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Original Article

LMO4 mediates trastuzumab resistance in HER2 positive breast cancer cells

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Abstract: Breast cancer is the leading cause of cancer-related mortality in women worldwide. Trastuzumab (Herceptin) is an effective antibody drug for HER2 positive breast cancer; *de novo* or acquired trastuzumab resistance retarded the use of trastuzumab for at least 70% of HER2 positive breast cancers. In this study, we reported LMO4 (a member of LIM-only proteins) promoted trastuzumab resistance in human breast cancer cells. Over-expression of LMO4 was observed in acquired trastuzumab resistance breast cancer cells SKBR3 HR and BT474 HR. Depletion of LMO4 partly abolished the trastuzumab resistance of SKBR3 HR and BT474 HR cells. Forced expression of LMO4 significantly increased trastuzumab resistance of HER2 positive breast cancer cells both *in vitro* and *in vivo*. BCL-2 was regulated by LMO4 and mediated the promoting role of LMO4 in trastuzumab resistance of HER2 positive breast cancer cells. High level of LMO4 was associated with worse clinicopathological parameters (including tumor size and histological grade) and lower survival rate in HER2 positive breast cancer patients. LMO4 therefore could be used as a target to develop diagnostic and therapeutic methods for human HER2 positive breast cancer.

Keywords: LMO4, BCL-2, HER2, trastuzumab resistance, breast cancer

Introduction

Breast cancer is the most common form of cancer and the leading cause of cancer-related mortality in women worldwide [1, 2]. The traditional treatment for breast cancer included surgery, chemotherapy, radiation therapy, assistant hormone therapy, and targeted therapy [3]. Epidermal growth factor receptor HER2 (ERBB2) belongs to the receptor tyrosine kinase family [4]. HER2 is amplified in approximately 20-30% of human breast cancers and associated with high invasive property, high degree of malignancy and poor prognosis in breast cancer patients [5-7]. The humanized anti-HER2 antibody, trastuzumab (Herceptin), interrupting HER2-mediated downstream signaling has been approved for clinical use in breast cancer patients with HER2 amplification since 1998 and is known as a successful example of targeted therapy for human cancer [3, 8-10].

Despite some clinical success of trastuzumab used for HER2 positive breast cancer patients, *de novo* or acquired trastuzumab resistance retarded the use of trastuzumab for at least 70% of HER2 positive breast cancers [5, 11, 12]. Previous studies revealed complex molecular mechanisms contributing to trastuzumab resistance; these molecules involved PI3K/AKT/mTOR, IGF-1R, PTEN, MET, VEGF etc. [13-18]. In addition, Lu X et al. reported that miR-129-5p promoted the sensitivity of HER2 positive breast cancer to trastuzumab by negatively regulation of Rps6 [6]. DUSP4 was documented to enhance resistance against anti-HER2 therapy in breast cancer [19]. Ma T et al. demonstrated that down regulation of miR-542-3p promoted trastuzumab resistance in breast cancer cells by activation of AKT [20]. Therefore, the detailed and varied molecular mechanisms of trastuzumab resistance in human breast cancer are desirable for further investigation.

LMO4 is a member of LIM-only family proteins (LMO). Other main members of LIM-only family proteins are LMO1, LMO2 and LMO3, which directly influence the fate and differentiation of cells at embryonic stage [21]. It has been reported that over-expression of LMO1 or LMO2 was associated with T cell leukemia, and they were considered as oncogenes in T cell leukemia [22-24]. LMO3 has been reported to be oncogenic in human neuroblastoma [25, 26]. LMO4 was also demonstrated to be an oncogene in T cell leukemia [27]. In addition, LMO4 was associated with malignant phenotype of pancreatic cancer, non-small-cell lung cancer, head and neck cancer etc. [28-30]. In breast cancer, over-expression of LMO4 suppressed the differentiation of mammary epithelial cells and promoted initiation and development of breast cancer [31, 32]. Moreover, LMO4 was involved in HER2 signaling pathway regulation in HER2 positive breast cancer cells [33]. However, the role of LMO4 in trastuzumab resistance of HER2 positive breast cancer cells remains unclear.

In this study, we determined LMO4 promoted trastuzumab resistance of human HER2 positive breast cancer cells both *in vitro* and *in vivo*. BCL-2 was specifically regulated by LMO4 and mediated the promoting role of LMO4 in trastuzumab resistance of HER2 positive breast cancer cells. Over-expression of LMO4 was significantly associated with clinicopathological parameters and survival rates in HER2 positive breast cancer patients. Therefore, LMO4 could be used as a new bio-marker for predicting the prognosis of HER2 positive breast cancer patients. Developing functional-inhibitors targeting LMO4 would be a good adjuvant therapeutic approach to enhance trastuzumab efficacy in HER2 positive breast cancer patients.

Materials and methods

Cell lines and cell culture

Human HER2 positive cell lines SKBR3 and BT474 were used in this study, which were obtained from the ATCC (American Type Culture Collection) and were cultured at 37°C in a humidified atmosphere of 5% CO_2 as recommended.

Trastuzumab resistant cell lines (SKBR3 HR and BT474 HR) were generated as described earlier [13, 34]. Briefly, SKBR3 and BT474 cells were treated continuously with 10 μg/ml trastuzumab for at least 6 months until significant trastuzumab resistance were examined by assessing cell viability after treated with trastuzumab for 6 days.

Plasmid constructs and transfection

Mammalian expression vector pIRESneo3 (Invitrogen) was used for plasmid construction. Human LMO4 coding sequence transcript (GenBank accession no.NM_006769.3) was cloned into pIRESneo3 plasmid and it was designated as pIRESneo3-LMO4. We used lip2000 (QIAGEN) for plasmid transfection as recommended [35, 36].

RNA oligonucleotides and transfection

siRNAs used in this study including siLMO4, siBCL-2 and siNC were designed and synthesized by GenePharma (Shanghai, China). We used lip2000 (QIAGEN) for siRNA transfection as recommended [35, 36]. The siRNA sequences were as follows: siLMO4, 5'-GCAUGAUCCUUUGCA GAAATT-3'; siBCL-2, 5'-UCACCCCGUCCCUGAAGAGTT-3'; siNC, 5'-UUCUCCGAACGUGUCACG UTT-3'.

RT-quantitative PCR (qPCR)

RT-qPCR was performed using SYBR green Master MIX (Applied Biosystem) as described previously [13, 36]. GAPDH was examined as an endogenous control. The primers used were: LMO4, forward 5'-ACATCGGCACGTCCTGTTACA-3' and reverse 5'-CTCATGACGAGTTCACTCGCAG-3'; BCL-2, forward 5'-TCCGCATCAGGAAGGCTAGA-3' and reverse 5'-AGGACCAGGCCTCCAAGCT-3'; GAPDH, forward 5'-TGCACC ACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGT GGTCATGAG-3'.

Western blot analysis

Protein levels of LMO4 and BCL-2 were detected using Western blot and were performed as described earlier [13, 36]. Rabbit polyclonal antibody against LMO4 (Origene, TA324369), mouse monoclonal antibody against BCL-2 (Santa Cruz Biotechnology sc-509) and mouse monoclonal antibody against β-actin (Sigma) were used. β-actin was examined as an endogenous control.

Cell function assays

Cell Function Assays including MTT assay and soft agar colony formation assay were carried out as described earlier [19, 35]. For trastuzumab treated experiments, MTT assay and soft agar colony formation assay were examined after trastuzumab treated for 6 days.

