Article

Discovery Medicine 2024; 36(182): 559–570
https://doi.org/10.24976/Discov.Med.202436182.52

DNA Methylation-Regulated HOXC8's Role in HER2-Positive Breast Cancer Function and its Contribution to Herceptin Resistance

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Published: 20 March 2024

Background: The epidermal growth factor receptor 2 (*HER2*) is overexpressed in 30% of breast cancers, and this overexpression is strongly correlated with a poor prognosis. Herceptin is a common treatment for HER2-positive breast cancer; however, cancer cells tend to adapt gradually to the drug, rendering it ineffective. The study revealed an association between the methylation status of the Homeobox C8 (*HOXC8*) gene and tumor development. Therefore, it is of paramount importance to delve into the interaction between *HOXC8* and HER2-positive breast cancer, along with its molecular mechanisms. This exploration holds significant implications for a deeper understanding of the pathophysiological processes underlying HER2-positive breast cancer.

Method: Tumor tissue and pathological data from patients with HER2-positive breast cancer were systematically collected. Additionally, the human HER2-positive breast cancer cell line, SKBR3, was cultured *in vitro* to assess both the expression level of *HOXC8* and the degree of DNA methylation. The study aimed to explore the relationship between the relative expression of *HOXC8* and the clinical characteristics of breast cancer patients. The expression level of *HOXC8* and the promoter methylation of *HOXC8* were verified by methylation treatment of SKBR3 breast cancer cells. The regulation of *HOXC8* was meticulously carried out, leading to the division of the cells into distinct groups. The study further analyzed the expression levels and biological capabilities within each group. Finally, the *in vitro* and *in vivo* sensitivity of the cells to Herceptin, a common treatment for HER2-positive breast cancer, was measured to assess the efficacy of the drug.

Result: In HER2-positive breast cancer cases characterized by poor methylation, there was an up-regulation of *HOXC8*. Its expression was found to be correlated with key clinical factors such as tumor size, lymph node status, clinical tumor, node, metastasis (cTNM) staging, and Herceptin resistance ($p < 0.05$). Upon methylation of breast cancer cells, there was a significant decrease in *HOXC8* expression ($p < 0.05$). The study revealed that overexpression of *HOXC8* resulted in increased proliferation, cloning, and metastasis of HER2-positive breast cancer cells, along with a reduced apoptosis rate ($p < 0.05$). Conversely, interference with *HOXC8* expression reversed this scenario ($p < 0.05$). A Herceptin-resistant substrain, POOL2, was established using SKBR3 cells. Animal studies demonstrated that overexpressing *HOXC8* accelerated tumor development and enhanced POOL2 cells' resistance to Herceptin ($p < 0.05$). However, following interference with *HOXC8*, POOL2 cells exhibited increased responsiveness to Herceptin, leading to a gradual reduction in tumor size ($p < 0.05$).

Conclusions: In HER2-positive breast cancer, the expression of *HOXC8* is elevated in a manner dependent on DNA methylation, and this elevated expression is closely linked to the pathology of the patient. Interfering with *HOXC8* expression demonstrates the potential to partially inhibit the development and spread of breast cancer, as well as to alleviate resistance to Herceptin.

Keywords: HER2-positive breast cancer; methylation; HOXC8; herceptin; drug resistance

Introduction

Breast cancer continues to exhibit one of the highest fatality rates among female malignancies in recent years, despite significant progress in detection and therapy [1,2]. The traditional molecular subtypes of breast cancer encompass Luminal A, Luminal B, the epidermal growth factor receptor 2 (HER2)-positive, and triple-negative. Up to 30% of invasive breast cancer cases are characterized by the overexpression of *HER2* [3–5]. Within the epidermal growth factor receptor (HER) family—comprising HER1, HER2, HER3, and HER4—HER2 is a member. These receptors feature intracellular domains with tyrosine kinase catalytic activity, single-chain transmembrane sections, and cysteine-rich extracellular ligand binding sites.

The *HER2* oncogene is located on human chromosome 17q21 and encodes the *HER2* protein, a 185kD transmembrane protein with 1255 amino acids [6]. Upon ligand interaction, *HER2* forms an active *HER2/HER2* dimer or an epidermal growth factor receptor (*EGFR*)/*HER2* het-

erodimer. This interaction leads to the self-phosphorylation of tyrosine residues in the intracytoplasmic domain of the receptor, activating tyrosinase activity. Several signaling pathways, including the mitogen-activated protein kinase (*MAPK*) pathway, phosphatidylinositol 3-kinase (*PI3K*) pathway, and protein kinase C (*PKC*) pathway, are activated to promote tumor proliferation, angiogenesis of tumor vessels and lymphatics, and increased tumor cell invasiveness [7,8].

In clinical practice, *HER2* overexpression in breast cancer patients is frequently associated with a poor prognosis. Patients with this type of breast cancer often experience rapid disease progression, a short period of chemotherapeutic remission, a propensity for developing drug resistance, limited efficacy of endocrine therapy, and low recurrence-free survival and overall survival rates [9].

The introduction of Herceptin (trastuzumab) has significantly transformed the approach to treating HER2-positive breast cancer. Herceptin is a frequently prescribed medication for HER2-positive breast cancer and can be administered either alone or in combination with other chemotherapy medications such as doxorubicin, cyclophosphamide, paclitaxel, among others [10].

Despite its widespread use, a substantial number of women with breast cancer using Herceptin develop secondary resistance, and approximately 70% of HER2-positive patients experience initial resistance [11–13]. This challenges therapists with a limited selection of therapeutic options for these individuals. To address the issues associated with the poor prognosis of HER2-positive breast cancer, it is imperative to delve into the biological roles of molecules linked to HER2-positive breast cancer, explore potential targets, and identify molecular pathways to mitigate treatment resistance.

Methylation, a form of chemical alteration of genomic DNA involving the addition of methyl groups, plays a crucial role in the onset and progression of diseases, including cancers [14]. Abnormalities in methylation are common in cancer, contributing to uncontrolled cell proliferation, resistance to apoptosis, invasion, and metastasis [15]. Notably, research suggests that the aberrant expression of Homeobox C8 (*HOXC8*) is associated with various cancers and the development of medication resistance [16,17].

