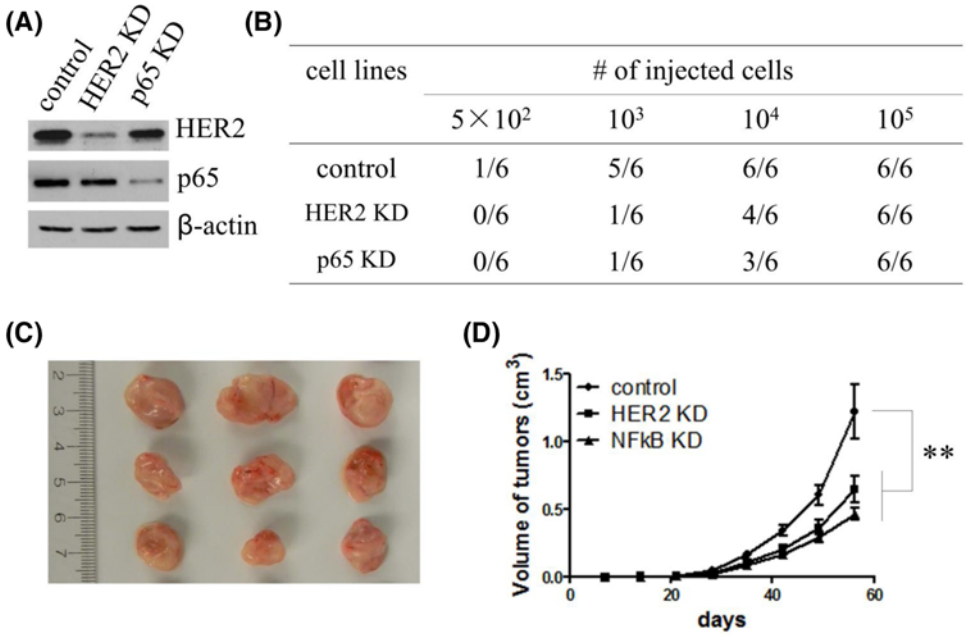

Figure 4. Inhibition of HER2 attenuates the tumorigenicity of ovarian cancer cells

(**A**) Generation of HER2 KD and NFκB knockdown cell lines. Expression of HER2 and p65 in HER2 and NFκB knockdown cell lines are examined by Western. (**B**–**D**) Knockdown of either HER2 or NFκB impairs tumor initiation and growth of ovarian cancer cells. Series of dilutions of SKOV-3 cells with HER2 or p65 knockdown and parental SKOV-3 cells were inoculated orthotopically into nude mice (n=6). The initiation of tumors was monitored weekly (B). Tumors' growth was monitored. Representative tumors (C) and tumors' growth curve (D). Data represent mean ± S.D. of ten tumors. $^{**}P<0.01$.

with 1.0 μM) and PTX (Supplementary Figure S3D, 3.7 compared with 10.7 nM and Supplementary Figure S3F, 20.7 compared with 46.2 nM). Considering the potential possibility for translation, the efficacy of combination of DOX or PTX with HER2 inhibitor lapatinib was evaluated. As shown in Figure 5C, treatment only with DOX or PTX increased CD44+/CD24− population. The percentage of cancer stem cells in combination of either DOX or PTX with lapatinib was much lower than single treatment (4.5 compared with 9.5% for DOX, 5.0 compared with 11.2% for PTX), and even lower than control group (4.5 compared with 6.5% for DOX, 5.0 compared with 6.5% for PTX). The same pattern was found when SFE was checked (Figure 5D). Combination of either DOX or PTX with lapatinib decreased SFE by more than 50% (9.5 compared with 20%, DOX) or ∼74% (6.8 compared with 26%, PTX).

To explore the possibility for clinical implication, correlation between HER2 and expression of cancer stem cells related genes were examined in human ovarian tumor tissues. Thirty-seven samples were classified into two groups based on HER2 expression level (Figure 6A). As shown in Figure 6B–D, expressions of SOX2 (Figure 6B), OCT4 (Figure 6C), and Nanog (Figure 6D) were, respectively, significantly higher in HER2-positive samples than in HER2-negative samples (0.007 compared with 0.017 for SOX2, 0.013 compared with 0.029 for OCT4, 0.011 compared with 0.024 for Nanog). Consistently, the 5-year tumor-free survival rate in HER2-positive patients was lower significantly than in HER2-negative patients (Figure 6E).