Xenograft analyses

Animal work in this study was done in accordance with the Institutional Animal Care and Use Committee guidelines (available at www.iacuc.org) and the approval by the Institutional Animal Care and Ethics Committee of Anhui Medical University was obtained before work. SKBR3 LMO4 and SKBR3 Vec cells (500×10^4 per 125 μl per site) were injected subcutaneously into the dorsal flank of 4-week-old female BALB/c-nu/nu mice (Shanghai Slaccas Co, Shanghai, China). Each cell was injected into 6 mice (12 sites). Both cells injected into mice formed palpable tumors after about one week and mice were randomized to receive trastuzumab (10 mg/kg) or vehicle as control 15 days after cell inoculation once a week. Tumor size was measured every 4 days and the formula (Volume (mm^3) = $L \times W^2 \times \Pi/6$) [37] was used to calculate tumor volume. Mice were sacrificed and tumors were harvested after 39 days and the tumors were made into paraffin sections for Ki-67 protein analysis by immunohistochemistry (IHC).

Clinical samples

One hundred paraffin-embedded HER2 (++/+++) breast cancer tissues were collected at the Department of Pathology in the First Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China). These tissues were from patients who underwent surgical resection between 2011 and 2012. Patients with other diseases or had underwent special therapies were excluded for the present study. The clinicopathological parameters of these patients were collected which were determined based on the 2003 World Health Organization (WHO) classification system. All of these patients were followed up for more than 5 years. Experiments for clinical tissues were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and the approval by the Institutional Review Boards of Anhui Medical University was obtained before work. Informed consent form was signed by every patient.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was performed using an UltraSensitive-SP kit (Maxin-Bio, Fuzhou, China) according to the kit's instructions. Protein levels of HER2 and LMO4 in human HER2 (++/+++) breast cancer tissues were detected and a mouse polyclonal antibody against HER2 (Maxin-Bio, Fuzhou, China) and a rabbit polyclonal antibody against LMO4 (Origene, TA350138) were used. Protein levels of Ki-67 in tumors formed in mice from SKBR3 LMO4 or SKBR3 Vec cells were detected and a mouse polyclonal antibody against Ki-67 (Zhongshan Goldenbridge Biotechnology Co, Beijing, China) was used.

Assessment of immunohistochemical staining

Immunohistochemical stained sections were reviewed and scored by two experienced pathologists using an Olympus microscope (Olympus America Inc., Melville, NY) independently. For HER2 staining in tissues from breast cancer patients, 0, 1, 2, 3 scoring system were used. For LMO4 staining in tissues from breast cancer patients, two-tier grading system was used to analyze the staining levels and classified them to high expression group and low expression group; cases were graded as LMO4 high when positive cells were above 20% and cases were graded as LMO4 low when positive cells were below 20%. For Ki-67 staining in tumors formed in mice, the percentage of Ki-67 positive cell population was calculated.

Statistical analyses

All of the results in this study represented the average of at least three independent repeating experiments. RT-qPCR, MTT assay, soft agar colony formation assay and tumor growth in xenograft were analyzed using unpaired two-tailed t test. Immunohistochemistry and clinicopathological parameters analysis were performed using Pearson's chi-square test. Survival rate analyses in patients (Kaplan-Meier curves) were carried out using log-rank test. $P<0.05$ was considered statistically significant.

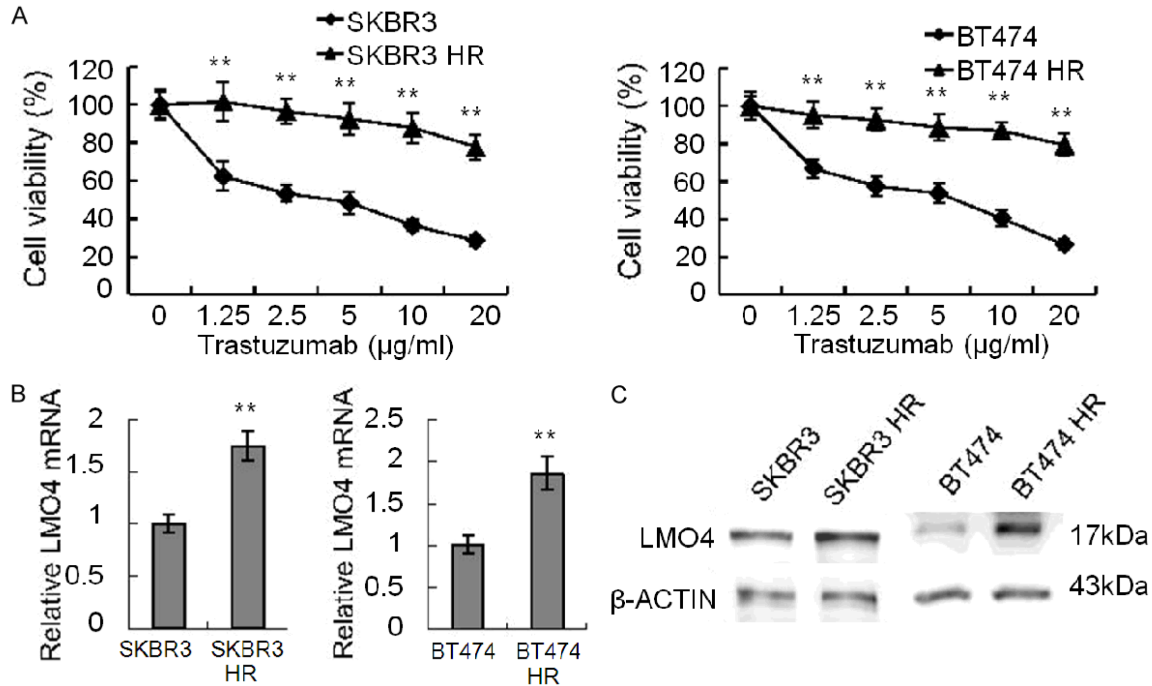


Figure 1. Expression of LMO4 in acquired trastuzumab resistant breast cancer cells. A. SKBR3, BT474 parental cells and trastuzumab resistant cells SKBR3 HR (10 μg/ml), BT474 HR (10 μg/ml) were treated with the indicated concentrations of trastuzumab for 6 days, MTT assay was performed to detected cell viability. B. mRNA levels of LMO4 in SKBR3/SKBR3 HR and BT474/BT474 HR cells were examined by RT-qPCR. GAPDH was used as an endogenous control. C. Protein levels of LMO4 in SKBR3/SKBR3 HR and BT474/BT474 HR cells were examined by western blot. β-actin was used as an endogenous control. **P*<0.05. ***P*<0.01.