The methylation state of the *HOXC8* gene is correlated with tumor incidence and progression, exhibiting diverse alterations across different types of tumors [18]. *HOXC8* has been shown to impact the self-renewal, differentiation, and transformation of breast cancer stem cells [19]. However, investigations into its precise mechanism of action in breast cancer cells and its influence on breast cancer treatment resistance are limited.

Therefore, our study focused on examining the expression and methylation status of the *HOXC8* gene in HER2-positive breast cancer. We explored the impact of *HOXC8* regulation on the biological behavior of HER2-positive breast cancer cells and its role in Herceptin resistance. This investigation aims to enhance our understanding of the pathophysiological processes underlying HER2-positive breast cancer and elucidate the molecular mechanisms associated with drug resistance. Ultimately, it provides a robust theoretical foundation for improving patient prognosis and identifying molecular targets to reduce medication resistance.

Methods

Clinical Sample

This study involved the selection of 30 pairs of cancer tissue and para-cancer tissue samples obtained from female patients diagnosed with HER2-positive breast cancer in the Department of Endocrinology at our hospital between May 2022 and May 2023. Inclusion criteria comprised a pathological diagnosis of HER2-positive breast cancer [20], absence of prior malignant tumor history, and the availability of complete clinical data. Additionally, patients should have exclusively received treatment with Herceptin. Exclusion criteria encompassed unclear diagnoses or carcinoma *in situ*, presence of other malignant tumors or breast cancer recurrence, and prior treatment with neoadjuvant chemotherapy, endocrine therapy, targeted therapy, radiotherapy, among others, with incomplete clinical data.

The study adhered to the principles outlined in the Declaration of Helsinki and received approval from the Ethics Committee of The First Affiliated Hospital, Hengyang Medical School, University of South China (approval number: P2022053). Informed consent was obtained from all participating patients, who willingly provided their consent by signing informed consent forms.

Cell Selection

The human HER2-positive breast cancer cell line SKBR3 (SNL-058, Wuhan Shanen Biotechnology Co., Ltd., Wuhan, China) was cultured in DMEM medium (SNM-004E, Wuhan Shanen Biotechnology Co., Ltd., Wuhan, China), supplemented with 15% fetal bovine serum (SNS-001, Wuhan Shanen Biotechnology Co., Ltd., Wuhan, China), and 1% penicillin/streptomycin solution (SNA-001, Wuhan Shanen Biotechnology Co., Ltd., Wuhan, China). Cultivation took place in a 5% CO_2 incubator at 37 °C, with the medium being changed every two days, and passage required every 5 to 7 days. Adhering cells were dissociated using 0.25% trypsin (SNT-001, Wuhan Shanen Biotechnology Co., Ltd., Wuhan, China) and then transferred to a petri dish containing fresh media.

All cells utilized in the experiment underwent identification through short tandem repeat (STR) analysis and were tested for mycoplasma, with no mycoplasma infection detected. All procedures strictly adhered to aseptic techniques to prevent cell contamination.

Table 1. Methylation primers.

Gene	Primers
HOXC8 methylation	F: 5′- AGGAAGGTTGGGAATGTGAG -3′ R: 5′- AACCCAACTACCAAACCACC -3′
HOXC8 non-methylation	F: 5′- TGTTTTTGTTGGGTTTGGTG -3′ R: 5′- AAACCCCACAACCCCAAAACA -3′

HOXC8, Homeobox C8.

Table 2. Primer sequence.

Gene	Primer
HOXC8	F: 5′- GGCGGCGGGTTTTCATGT -3′ R: 5′- AGCAGGGAGGTGGCAGTG -3′
GAPDH	F: 5′- CGGAGTCAACGGATTTGGTCGTAT -3′ R: 5′- AGCCTTCTCCATGGTGGTGAAGAC -3′

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Methylation Treatment: SKBR3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) culture solution containing 10% fetal bovine serum following the manufacturer's instructions. DNA methylase (R0639L, Beyotime, Shanghai, China) was introduced, and the methylation of the cells was assessed, comparing the SKBR3 Control group with the SKBR3 Methylation group.

Methylation Specificity PCR (MSP)

Genomic DNA from tissues and cells was isolated using the conventional phenol-chloroform extraction procedure. Subsequently, the DNA was converted using the EpiTect rapid DNA Bisulfite kit (59824, Shanghai Limin Industrial Co., Ltd., Shanghai, China). Following the conversion reaction, the kit's centrifugal column was employed for the purification and recovery of the products, which were then stored at –20 °C for subsequent use. The EpiTect methylation-specific PCR (MSP) kit (R100A, UNingwei Biotechnology Co., Ltd., Shanghai, China) was utilized to assess the methylation level of the *HOXC8* promoter region. The methylated and unmethylated primers are detailed in Table 1. Post-electrophoresis, the MSP products of the *HOXC8* gene promoter region displayed methylation (M band) and unmethylated (U band) bands. The M-band was indicative of positive methylation, with or without the presence of the U-band. Conversely, the amplification of the U band without the M band indicated negative methylation. The methylation rate = number of positive methylation specimens/total number of specimens × 100%.

Cell Grouping

SKBR3 cells in the logarithmic growth stage were divided into four groups: Control group (without the transfection of any sequence), NC group (transfection of *HOXC8* negative control sequence, purchased from Shanghai Gemma Bio Company), oe-HOXC8 group (transfection of oe-*HOXC8*, purchased from Shanghai Gemma Bio Company), and si-HOXC8 group (transfection of si-*HOXC8*, purchased from Shanghai Gemma Bio Company).

Twenty-four hours before transfection, the cells were inoculated into a 12-well plate, with 1.5 mL of complete culture solution without antibiotics added to each well. When the cell density reached approximately 60% at the time of infection, the cells were transfected with the respective sequences into SKBR3 using lipofectamine2000 (11668500, Invitrogen, Wilmington, MA, USA), and the medium was changed 6 hours post-transfection. After 48 hours of culture, cells were collected for subsequent experiments.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA from tissues and cells was extracted using TRIzol reagent (15596026CN, Invitrogen, Wilmington, Massachusetts, USA) following the manufacturer's protocol. The RNA's quality was assessed through UV analysis and formaldehyde denaturation electrophoresis to ensure high quality. Reverse transcription of 1 µg of RNA was performed using Avian Myeloblastosis Virus (AMV) reverse transcriptase to generate cDNA. Quantitative polymerase chain reaction (qPCR) was carried out using the SYBR Green technique. PCR primers were developed and produced by Shenggong BioEngineering (Shanghai) Co., Ltd. (Table 2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference for *HOXC8*. Each reaction tube's threshold cycle was acquired, and the analysis was done using the $2^{-\Delta\Delta Ct}$ technique. The multiple ratio of target gene expression between the experimental group and the control group was represented by the $2^{-\Delta\Delta Ct}$ statistic [21]. The formula is as follows: $\Delta\Delta Ct = [Ct \text{ (target gene)} - Ct \text{ (Refer to gene)}]_{\text{experimental group}} - [Ct \text{ (target gene)} - Ct \text{ (Refer to gene)}]_{\text{control group}}$.