Discussion

Tumor cells are heterogeneous in the tumor tissue. Some of them are tumorigenic and some of them are not, which constitutes the foundation of cancer stem cell hypothesis. Drug resistance and relapse are two major challenges during the fight against cancers. Increasing evidence shows that cancer stem cells are most likely the root for drug resistance and relapse. Obviously, identification of signaling pathways regulating cancer stem cells is thus the key for eradicating cancer stem cells and in turn harnessing drug resistance and relapse of tumors. To do this, the prerequisite is isolation of cancer stem cells. However, so far there are no specific markers for the identification of cancer stem cells in different type of cancers. For example, CD90 has been reported to be a specific marker for ovarian cancer stem cells [11]. Controversially, a recent study has shown that CD90 inhibits ovarian cancer formation by interacting with β3 integrin [12], which indicates that CD90 alone may not be enough for the identification of ovarian cancer

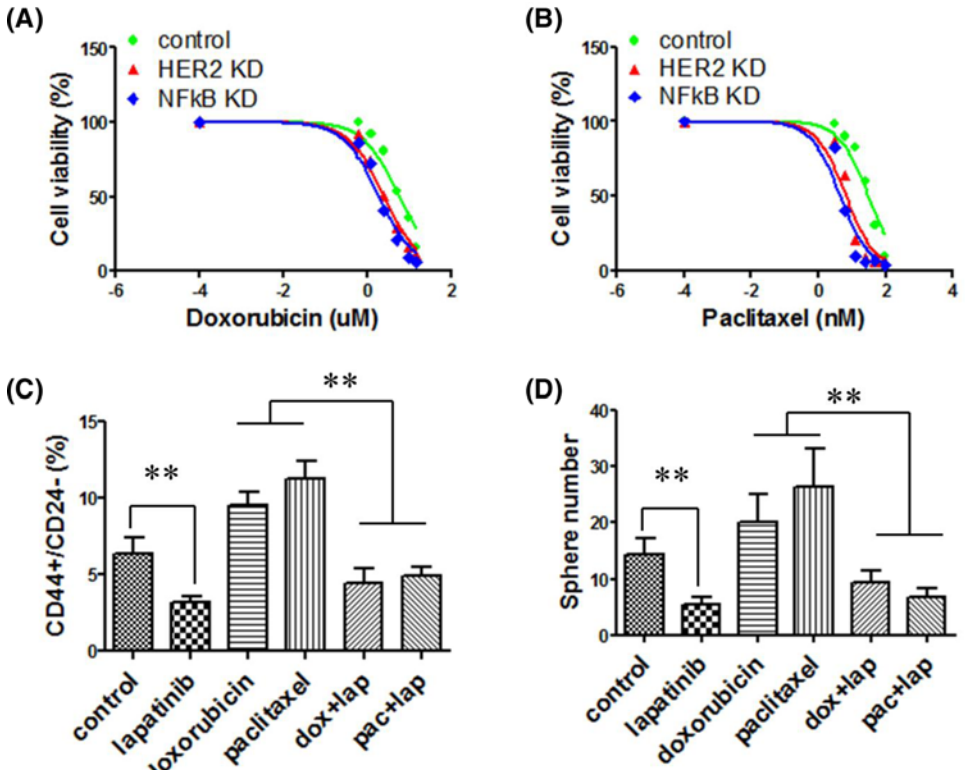

Figure 5. Inhibition of HER2 sensitizes ovarian cancer cells to chemotherapeutic drugs

(**A**,**B**) Knockdown of HER2 or NFκB sensitized ovarian cancer cells to DOX or PTX. SKOV3 cells with HER2 KD or NFκB KD and control cells were seeded into 96-well plate at a density of 10^4/well. Cells were treated with different concentrations of DOX (A) or PTX (B) for 3 days. Cell growth was then examined by MTT assay. (**C**,**D**) Combination of DOX with lapatinib or PTX with lapatinib synergistically decrease CD44+CD24− population (C) and sphere numbers (D). Cells were treated with different chemicals or combinations for 4 days. The cells were then collected for FACS (C) or evaluation of SFE by seeding cells in sphere-special medium for 10 more days (D). Data represent mean ± S.D. of three independent experiments. $^{**}P<0.01$.