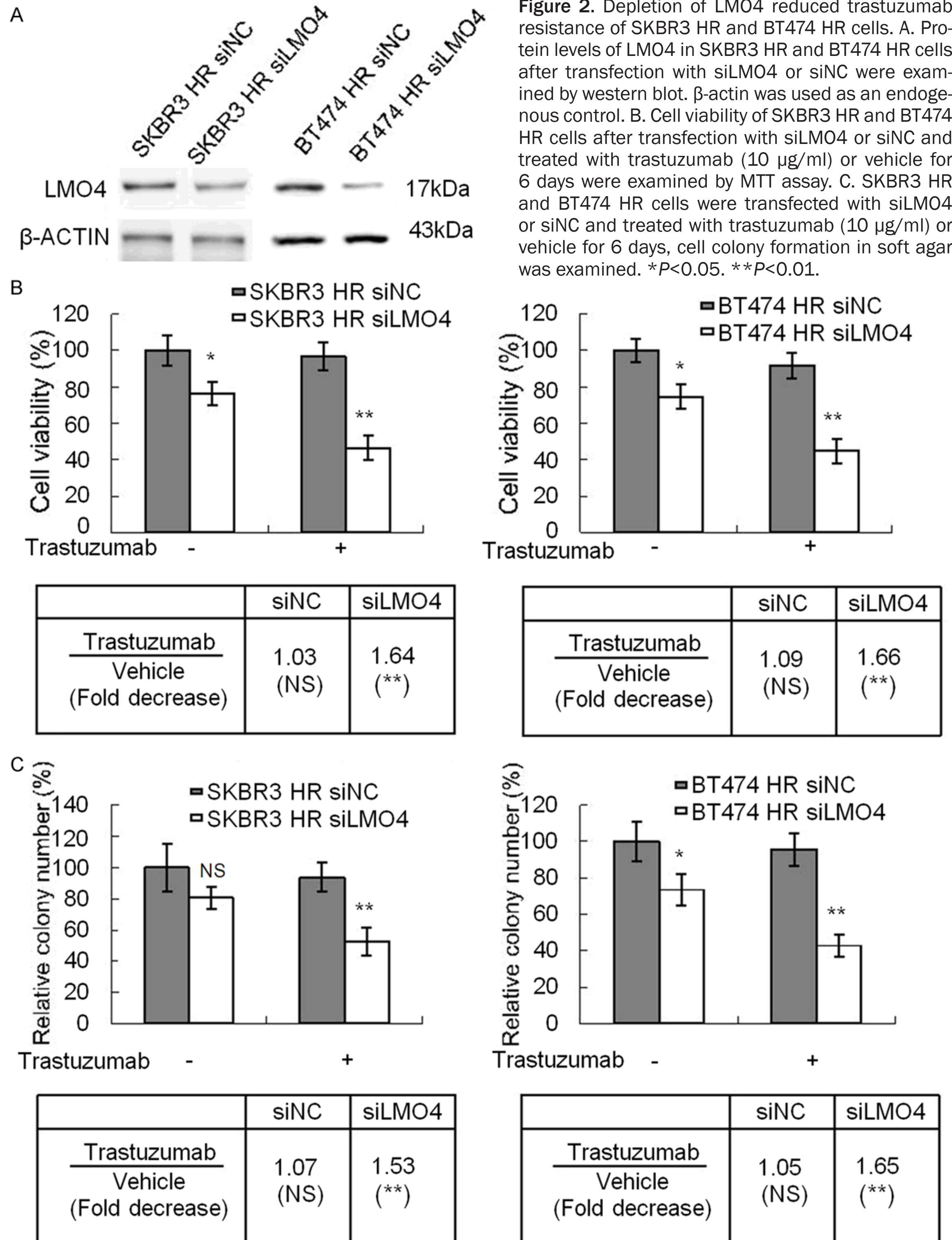
Results

LMO4 was over-expressed in human breast cancer cells with acquired trastuzumab resistance

Two HER2 positive human breast cancer cells SKBR3 and BT474 with acquired resistance to trastuzumab (10 μg/ml) were developed as described in "Materials and Methods", and were described as SKBR3 HR and BT474 HR respectively. Cell viability was detected using MTT assay to examine the resistance of these two cells to trastuzumab. As shown in **Figure 1A**, both SKBR3 HR and BT474 HR cells showed significant resistance to trastuzumab at 10 μg/ml and even at 20 μg/ml. We subsequently examined the expression levels of LMO4 in SKBR3 HR and BT474 HR and their parental cells. The mRNA levels of LMO4 were higher in both SKBR3 HR and BT474 HR cells compared with SKBR3 and BT474 cells respectively (**Figure 1B**). Concordantly, the protein levels of LMO4 were also dramatically higher in SKBR3 HR cells than SKBR3 cells and dramatically higher in BT474 HR cells than BT474 cells (**Figure 1C**). Therefore, acquired trastuzumab resistant breast cancer cells over-expressed LMO4 compared with trastuzumab sensitive cells.

Depletion of LMO4 sensitized acquired trastuzumab resistant breast cancer cells to trastuzumab

We next depleted the LMO4 levels in SKBR3 HR and BT474 HR cells with siLMO4. The protein levels of LMO4 decreased significantly in both SKBR3 HR and BT474 HR cells after transfection with siLMO4 compared with siNC (**Figure 2A**). As determined by MTT assay, there were no significant change of cell viability for SKBR3 HR siNC and BT474 HR siNC cells when treated with trastuzumab compared with vehicle control. However, the cell viability of both SKBR3 HR siLMO4 and BT474 HR siLMO4 cells decreased significantly on exposure to trastuzumab compared with vehicle control (**Figure 2B**). The fold decrease of trastuzumab treated group compared with vehicle treated group was 1.03



	siNC	siLMO4
Trastuzumab / Vehicle (Fold decrease)	1.03 (NS)	1.64 (**)

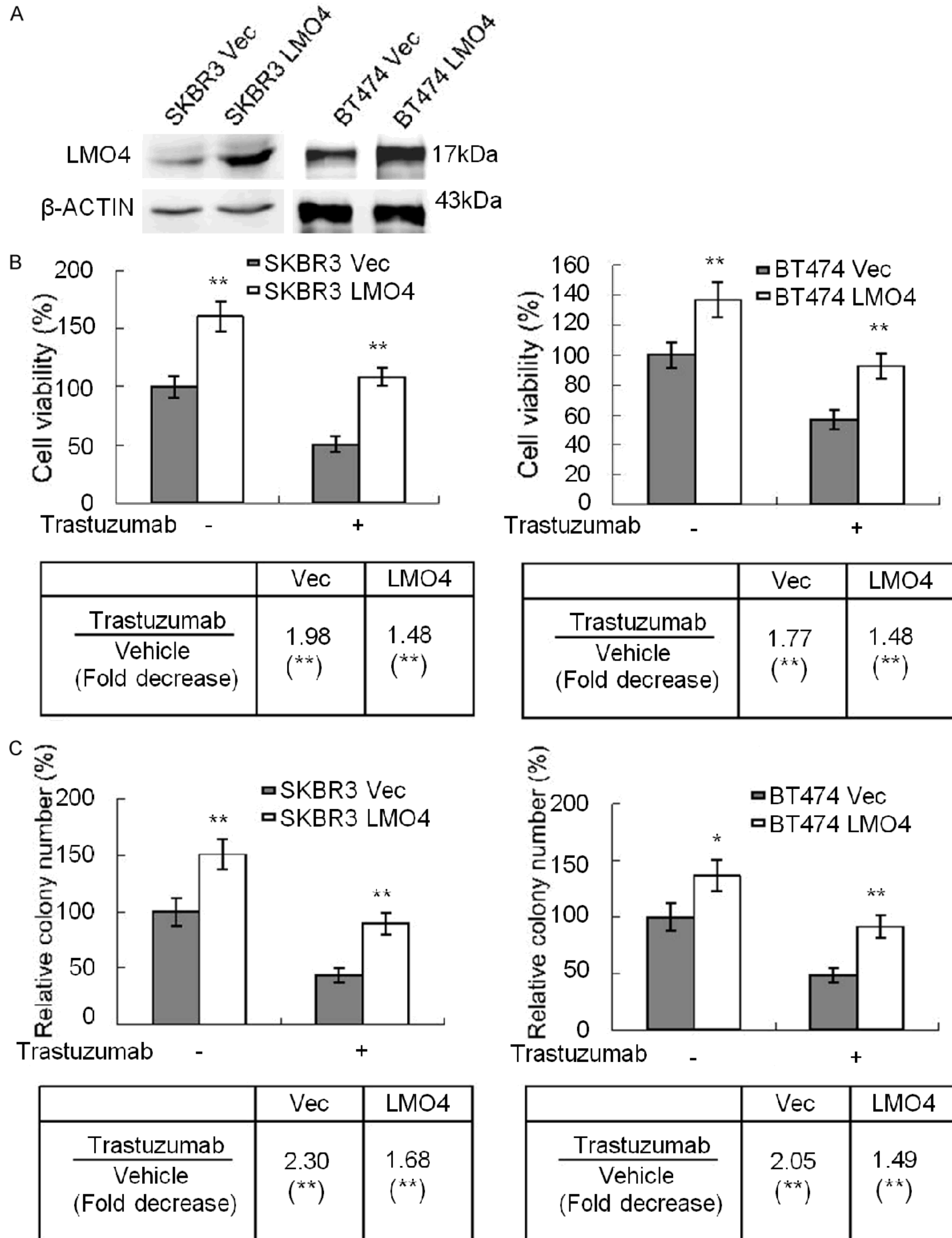
	siNC	siLMO4
Trastuzumab / Vehicle (Fold decrease)	1.09 (NS)	1.66 (**)

	siNC	siLMO4
Trastuzumab / Vehicle (Fold decrease)	1.07 (NS)	1.53 (**)

	siNC	siLMO4
Trastuzumab / Vehicle (Fold decrease)	1.05 (NS)	1.65 (**)