Western Blot

Tissues and cells were harvested for their protein content, which was quantified using the bicinchoninic acid (BCA) kit following the provided instructions (AR0197, Boster, Wuhan, China). With 30 µg of protein per well, the extracted protein was mixed with sample buffer and heated at 95 °C for 10 minutes. Protein electrophoresis was performed using a 10% polyacrylamide gel, followed by PVDF transfer.

After blocking with 5% BSA at room temperature for 1 hour, primary antibodies HOXC8 (A15066, 1:500 dilution, Abclonal, Wuhan, China) and GAPDH (AC001, 1:3000 dilution, Abclonal, Wuhan, China) were added and incubated overnight at 4 °C. Subsequently, TBST washing was conducted three times for 5 minutes each. Corresponding secondary antibodies (Horseradish Peroxidase (HRP) Goat Anti-Rabbit Immunoglobulin G (IgG), AS014, 1:2000, Abclonal, Wuhan, China) were then incubated at room temperature for 1 hour, followed by three washes with

film for 5 minutes each. Chemiluminescent reagent was applied for development, with GAPDH serving as the internal control. The Bio-Rad Gel Doc EZ imager (Bio-Rad, Hercules, CA, USA) was employed for development, and the target bands were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Cell Counting Kit-8 (CCK-8) Assay

Each group's cell suspension was appropriately diluted before being dispensed onto a 96-well plate at a density of 1×10^3 cells per 100 µL in each well. The cells were cultured for 1 day, 2 days, 3 days, and 4 days, with triplicate wells for each time point. As a blank control, Cell Counting Kit-8 (CCK-8) (96992, Sigma, St. Louis, MO, USA) was added to cell-free medium. The culture plates were then incubated at 37 °C and 5% CO_2. At each time point, 10 µL of CCK-8 solution was added to the corresponding wells, and the plates were further incubated in the incubator for 4 hours. Subsequently, the optical density (OD) value of each well was measured using an enzyme-labeled instrument at a wavelength of 490 nm.

Plate Cloning Experiment

After pancreatic enzyme digestion of the cells in each group, 200 cells from each group were seeded into a culture dish. The dish was gently shaken to ensure an even distribution of cells, and they were cultivated for two to three weeks. When visible cell clones appeared, the culture was terminated. The culture medium was discarded, and the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, and then washed with PBS three times.

Subsequently, the cells were stained with Giemsa application solution for 60 minutes, slowly washed with running water, and air-dried. The number of cell clones was then counted under a microscope.

Flow Cytometry

Cells were suspended, washed with PBS, and their concentration was adjusted to 1×10^6 cells per milliliter. Subsequently, 200 µL of this cell solution was collected, washed twice with 1 mL of pre-cooled PBS, and centrifuged. After gentle mixing, the cells were resuspended in 100 µL of binding buffer containing 2 µL of Annexin-V-FITC (20 µg/mL, P-CA-204, Procell, Wuhan, China). The suspension was kept on ice, protected from light, for 15 minutes.

Following incubation, the cells were transferred to a flow detection tube, and 300 µL of PBS was added. Prior to loading, 1 µL of PI (50 µg/mL, P-CA-204, Procell, Wuhan, China) was added to each sample. The flow cytometer was used for testing within 30 minutes. The result criteria are as follows: The horizontal axis represents annexin-V, and the vertical axis represents PI. Injured cells can be observed in the top left quadrant, while late apoptotic or necrotic cells may be seen in the top right quadrant. Negative normal cells are visible in the lower left quadrant, and early apoptotic cells are observed in the lower right quadrant.

Transwell

After the digestion of cells in each group, the cell density was adjusted to 1×10^5/mL. Subsequently, 200 µL of the cell suspension was injected into the upper portion of the transwell chamber. Serum-free media were added to the upper chamber, while complete medium containing 10% serum was added to the lower portion of the transwell chamber. The cells were then incubated at 37 °C in a 5% CO_2 incubator.

After 24 hours, non-migrating cells from the top layer of the membrane were removed using a cotton swab. The cells were fixed with formaldehyde at 37 °C for 30 minutes, and the fixing solution was then discarded. Migratory cells were stained with 0.5% crystal violet for 20 minutes. This process was repeated three times per set. For the cell invasion experiment, the transwell chamber was pre-coated with a 50 µL Matrigel solution.

Preparation of Herceptin Resistant Cells

The Herceptin-resistant POOL2 cell model was induced through a combination of high-dose shock and progressive dose increase. To establish a steady growth of SKBR3 cells under the influence of 0.5 µg/mL trastuzumab, 0.5 µg/mL Herceptin, a concentration approximately 10 times the 50% inhibitory dose, was added to the entire medium. Subsequently, the drug concentration was incrementally increased to induce cell expansion under 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 µg/mL Herceptin. After sustained growth and passage under 8 µg/mL trastuzumab for one month, POOL2 cells were generated [22]. The complete induction of drug resistance took approximately 8 months.

To assess the sensitivity of POOL2 cells to Herceptin (0, 10, 50, 100, 200 µg/mL), the MTS method was employed. Absorbance, representing cell viability, was recorded at 490 nm per well, and cell viability at each time point was calculated. POOL2 cells were then categorized into four groups following the SKBR3 cell grouping method described earlier: Control group, NC group, oe-HOXC8 group, and si-HOXC8 group. These groups were subsequently subjected to experiments and detection.