stem cells. Shortage of specific markers prompts us to explore new markers for identification of ovarian cancer stem cells. It is well known that CD44+/CD24– population bears stem cell-like property in breast cancer [13]. Given that CD44+/CD24– population represents breast cancer stem cells, one of the main purposes of this paper is whether CD44+/CD24– phenotype can be used as the marker for ovarian cancer stem cells.

It is well known that HER2 induces cancer stem cells in breast cancer [7]. In ovarian cancer, HER2 status is closely associated with metastasis and relapse of tumors [3,5,6], indicating that HER2 may regulate ovarian cancer stem cells. Tumorigenicity is one of the most important properties of cancer stem cells. Our data showed that HER2 KD impaired greatly the potential of SKOV3 cells to initiate tumors in nude mice, indicating that HER2 is involved in the regulation of ovarian cancer stem cells.

To explore the possibility that CD44+/CD24– population bears cancer stem cell-like property, we examined the correlation between HER2 and CD44+/CD24– population by overexpressing or knocking down HER2 in different ovarian cancer cell lines. As expected, HER2 OE increased the percentage of CD44+/CD24− population in A2780 and OVCAR-5, while HER2 KD decreased the population in SKOV3 and IGROV-1. Further, the difference of SFE between parental cells and cells with HER2 OE or KD confirmed the stem cell-like property of CD44+/CD24− population. In-line with the *in vitro* data, tumor initiation efficiency in nude mice further confirmed that CD44+/CD24− population represented the cells with stem cell-like property. Collectively, our *in vitro* data indicate that CD44+/CD24− population may represent ovarian cancer stem cells.

Of note, one of the caveats of our study is that only limited cell lines were used. Therefore more work should be done to validate the possibility that CD44+/CD24− serves as an ovarian cancer stem cells marker by using more cell lines and tumor tissues. In addition, HER2 was used as the inducer of cancer stem cells in the present study. It is not clear whether CD44+/CD24− population represents ovarian cancer stem cells only in HER2-induced cancer stem

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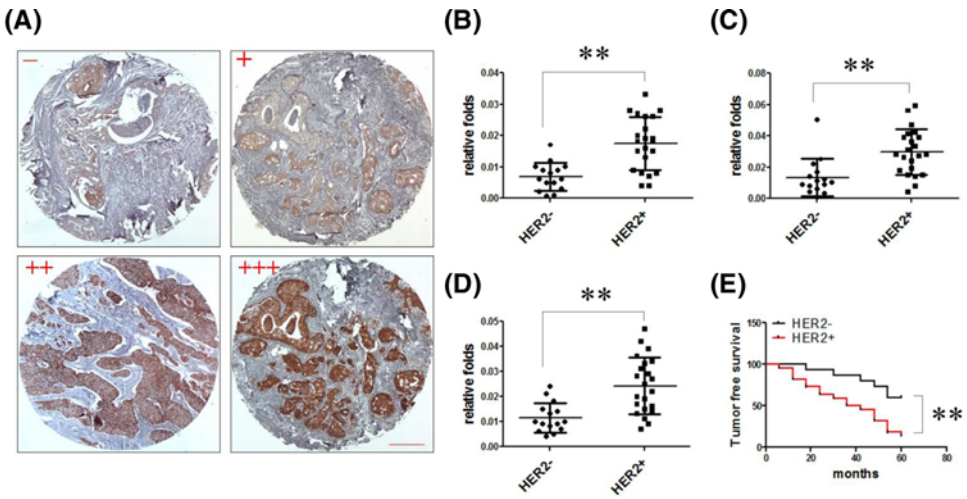

Figure 6. HER2 status is closely correlated with cancer stem cells related genes expression and 5-year tumor-free survival of patients