Figure 2. Depletion of LMO4 reduced trastuzumab resistance of SKBR3 HR and BT474 HR cells. A. Protein levels of LMO4 in SKBR3 HR and BT474 HR cells after transfection with siLMO4 or siNC were examined by western blot. β-actin was used as an endogenous control. B. Cell viability of SKBR3 HR and BT474 HR cells after transfection with siLMO4 or siNC and treated with trastuzumab (10 μg/ml) or vehicle for 6 days were examined by MTT assay. C. SKBR3 HR and BT474 HR cells were transfected with siLMO4 or siNC and treated with trastuzumab (10 μg/ml) or vehicle for 6 days, cell colony formation in soft agar was examined. *$P<0.05$. **$P<0.01$.

($P>0.05$) in SKBR3 HR siNC cells, 1.64 ($P<0.01$) in SKBR3 HR siLMO4 cells, 1.09 ($P>0.05$) in BT474 HR siNC cells and 1.66 ($P<0.01$) in BT474 HR siLMO4 cells (**Figure 2B**). Moreover, siLMO4 dramatically decreased cell colony formation in soft agar on exposure to trastuzumab in SKBR3 HR and BT474 HR cells (fold decrease of trastuzumab/vehicle: SKBR3 HR siNC 1.07



	Vec	LMO4
Trastuzumab / Vehicle (Fold decrease)	1.98 (**)	1.48 (**)

	Vec	LMO4
Trastuzumab / Vehicle (Fold decrease)	1.77 (**)	1.48 (**)

	Vec	LMO4
Trastuzumab / Vehicle (Fold decrease)	2.30 (**)	1.68 (**)

	Vec	LMO4
Trastuzumab / Vehicle (Fold decrease)	2.05 (**)	1.49 (**)

Figure 3. Forced expression of LMO4 reduced trastuzumab sensitivity of SKBR3 and BT474 cells *in vitro*. A. Protein levels of LMO4 in SKBR3 and BT474 cells after transfection with LMO4 plasmid or Vec control were examined by western blot. β-actin was used as an endogenous control. B. Cell viability of SKBR3 and BT474 cells after transfection with LMO4 plasmid or Vec control and treated with trastuzumab (1.25 μg/ml) or vehicle for 6 days were examined by MTT assay. C. SKBR3 and BT474 cells were transfected with LMO4 plasmid or Vec control and treated with trastuzumab (1.25 μg/ml) or vehicle for 6 days, cell colony formation in soft agar were examined. **P*<0.05. ***P*<0.01.

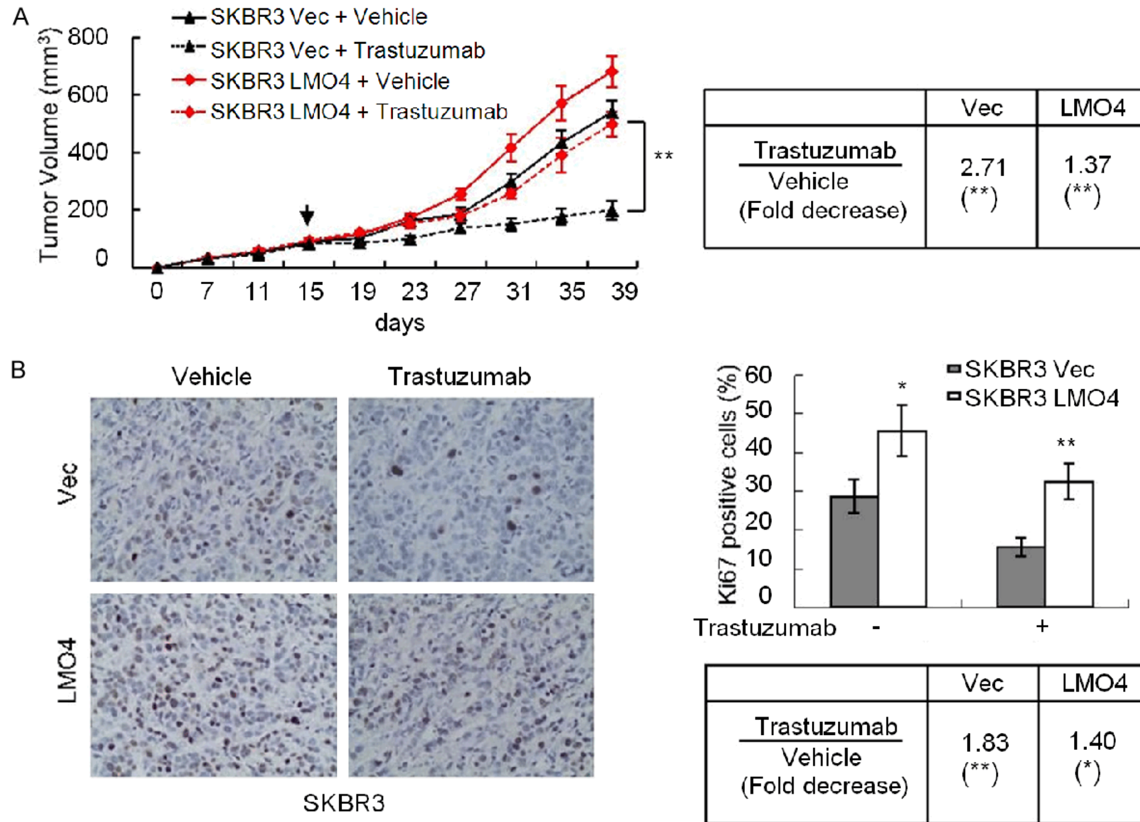


Figure 4. Forced expression of LMO4 reduced trastuzumab sensitivity of breast cancer cells *in vivo*. A. SKBR3 LMO4 and SKBR3 Vec cells were injected subcutaneously into the dorsal flank of 4-week-old female mice. At 15 days after cell inoculation, these tumors reached about 100 mm^3 and mice were randomized to receive trastuzumab (10 mg/kg) or vehicle as control once a week. Mice were sacrificed and tumors were harvested after 39 days. Growth curves of tumors were calculated. B. Ki-67 staining of tumor sections derived from mice injected with SKBR3 LMO4 or SKBR3 Vec cells and received trastuzumab or vehicle control. **P*<0.05. ***P*<0.01.

(*P*>0.05), SKBR3 HR siLMO4 1.53 (*P*<0.01); BT474 HR siNC 1.05 (*P*>0.05), BT474 HR siLMO4 1.65 (*P*<0.01). Therefore, depletion of LMO4 with siRNA dramatically re-sensitized the trastuzumab resistant cells SKBR3 HR and BT474 HR to trastuzumab.