Tumor Implantation in Nude Mice

Twenty-four male BALB/c-nude mice, weight 20–25 g, aged 4–6 weeks, were procured from Hunan Slack Jingda Experimental Animal Co., Ltd. The mice were housed in a specific-pathogen-free (SPF) environment, provided with sterile standard feed and water ad libitum. The mice were categorized into eight groups (SKBR3-Before adding medicine (BM), SKBR3-After adding medicine (AM), POOL2-BM, POOL2-AM, control group, negative control (NC) group, oe-HOXC8 group, si-HOXC8 group), each comprising three animals.

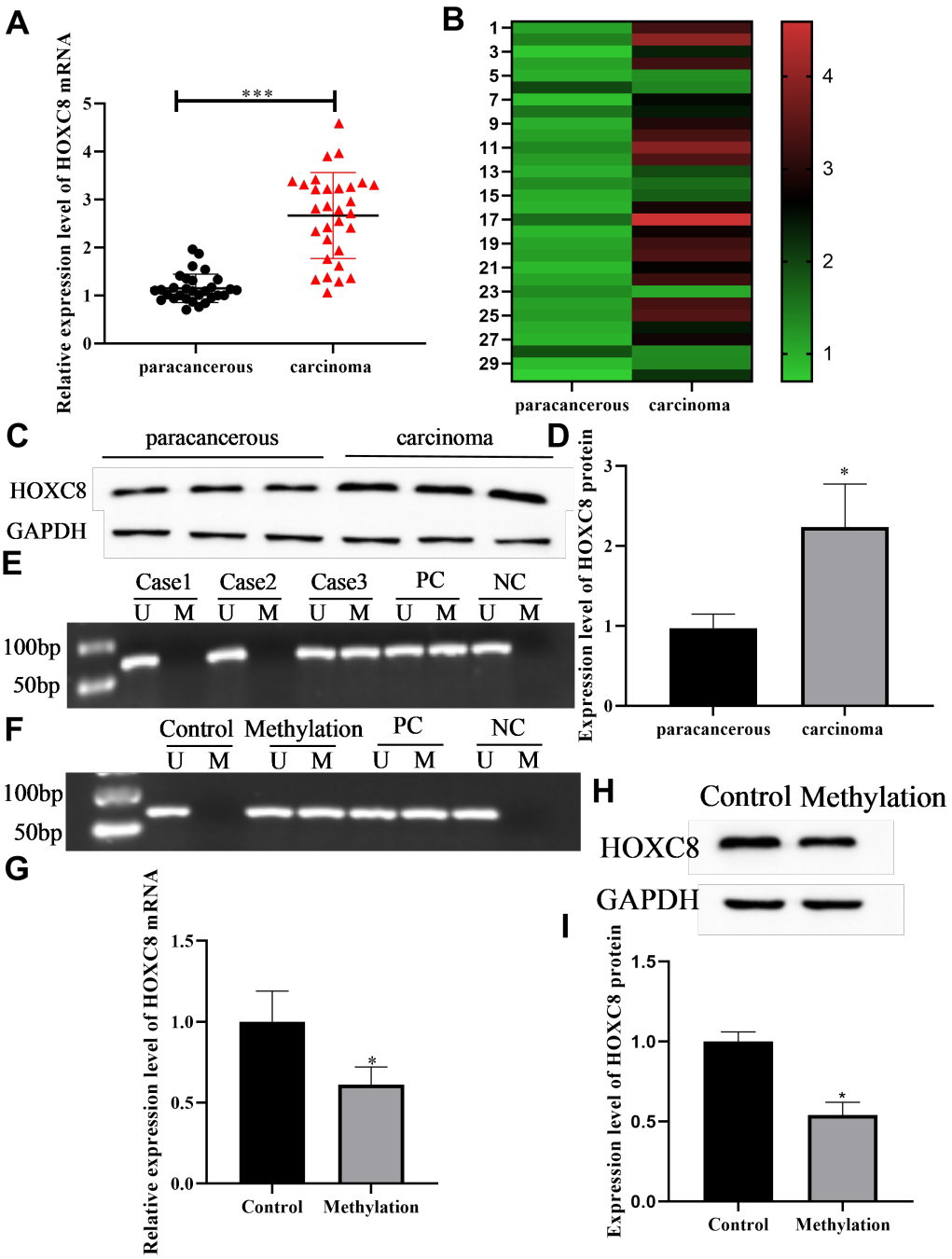

Fig. 1. Expression level and methylation degree of *HOXC8* were analyzed. Note: (A) Levels of *HOXC8* mRNA expression in clinical tissues, ***$p < 0.01$. (B) Heat map of *HOXC8* mRNA expression levels. (C,D) Expression level of HOXC8 protein in clinical tissues, *$p < 0.05$ vs paracancerous. (E) The methylation level of *HOXC8* in clinical tissues, U is the unmethylated band, M is the methylated band, NC is the negative control, and PC is the positive control. Case 1–3 represents the three representative cases selected. (F) Methylation degree of *HOXC8* in cells after methylation treatment, U is the unmethylated band, M is the methylated band, NC is the negative control, PC is the positive control. (G) *HOXC8* mRNA expression level in cells. (H,I) The expression level of HOXC8 protein in cells; *$p <$ 0.05 vs control; The experiment was repeated three times.

SK-BR-3, POOL2, and transfected cells (control group, NC group, oe-HOXC8 group, and si-HOXC8 group) in a healthy growth state were trypsinized, resuspended, and the cell number adjusted to 4×10^6 cells/0.1 mL PBS solution after cell counting. Subsequently, the cells were inoculated into the back of nude mice at a concentration of