(**A**) HER2 expression in 37 human ovarian tumor tissues. Representative images with different HER2 expression level. − denotes negative; + denotes weakly positive; ++ denotes moderately positive; and +++ denotes strongly positive. Scale bar, 500 μm. SOX2 (**B**), OCT4 (**C**), and Nanog (**D**) expression in HER2− samples (including HER2 negative and weakly positive samples) and HER2+ samples (including HER2 moderately positive and strongly positive samples). Data represent mean ± S.D. of three independent experiments. $^{**}P<0.01$. (**E**) Five-year tumor-free survival rate. Thirty-seven ovarian cancer patients were classified into two groups: HER2− and HER2+. The 5-year tumor-free survival rate for each group was evaluated using Kaplan–Meier method. $^{**}P<0.01$.

cells model. Other models need to be tested to validate the universality of CD44+/CD24− population as the marker of ovarian cancer stem cells.

Our *in vitro* data supported that HER2 regulated ovarian cancer stem cells. Further, we were interested in whether HER2 was correlated with cancer stem cells *in vivo*. Due to the lack of fresh ovarian tumor tissues for measurement of CD44+/CD24− population, we examined the correlation between HER2 and stem cell-related genes in tumor tissues. SOX2 [14–16], OCT4 [17–19], and [20–22] are genes playing important roles in self-renewal and proliferation of cancer stem cells. We found that there was a close correlation between HER2 status and the expression of these three genes in human ovarian tumor tissues. Taken together, our data indicate HER2 can induce cancer stem cells in ovarian cancer.

Increasing evidence shows that cancer stem cells are more resistant to chemotherapy drugs [23,24]. It is therefore reasonable to hypothesize that HER2 can decrease the sensitivity of ovarian cancer cells to chemotherapy drugs (such as PTX and DOX) via induction of cancer stem cells. Indeed, our data showed that knockdown of HER2 decreased the IC_{50} of DOX and PTX in SKOV3 and IGVOA-1. In contrast, HER2 OE desensitized A2780 and OVCAR-5 to DOX and PTX, which is consistent with the role of HER2 in breast cancer [25].

For the translation purpose, we tested whether HER2 inhibition could sensitize tumor cells to chemotherapy. Lapatinib is a dual tyrosine kinase inhibitor which interrupts the HER2 and epidermal growth factor receptor (EGFR) pathways. Lapatinib is broadly used for treatment of HER2+ breast cancer patients [26]. Like many small molecule tyrosine kinase inhibitors, lapatinib is regarded as well tolerated [27,28]. More importantly, sensitivity of lapatinib for tumor cell growth inhibition is 10–75 folds higher than that of normal human fibroblast [29]. Although recent studies have shown the contribution of lapatinib to the clinical outcome is controversial maybe due to the small size of subjects [30–32], our data supported that lapatinib improved the sensitivity of ovarian cancer cells to DOX and PTX *in vitro* via suppressing the small side population with stem cell-like property. Combination of conventional chemotherapy drug DOX or PTX with lapatinib significantly decreased the IC_{50} of DOX and PTX in several cell lines. Single treatment with DOX or PTX even increased the percentage of cancer stem cells most likely due to the lower sensitivity of this specific population, which may be, at least partially, the underlying mechanism for the synergistic effect of lapatinib with DOX or PTX in SKOV3. Our *in vitro* data indicate that HER2 may be another promising target for HER2-positive ovarian cancer.

Interestingly, recent studies have shown that blockade of HER2 signaling by antibody (trastuzumab) benefits not only HER2-positve, but also HER2-negative breast cancer patients [33,34]. One of the possible explanations is that

there exists a very small HER2-positive population (CD44+/CD24−) with stem cell-like property in HER2-negative breast cancer patients [35]. Given that trastuzumab also benefits not only HER2-positive, but also HER2-negative ovarian cancer patients, our findings provide a mechanistic explanation for the clinical observation.

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Author contribution

W.W. was responsible for conception and design. Y.G., J.H., J.Y., and S.D. were responsible for acquisition and analysis of data. W.W. was also responsble for writing, review, and/or revision of the manuscript.

Competing interests

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Abbreviations

DOX, doxorubicin; HER2, human epidermal growth factor receptor 2; HER2 KD, HER2 knockdown; HER2 OE, HER2 overexpression; NFκB, Nuclear factor kB; PTX, paclitaxel; SFE, sphere formation efficiency.

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