Forced expression of LMO4 reduced the sensitivity of human breast cancer cells to trastuzumab in vitro

For further study, we transfected SKBR3 and BT474 cells with LMO4 over-expressing plasmid. Protein levels of LMO4 were significantly increased after transfection with LMO4 plasmid compared with Vec control both in SKBR3 and BT474 cells (**Figure 3A**). As shown in **Figure 3B** and **3C**, cell viability and cell soft agar colony formation of SKBR3 and BT474 cells increased after transfection with LMO4 plasmid compared with Vec control. Treatment with trastuzumab dramatically reduced cell viability and soft agar colony formation of SKBR3 Vec and BT474 Vec cells, whereas SKBR3 and BT474 cells with forced expression of LMO4 exerted less significant inhibitory effects on cell viability and soft agar colony formation on exposure to trastuzumab (fold decrease of trastuzumab/vehicle for cell viability: SKBR3 Vec 1.98 (*P*<0.01), SKBR3 LMO4 1.48 (*P*<0.01); BT474 Vec 1.77 (*P*<0.01), BT474 LMO4 1.48 (*P*<0.01). Fold decrease of trastuzumab/vehicle for soft agar colony formation: SKBR3 Vec 2.30 (*P*<0.01), SKBR3 LMO4 1.68 (*P*<0.01); BT474 Vec 2.05 (*P*<0.01), BT474 LMO4 1.49 (*P*<0.01)) (**Figure 3B**, **3C**). Hence, forced expression of LMO4 decreased the sensitivity of SKBR3 and BT474 cells to trastuzumab *in vitro*.

Forced expression of LMO4 reduced trastuzumab sensitivity of human breast cancer cells in vivo

To determine the role of LMO4 on trastuzumab sensitivity *in vivo*, SKBR3 LMO4 and SKBR3 Vec cells were injected subcutaneously into the

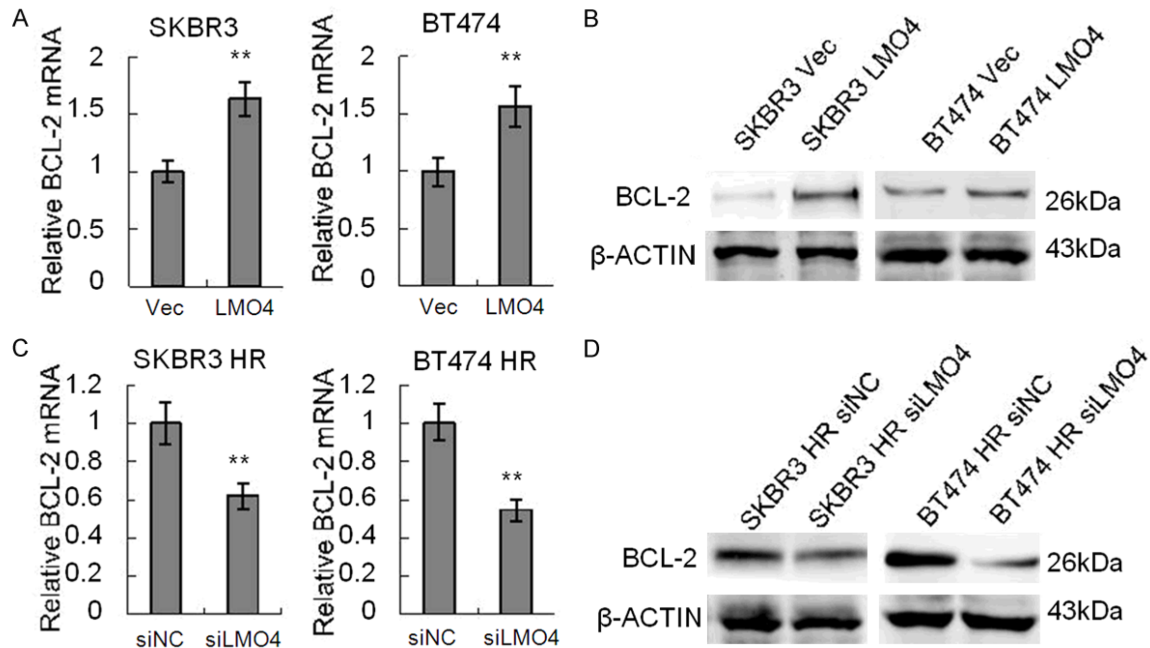


Figure 5. BCL-2 was regulated by LMO4 in parental and trastuzumab resistant breast cancer cells. A. mRNA levels of BCL-2 in SKBR3 and BT474 cells after transfection with LMO4 plasmid or Vec control were examined by RT-qPCR. GAPDH was used as an endogenous control. B. Protein levels of BCL-2 in SKBR3 and BT474 cells after transfection with LMO4 plasmid or Vec control were examined by western blot. β-actin was used as an endogenous control. C. mRNA levels of BCL-2 in SKBR3 HR and BT474 HR cells after transfection with siLMO4 or siNC were examined by RT-qPCR. D. Protein levels of BCL-2 in SKBR3 HR and BT474 HR cells after transfection with siLMO4 or siNC were examined by western blot. *$P<0.05$. **$P<0.01$.

dorsal flank of 4-week-old female BALB/c-nu/nu mice. Both cells formed palpable and measurable tumors (about 100 mm^3) after 15 days and the respective group of mice were randomized to receive either trastuzumab or vehicle as control. Tumor sizes were measured every 4 days and tumor growth curves were analyzed in the end at the 39th day. As shown in **Figure 4A**, tumors formed by SKBR3 LMO4 cells grew much faster than the tumors formed by SKBR3 Vec cells. Treatment with trastuzumab decreased tumor growth in mice with tumors formed by both SKBR3 LMO4 and SKBR3 Vec cells, but SKBR3 LMO4 tumors showed dramatically less decrease on exposure to trastuzumab with an effectually growth curve similar with SKBR3 Vec cells in the absence of trastuzumab. The fold decrease of tumor volumes for trastuzumab treated group compared with vehicle treated group was 2.71 ($P<0.01$) in SKBR3 Vec cells and was 1.37 ($P<0.01$) in SKBR3 LMO4 cells (**Figure 4A**). In addition, tumors were harvested for histological examination. Ki-67 levels were detected on the tumor sections by Immunohistochemistry to examine the proliferation of tumor cells. Accordant with tumor growth curves, Ki-67 positive cell population in tumors formed by SKBR3 LMO4 cells was higher than the tumors formed by SKBR3 Vec cells. Trastuzumab reduced Ki-67 positivity in tumors formed by both SKBR3 LMO4 and SKBR3 Vec cells, but tumors formed by SKBR3 LMO4 cells maintained cell proliferation and Ki-67 positivity on exposure to trastuzumab comparative with vehicle treated tumors derived from SKBR3 Vec cells (**Figure 4B**). As a result, forced expression of LMO4 reduced relative sensitivity of breast cancer cells to trastuzumab *in vivo*.