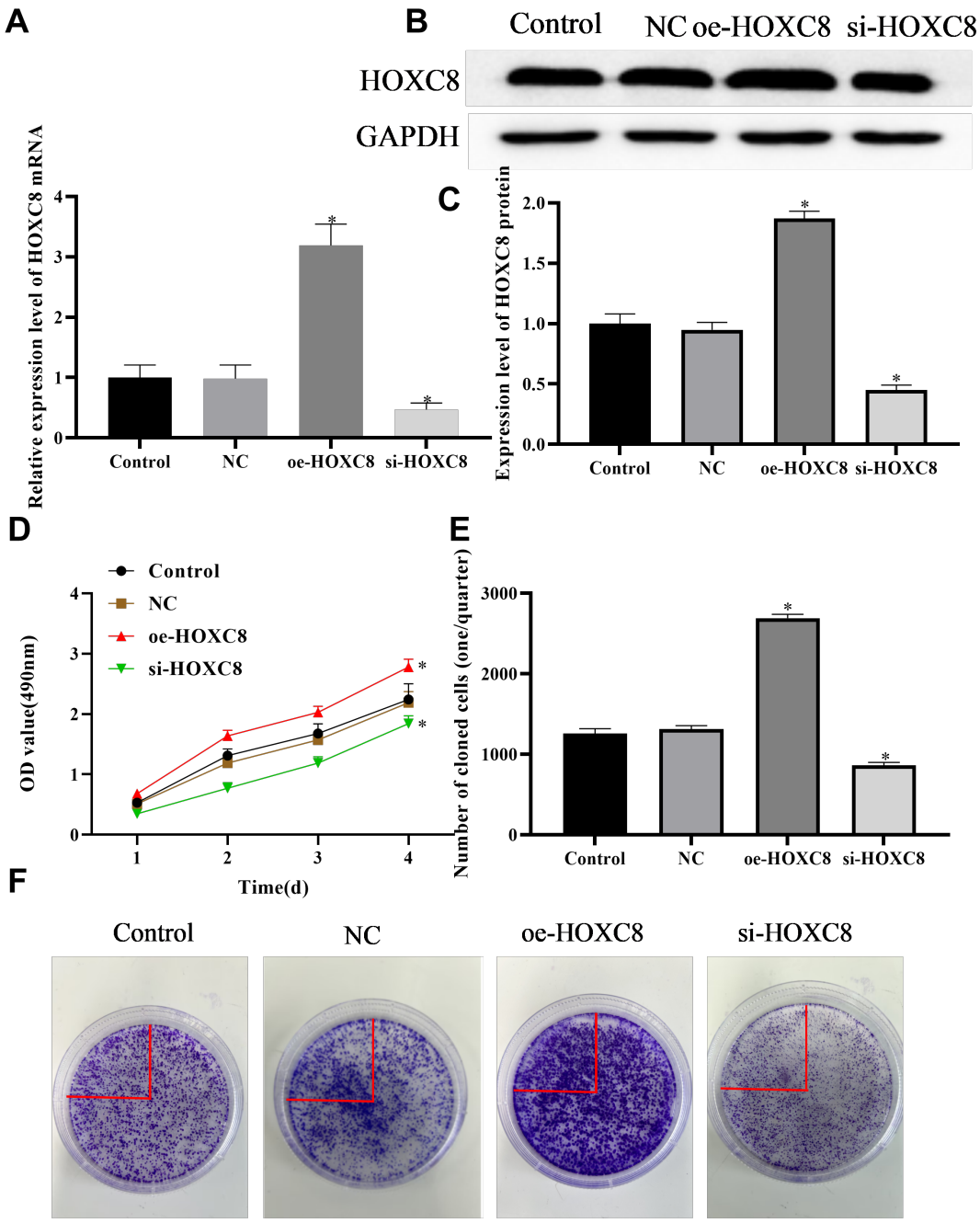

Fig. 2. Effect of *HOXC8* on proliferation and cloning ability of breast cancer cells. Note: (A) Expression levels of *HOXC8* mRNA. (B,C) Expression level of HOXC8 protein. (D) Cell proliferative potential. (E,F) Cell clonability; *$p < 0.05$ vs NC; The experiment was repeated three times.

4×10^6 cells per mouse. SKBR3 and POOL2 cells were transplanted into mice to observe whether or not they were injected with Herceptin. The mice were monitored every 5 days for tumor size measurements and weight assessments.

Tumor volume was calculated using the formula V = L × I (where L represents the longest diameter of the tumor, and I represents the shortest diameter of the tumor). Thirty days later, the nude mice were euthanized (after intraperitoneal injection of 1% pentobarbital sodium at 140 mg/kg, Ayrton Saunders, Cheshire, UK), and tumor tissues were excised and weighed. This study received approval from the Animal Ethics Committee of The First Affiliated Hospital, Hengyang Medical School, University of South China (D2022-1165), and all animal experiments adhered to the "3R" principle.

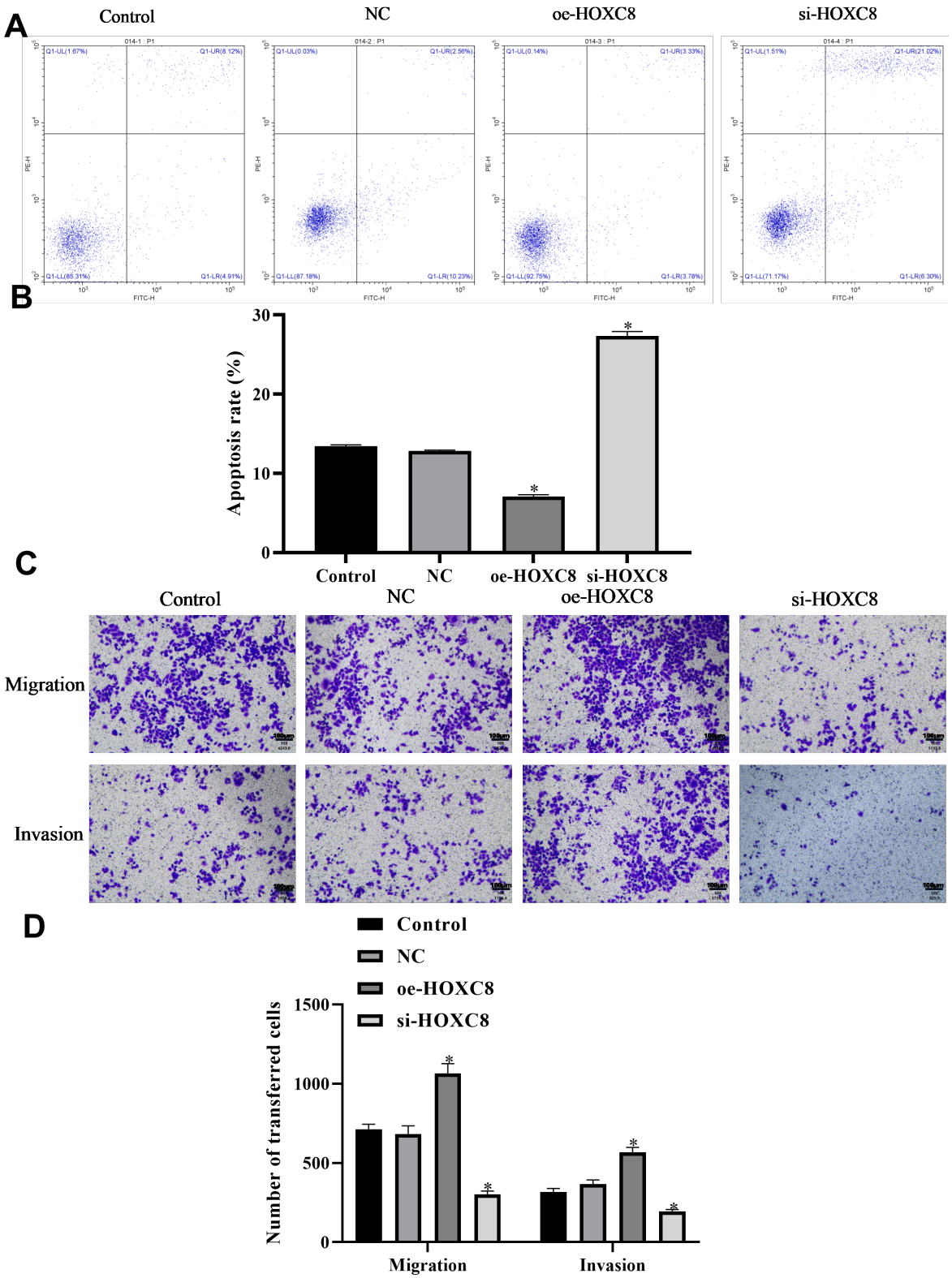

Fig. 3. Effect of *HOXC8* on apoptosis and metastasis of breast cancer cells. Note: (A,B) Apoptosis rate. (C,D) Cell migration and invasion ability, 100 μm ; *$p < 0.05$ vs NC; The experiment was repeated three times.

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software (IBM SPSS statistics, Chicago, IL, USA). The normal distribution of data was confirmed by the Kolmogorov-Smirnov test, and the results were presented as mean ± standard deviation. Group comparisons were conducted using the *t*-test for two groups and One-Way

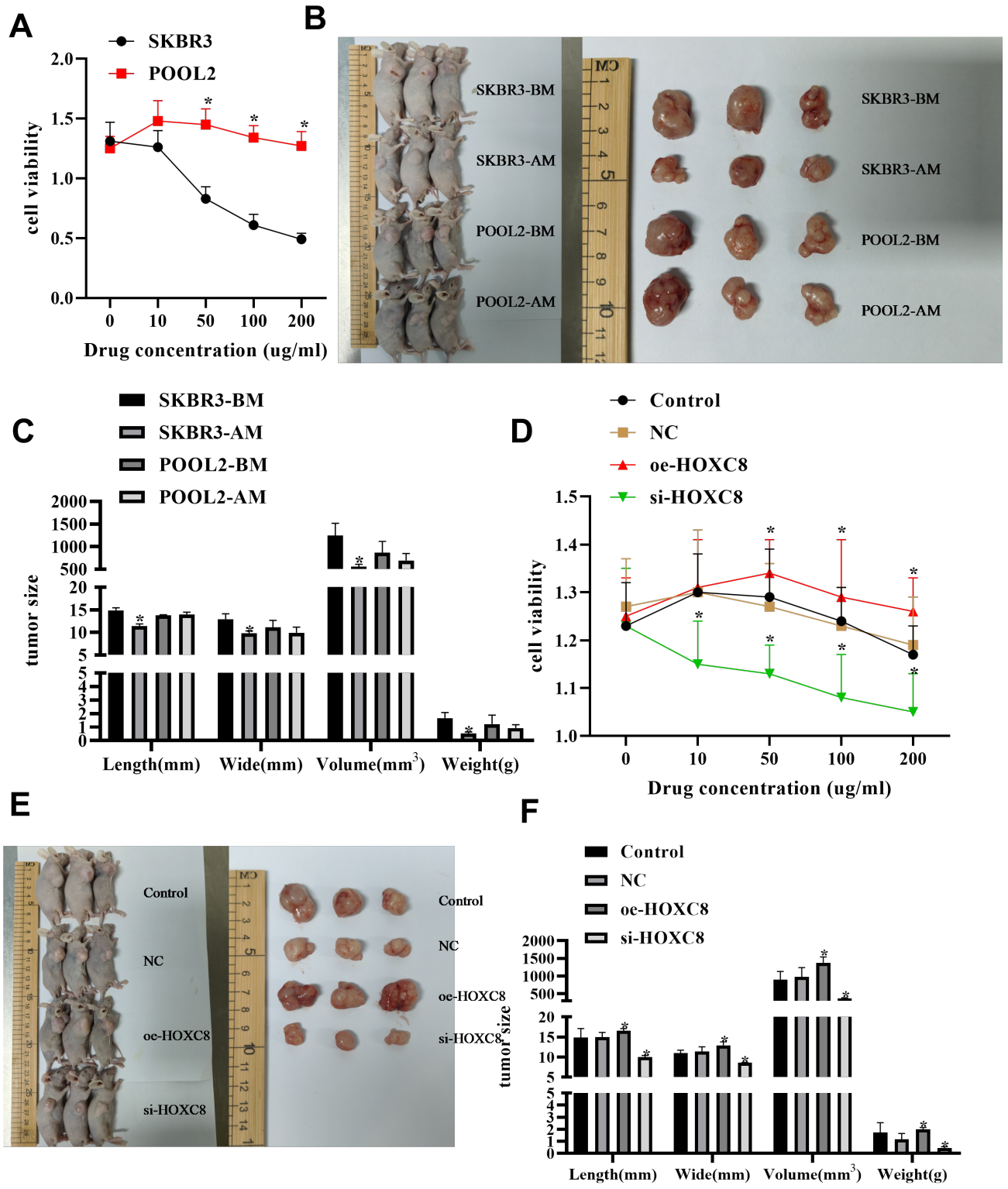

Fig. 4. Effect of *HOXC8* on tumor growth and Herceptin sensitivity. Note: (A) Cell viability, *$p < 0.05$ vs SKBR3. (B,C) Tumor size, *$p < 0.05$ vs SKBR3-BM. (D) Cell viability, *$p < 0.05$ vs NC. (E,F) Tumor size, *$p < 0.05$ vs NC. BM, Before adding medicine; AM, After adding medicine. The experiment was repeated three times.

analysis of variance (ANOVA) for multiple groups. Post hoc pairwise comparisons following ANOVA were carried out using Fisher's least significant difference *t*-test (LSD-t). The Chi-square test was employed to assess the relationship between *HOXC8* expression and the clinicopathological features of HER2-positive breast cancer. A significance level of $p < 0.05$ was considered statistically significant.