LMO4 enhanced trastuzumab resistance of human breast cancer cells through BCL-2

As reported previously, BCL-2 was an important gene that contributed to trastuzumab resistance of human breast cancer cells [13]. We examined the mRNA levels of BCL-2 by RT-qPCR in SKBR3 and BT474 cells with forced expression of LMO4. The mRNA levels of BCL-2 increased significantly in SKBR3 LMO4 and BT474 LMO4 cells compared with SKBR3 Vec

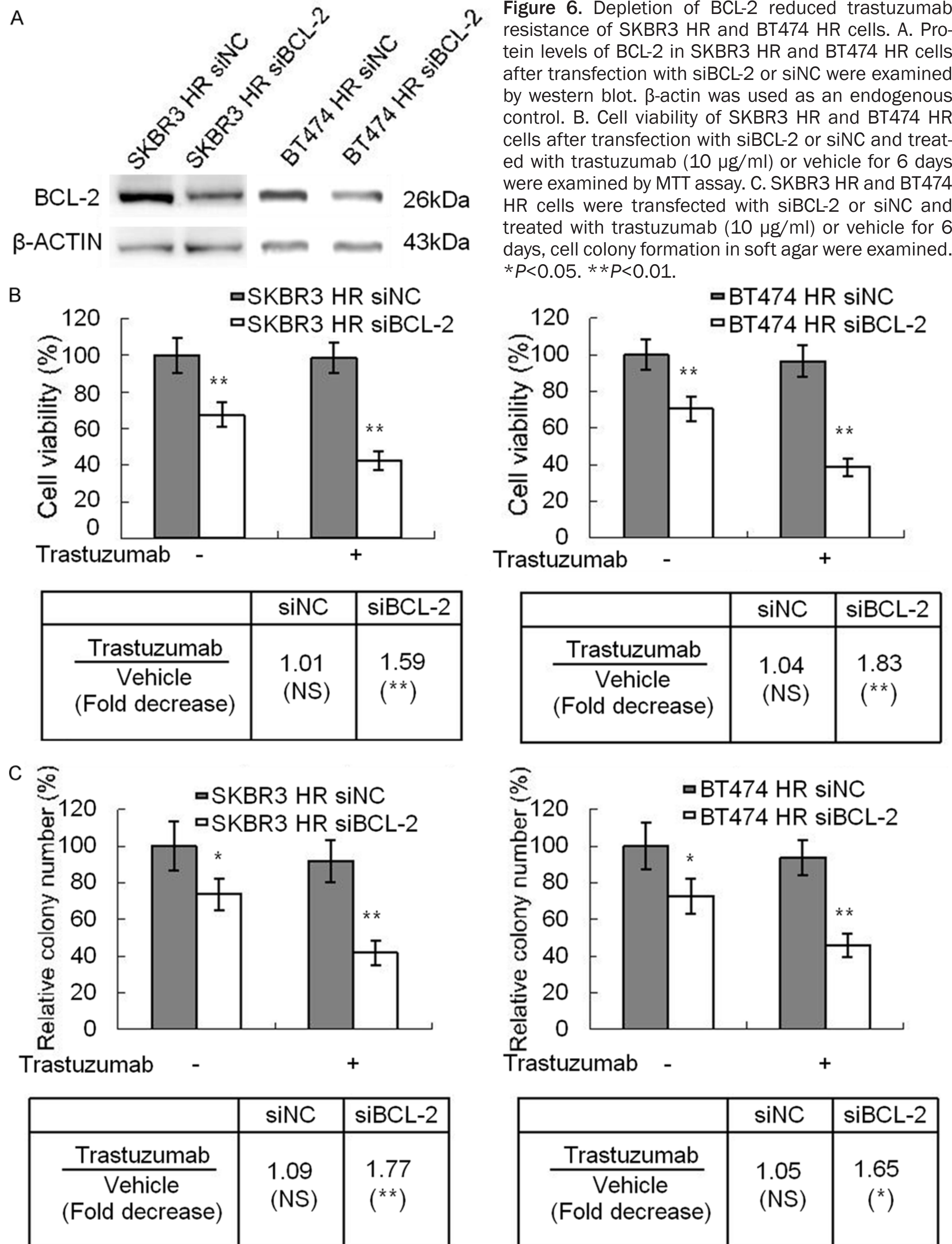
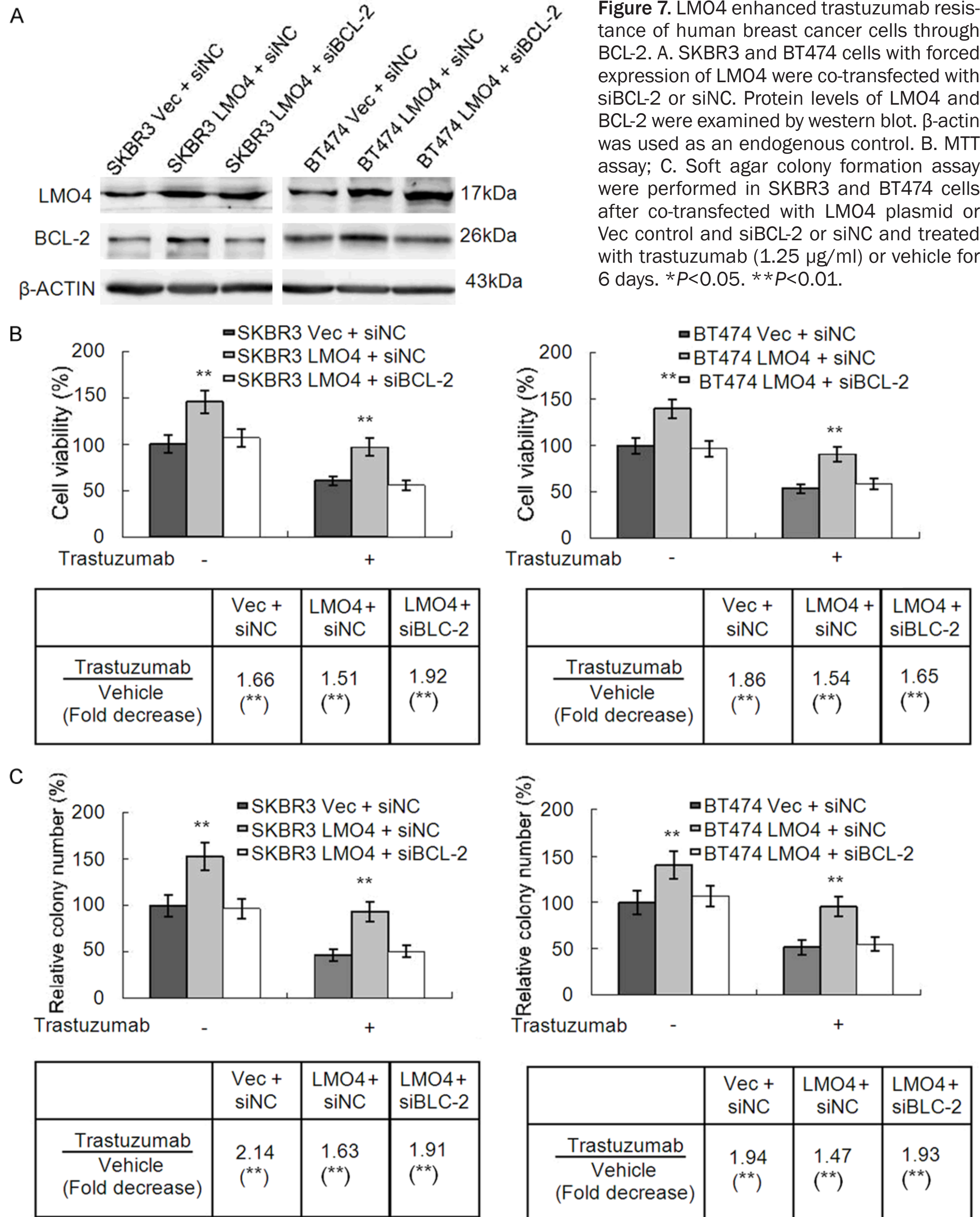


Figure 6. Depletion of BCL-2 reduced trastuzumab resistance of SKBR3 HR and BT474 HR cells. A. Protein levels of BCL-2 in SKBR3 HR and BT474 HR cells after transfection with siBCL-2 or siNC were examined by western blot. β-actin was used as an endogenous control. B. Cell viability of SKBR3 HR and BT474 HR cells after transfection with siBCL-2 or siNC and treated with trastuzumab (10 μg/ml) or vehicle for 6 days were examined by MTT assay. C. SKBR3 HR and BT474 HR cells were transfected with siBCL-2 or siNC and treated with trastuzumab (10 μg/ml) or vehicle for 6 days, cell colony formation in soft agar were examined. *$P<0.05$. **$P<0.01$.

and BT474 Vec cells respectively (**Figure 5A**). Concordantly, protein levels of BCL-2 also increased significantly in both SKBR3 and BT474 cells with forced expression of LMO4 compared with control respectively (**Figure 5B**).