Table 3. Relationship between *HOXC8* expression level and clinicopathology in the epidermal growth factor receptor 2 (HER2)-positive breast cancer patients (Mean ± standard deviation, n, %).

Basic information	Total number (n = 30)	*HOXC8* mRNA	*t/F*	*p* value
Average age (years)				
≤50	16 (53.33%)	2.72 ± 0.99	0.360	0.722
>50	14 (46.67%)	2.60 ± 0.81		
Menstrual status				
Premenopausal	18 (60.00%)	2.56 ± 0.91	0.777	0.444
Postmenopausal	12 (40.00%)	2.82 ± 0.88		
Family history of tumors				
Yes	3 (10.00%)	2.60 ± 0.93	0.705	0.487
No	27 (90.00%)	2.99 ± 0.57		
Tumor size				
cT1	2 (6.67%)	1.96 ± 0.89	3.059	0.046
cT2	16 (53.33%)	2.23 ± 0.76		
cT3	7 (23.33%)	2.98 ± 0.61		
cT4	5 (16.67%)	3.02 ± 0.64		
Lymph node status				
cN0	8 (26.67%)	2.16 ± 0.65	3.033	0.047
cN1	7 (23.33%)	2.37 ± 0.69		
cN2	9 (30.00%)	2.98 ± 0.86		
cN3	6 (20.00%)	3.14 ± 0.67		
cTNM staging				
I	1 (3.33%)	1.94 ± 0.00	3.594	0.041
II	17 (56.67%)	2.40 ± 0.51		
III	12 (40.00%)	2.96 ± 0.73		
Histological grading				
I	10 (33.33%)	2.71 ± 0.00	0.209	0.813
II	11 (36.67%)	2.84 ± 0.71		
III	9 (30.00%)	2.93 ± 0.78		
Ki67				
<30%	14 (46.67%)	2.66 ± 0.75	0.688	0.497
≥30%	16 (53.33%)	2.86 ± 0.83		
Drug resistance				
Yes	12 (40.00%)	2.93 ± 0.71	2.088	0.046
No	18 (60.00%)	2.41 ± 0.64		

cTNM, clinical tumor, node, metastasis; Ki67, Kiel 67.

Result

Expression Level and Methylation Degree of HOXC8 were Analyzed

When *HOXC8* expression was measured in breast cancer tissues that were HER2-positive, it was discovered that these tissues had higher levels of *HOXC8* than the paracancerous did ($p < 0.05$) (Fig. 1A–D).

In HER2-positive breast cancer tissue samples, the CpG island in the *HOXC8* promoter region exhibited poor methylation. The results indicated a significantly lower CpG island methylation rate in HER2-positive breast cancer tissue compared to paracancerous tissue (36.67% (11/30) vs. 63.33% (19/30), $p < 0.05$) (Fig. 1E).

The promoter methylation of *HOXC8* and the expression level of *HOXC8* were both shown to be methylated in the human HER2-positive breast cancer cell line SKBR3. After methylation, it was discovered that *HOXC8* expression levels reduced ($p < 0.05$) (Fig. 1F–I).

Relationship between HOXC8 Expression Level and Clinicopathology in HER2-Positive Breast Cancer Patients

Analysis of the connection between *HOXC8* expression and clinicopathology revealed that it was connected to tumor size, Lymph node status, clinical tumor, node, metastasis (cTNM) staging and Herceptin resistance ($p < 0.05$) (Table 3).

Effect of HOXC8 on Proliferation and Cloning Ability of Breast Cancer Cells

After the regulation of *HOXC8*, *HOXC8* expression increased in the overexpressing *HOXC8* group and decreased in the interfering *HOXC8* group ($p < 0.05$) (Fig. 2A–C). The proliferation and cloning ability of SKBR3 cells were assessed. Compared with the NC group, the proliferation and cloning ability of SKBR3 cells increased in the oe-HOXC8 group ($p < 0.05$) and decreased in the si-HOXC8 group ($p < 0.05$) (Fig. 2D–F).

Effect of HOXC8 on Apoptosis and Metastasis of Breast Cancer Cells

The metastatic potential and apoptosis rate of SKBR3 cells were assessed. In comparison to the NC group, the oe-HOXC8 group exhibited a lower SKBR3 cell apoptosis rate and a stronger capacity for metastasis ($p < 0.05$), whereas the si-HOXC8 group showed a higher SKBR3 cell apoptosis rate and a weaker capacity for metastasis ($p < 0.05$) (Fig. 3A–D).

Effect of HOXC8 on Tumor Growth and Herceptin Sensitivity

The sensitivity to Herceptin decreased upon the successful development of Herceptin resistance in POOL2 cells (Fig. 4A). Notably, no significant difference was observed between SKBR3 and POOL2 cells without Herceptin when SKBR3 cells were subcutaneously implanted into nude mice ($p > 0.05$). However, after the administration of Herceptin, the growth of SKBR3 cell grafts slowed down, and the volume decreased ($p < 0.05$), whereas no statistical difference was observed between POOL2 cell grafts and the non-Herceptin group ($p > 0.05$) (Fig. 4B,C).

Following the regulation of *HOXC8*, POOL2 cells were assessed for Herceptin sensitivity. The results indicated that, compared to the NC group, the oe-HOXC8 group exhibited lower sensitivity, faster tumor growth, and a larger tumor volume. Conversely, the si-HOXC8 group gradually developed sensitivity to Herceptin, leading to a gradual slowdown in tumor growth and a reduction in tumor volume ($p < 0.05$) (Fig. 4D–F).