In addition, both mRNA (**Figure 5C**) and protein levels (**Figure 5D**) of BCL-2 dramatically decreased after transfection with siLMO4 in SKBR3 HR and BT474 HR cells compared with siNC. Therefore, BCL-2 was positively regulated



Trastuzumab / Vehicle (Fold decrease)	Vec + siNC	LMO4+ siNC	LMO4+ siBLC-2
B, SKBR3	1.66 (**)	1.51 (**)	1.92 (**)
B, BT474	1.86 (**)	1.54 (**)	1.65 (**)
C, SKBR3	2.14 (**)	1.63 (**)	1.91 (**)
C, BT474	1.94 (**)	1.47 (**)	1.93 (**)

Figure 7. LMO4 enhanced trastuzumab resistance of human breast cancer cells through BCL-2. A. SKBR3 and BT474 cells with forced expression of LMO4 were co-transfected with siBCL-2 or siNC. Protein levels of LMO4 and BCL-2 were examined by western blot. β-actin was used as an endogenous control. B. MTT assay; C. Soft agar colony formation assay were performed in SKBR3 and BT474 cells after co-transfected with LMO4 plasmid or Vec control and siBCL-2 or siNC and treated with trastuzumab (1.25 μg/ml) or vehicle for 6 days. *$P<0.05$. **$P<0.01$.

by LMO4 in parental and trastuzumab resistant breast cancer cells.

To examine the role of BCL-2 in acquired trastuzumab resistant breast cancer cells, siBCL-2 was used to deplete the endogenous BCL-2 of SKBR3 HR and BT474 HR cells. Protein levels of BCL-2 decreased significantly in both SKBR3 HR and BT474 HR cells after transfection with siBCL-2 compared with control siNC (**Figure 6A**). MTT assay and soft agar colony formation assay were carried out in SKBR3 HR and BT474 HR cells after transfection with siBCL-2 or siNC and treated with trastuzumab or vehicle. As

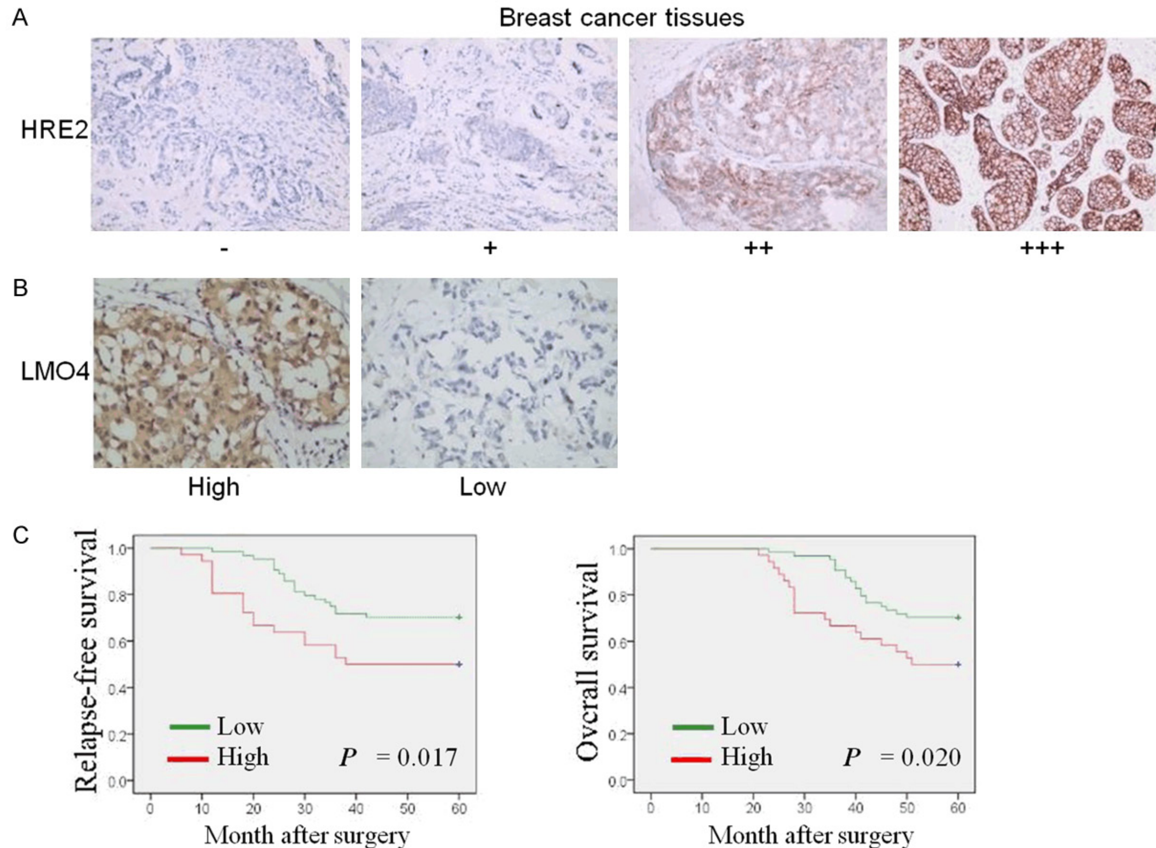


Figure 8. Expression of HER2 and LMO4 in human breast cancer tissues and patient survival analysis. A. Protein levels of HER2 in breast cancer tissues were examined using immunohistochemistry. B. Protein levels of LMO4 in HER2++/+++ breast cancer tissues were examined using immunohistochemistry. The magnifications of the images were 200. C. The RFS and OS rates of HER2 positive breast cancer patients with different LMO4 expression levels were analyzed by Kaplan-Meier curves.

shown in **Figure 6B** and **6C**, depletion of BCL-2 significantly increased the sensitivity of SK-BR3 HR and BT474 HR cells to trastuzumab. As a result, BCL-2 contributed to trastuzumab resistance of human breast cancer cells SKBR3 HR and BT474 HR, depletion of BCL-2 re-sensitized the trastuzumab resistant cells to trastuzumab.

To determine whether LMO4 enhanced trastuzumab resistance of human breast cancer cells were mediated by BCL-2, cell function experiments were performed in SKBR3 and BT474 cells with forced expression of LMO4 and BCL-2 depletion by siBCL-2 on exposure to trastuzumab. In SKBR3 and BT474 cells, protein levels of LMO4 increased significantly after transfection with LMO4 plasmid + siNC or LMO4 plasmid + siBCL-2 compared with controls. Whereas protein levels of BCL-2 increased significantly after transfection with LMO4 plasmid + siNC compared with control, this increase was abrogated by transfection with LMO4 plasmid + siBCL-2 (**Figure 7A**). Consistent with former results, forced expression of LMO4 significantly enhanced trastuzumab resistance of SKBR3 and BT474 cells as determined using MTT assay and soft agar colony formation assay. However, depletion of BCL-2 specifically abolished the enhanced trastuzumab resistance of SKBR3 and BT474 cells as a consequence of the forced expression of LMO4 (**Figure 7B**, **7C**). Therefore, we conclude that LMO4 enhanced trastuzumab resistance of human breast cancer cells partly through specific regulation of BCL-2 expression.