Discussion

Breast cancer accounts for approximately 15% of all new cancer cases in women, making it the most prevalent malignancy among women globally. It is also the leading cause of mortality in females under the age of 45 [23,24]. Among the various subtypes of breast cancer, HER2-positive breast cancer is particularly aggressive, constituting 15% to 20% of all breast cancer cases [25]. With continuous advancements in clinical diagnostics and treatment techniques, there has been an improvement in patients' awareness of health.

HER2-positive breast cancer is associated with a poor prognosis, necessitating comprehensive care that not only eliminates lesions but also meets the evolving standards of physical appearance and quality of life desired by modern women. Clinical professionals advocate for breast preservation in these patients, aiming to maintain an attractive appearance and ensure a normal life. However, irrespective of the type of surgery, patients with HER2-positive breast cancer are impacted by both the disease and the physical constraints of the surgical procedure.

HER2-positive breast cancer exhibits tumor heterogeneity, meaning that the same tumor can yield different therapeutic effects and prognoses across different patients. Even within the same patient, tumor cells may exhibit variations, highlighting one of the key characteristics of malignant tumors [26,27]. This heterogeneity reflects the significant complexity and diversity of malignant tumors throughout their evolutionary processes.

The study of carcinogenesis and tumor development has long been impeded by the intricate nature of tumors. One substantial challenge in the practical application of targeted treatment for breast cancer is the persistence of both primary and acquired resistance to Herceptin in HER2-positive breast cancer [28]. Recognizing the need for personalized approaches, the National Institutes of Health (NIH) introduced the concept of precision medicine and initiated the "precision medicine program". This program aims to consider genetic traits, individual living conditions, lifestyle, and other differences in developing disease prevention and treatment strategies [29].

At the core of precision medicine for malignant tumors is molecular targeted therapy. This approach involves directing active components such as medications and antibodies to specific molecular targets, aiming to modulate tumor-related molecules and achieve therapeutic objectives.

The Homeobox (*HOX*) gene family, with *HOXC8* as one of its members, plays a crucial role in regulating both embryonic and adult tissue development [30]. This gene family encodes transcription factors that contribute to the embryonic development of tissues and organs in various parts of the body. Typically, the expression of HOX genes is reduced in adults but can be reactivated under various physiological and pathological conditions [31,32]. Research indicates that the expression levels of *HOXC8* in specific tumor types may deviate from the norm, being either unusually high or low, and this aberrant expression is associated with the growth and metastasis of tumors [33,34].

Abnormal fluctuations in *HOXC8* DNA methylation levels have been observed in various malignancies, leading to either increased or decreased methylation levels, resulting in abnormal expression of the *HOXC8* gene [35]. In this study, 30 cases of HER2-positive breast cancer were examined to investigate the expression and methylation of *HOXC8*. The findings revealed low levels of methylation and up-regulation of *HOXC8* in the cancer tissues of HER2-positive breast cancer patients. Furthermore, a correlation was identified between *HOXC8* expression and histological

grade, level of differentiation, distant metastases, Herceptin resistance, and patient prognosis. These findings provide significant momentum for our ongoing research.

Studies suggested that aberrant methylation levels of the *HOXC8* gene may influence its transcriptional activity, subsequently impacting linked signaling pathways, altering cell proliferation and fate determination, and either promoting or inhibiting the growth of malignancies [36]. In the context of HER2-positive breast cancer, SKBR3 cells were subjected to DNA methylase treatment to modulate the methylation level, confirming that changes in *HOXC8* methylation levels influence the transcriptional activity of the gene, leading to altered expression in SKBR3 cells.

To further explore the impact of the *HOXC8* gene on tumor cell function, the study manipulated *HOXC8* expression and conducted relevant tests on the biological behavior of cells. The results indicated that overexpression of *HOXC8* enhanced the proliferation, cloning, and metastasis ability of HER2-positive breast cancer cells while weakening apoptosis rates. Conversely, interference with *HOXC8* expression reversed these effects. These findings suggested that interfering with *HOXC8* can potentially inhibit the growth, metastasis, and apoptosis of HER2-positive breast cancer cells, making it a promising therapeutic target for HER2-positive breast cancer.

To test the hypothesis that *HOXC8* controls Herceptin resistance in HER2-positive breast cancer, a Herceptin-resistant cell line named POOL2 was developed over an 8-month period. *In vivo* experiments demonstrated that, following the regulation of *HOXC8* in POOL2 cells, the oe-HOXC8 group exhibited lower sensitivity to Herceptin, faster tumor growth, and a larger tumor size compared to the NC group. In contrast, the si-HOXC8 group gradually became sensitive to Herceptin, resulting in slower tumor growth and a smaller tumor size. These findings suggested that interfering with *HOXC8* could enhance tumor cells' sensitivity to Herceptin.

The potential mechanism behind this phenomenon could be attributed to *HOXC8*'s role as a transcription factor controlling the production of specific drug-metabolizing enzymes, such as the cytochrome P450 enzyme. This control influences the rate at which medications are metabolized, subsequently affecting the sensitivity of tumor cells to various treatments [37]. Further experimental research is warranted to comprehensively understand the specific mechanism of drug resistance.

Conclusions

The expression of *HOXC8*, regulated by DNA methylation, is elevated in HER2-positive breast cancers, and it is linked to the pathophysiology of the patients. Inhibiting *HOXC8* expression can partially reduce Herceptin resistance, as well as impede the development and metastasis of breast cancer.

Availability of Data and Materials

The data used and/or analyzed during the current study are available from the corresponding author.

Author Contributions

XX conceived and designed the research study, acquired and analyzed the data, and wrote the initial draft of the manuscript. ST provided technical support for the acquisition and analysis of the data, performed statistical analysis, and contributed to the interpretation of the data and the manuscript revisions. ZY provided expertise in the field of study, contributed to the interpretation of the data, and critically revised the manuscript for important intellectual content. All authors have read and approved the final manuscript, and have participated sufficiently in the work to take public responsibility for appropriate portions of the content.

Ethics Approval and Consent to Participate

This study was carried out in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of The First Affiliated Hospital, Hengyang Medical School, University of South China (approval number: P2022053). All patients gave informed consent and signed informed consent forms. This study has been approved by the Animal Ethics Committee of The First Affiliated Hospital, Hengyang Medical School, University of South China (D2022-1165), and all animal experiments follow the "3R" principle.

Acknowledgment

We would like to express our gratitude to the participants who generously gave their time and effort to make this study possible.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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