LMO4 was associated with poor clinicopathological parameters and survival of HER2 positive breast cancer patients

For further study, 100 paraffin-embedded HER2 (++/+++) breast cancer tissues were col-

Table 1. Association of LMO4 expression with clinicopathological parameters of HER2++/+++ breast cancer patients

Parameter	*n*	LMO4 expression (n (%))		*P*	χ^2
		Low	High		
Age (years)					
≤50	55	36 (65.5)	19 (34.5)	0.738	0.112
>50	45	28 (62.2)	17 (37.8)		
Tumor size (cm)					
≤2	38	29 (76.3)	9 (23.7)	0.045	4.035
>2	62	35 (56.5)	27 (43.5)		
Lymph node metastasis					
No	46	30 (65.2)	16 (34.8)	0.815	0.055
Yes	54	34 (63.0)	20 (37.0)		
Grade					
I-II	65	47 (72.3)	18 (27.7)	0.018	5.563
III	35	17 (48.6)	18 (51.4)		
Stage					
I-II	79	52 (65.8)	27 (34.2)	0.461	0.542
III-IV	21	12 (57.1)	9 (42.9)		

lected. Protein levels of HER2 in these breast cancer tissues were examined using IHC to confirm they were all over-expression of HER2 (++/+++) (**Figure 8A**). We next examined the protein levels of LMO4 in these HER2 positive breast cancer tissues and analyzed the association of LMO4 levels with clinicopathological parameters including patients' age, tumor size, lymph node metastasis, histological grade and clinical stage. As shown in **Figure 8B** and **Table 1**, the expression of LMO4 in HER2 positive breast cancer tissues was positively correlated with patient tumor size (P=0.045) and histological grade (P=0.018). However, there was no significant correlation between LMO4 expression and patients' age, lymph node metastasis or clinical stage (P>0.05). Furthermore, we followed up these breast cancer patients for more than 5 years, and the association of LMO4 levels with their survival rate was analyzed using Kaplan-Meier analysis. Dramatically, patients with high LMO4 levels showed both lower RFS rate (P=0.017) and OS rate (P=0.020) compared with patients with low LMO4 levels (**Figure 8C**). Hence, LMO4 was associated with poor prognosis in HER2 positive breast cancer patients.

Discussion

In this study, we systematically examined the role of LMO4 in trastuzumab resistance of human HER2 positive breast cancer cells. LMO4 was over-expressed in aquired trastuzumab resistant breast cancer cells SKBR3 HR and BT474 HR. Depletion of LMO4 dramatically decreased the trastuzumab resistance of SKBR3 HR and BT474 HR cells. In parental HER2 positive breast cancer cells SKBR3 and BT474, forced expression of LMO4 enhanced their resistance to trastuzumab *in vitro*. Moreover, forced expression of LMO4 but also increased the trastuzumab resistance of SKBR3 cells in xenograft models *in vivo*. BCL-2 was identified to be a downstream gene regulated by LMO4. Combinatorial function experiments revealed that the promoting role of LMO4 in trastuzumab resistance of human HER2 positive breast cancer cells was mediated by BCL-2. In addition, high levels of LMO4 in HER2 positive breast cancer tissues were associated with patient tumor size and histological grade. High levels of LMO4 were also associated with both low RFS and OS rates of HER2 positive breast cancer patients. This study expanded our understanding of LMO4 in HER2 positive breast cancer and the trastuzumab resistance.

LMO4 has been reported to be oncogenic in various human cancers. Grutz G et al. demonstrated the abnormality of LMO4 would influence T cell differentiation and contribute to the occurrence of T cell acute leukaemia [27]. Mizunuma H et al. determined LMO4 and LDB1 interacted with each other and over-expressed in both patient tissues and tumor cell lines of squamous cell carcinomas of the oral cavity [38]. LMO4 was also over-expressed in late stage pancreas cancer as reported previously [28]. High levels of LMO4 were associated with disease progression of squamous cell carcinoma of the anterior tongue [39]. In human head and neck cancer, LMO4 in association with LDB1 (LIM domain binding protein 1) promoted tumor growth and metastasis [30]. In human non-small-cell lung cancer, LMO4 was over-expressed in both tumor cell lines and tumor tissues and correlated to low survival rate of patients; LMO4 regulated migration and invasion of non-small-cell lung cancer cells through

AKT/PI3K pathway [29]. Therefore, LMO4 was an acceptable oncogene in many kinds of human cancers. Especially, LMO4 was well studied in human breast cancer and determined to be an important tumor promoter. Visvader JE et al. firstly reported LMO4 was over-expressed in human breast cancer cell lines and tissue specimens and demonstrated deregulation of LMO4 might contribute to breast tumorigenesis [31]. Over-expression of LMO4 in human breast cancer and the oncogenic role of LMO4 in human breast cancer cells were further confirmed in Wittlin S et al.'s study and Sum EY et al.'s study [32, 40]. Montañez-Wiscovich ME et al. documented that aberrant expression of LMO4 induced centrosome amplification and mitotic spindle abnormalities in breast cancer cells and the impact of dysregulated LMO4 on the centrosome cycle might promote LMO4-induced tumor formation [41]. Recently, HBXIP, DEAF1 and p53 were demonstrated to be involved in LMO4 pathway and contributed to human breast cancer proliferation and progression [42-44]. In the current study, we have also determined LMO4 promoted cell proliferation and tumor growth both *in vitro* and *in vivo*. Moreover, we have examined LMO4 but also promoted trastuzumab resistance in human HER2 positive breast cancer. As reported previously, LMO4 was a downstream target of HER2 and PI3K in HER2-dependent breast cancer cells; LMO4 (as a cell cycle regulator) mediated HER2-induced proliferation in human breast cancer [33]. It was concordant that aberrant expression of LMO4 contributed to the resistance of human HER2 positive breast cancer cells toward the anti-HER2 antibody drug trastuzumab.

BCL-2 was identified to be regulated by LMO4 and mediated the promoting role of LMO4 in trastuzumab resistance of human HER2 positive breast cancer cells. As reported previously, BCL-2 was a famous oncogene through suppressing apoptosis in various human cancers including breast cancer [45-48]. BCL-2 mediated multi-drug resistance in gastric cancer [49], breast cancer [50], prostate cancer [51] etc. Furthermore, Banerjee A et al. demonstrated activation of TWIST1-BCL-2 pathway stimulated radio- and chemo-resistance of human breast cancers [52]. As reported in our previous study, BCL-2 was also a downstream gene regulated by ARTN and regulated cancer stem cell like behaviors resulted in enhanced trastuzumab resistance in HER2 positive breast cancer cells [13]. These results all are in line with our present study. In addition, Salmans ML et al. determined Clim2, in a complex with LMO4, supported mammary stem cells by directly targeting the Fgfr2 promoter in basal cells and contributed to breast tumorigenesis [53]. Since the breast cancer stem cell regulator BCL-2 was exactly up-regulated by LMO4, LMO4 therefore might increase trastuzumab resistance of human HER2 positive breast cancer cells partly through activating breast cancer stem cells.

Moreover, clinical study was performed in our study. Over-expression of LMO4 was positively correlated with pathological features including larger tumor size and higher histological grade, and lower patient survival rates in HER2 positive breast cancer patients. In previous studies, it was also reported LMO4 levels were high in high-grade/less differentiated breast cancers, which were characteristically highly proliferative [33]. These results were concordant with ours. Hence, LMO4 could be used as a prognostic indicator for HER2 positive breast cancer patients.

In summary, we herein defined the functional roles of LMO4 in trastuzumab resistance of human HER2 positive breast cancer cells and unveiled the clinicopathological significance of LMO4 in HER2 positive breast cancer patients. LMO4 might be a potential diagnostic and adjuvant therapeutic target in human HER2 positive breast cancer.

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Disclosure of conflict of interest

None.

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