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ABOUT COVER

AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

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ORIGINAL ARTICLE

Basic Study

BIRC3 induces the phosphoinositide 3-kinase-Akt pathway activation to promote trastuzumab resistance in human epidermal growth factor receptor 2-positive gastric cancer

Shu-Liang Li, Pei-Yao Wang, Yang-Pu Jia, Zhao-Xiong Zhang, Hao-Yu He, Peng-Yu Chen, Xin Liu, Bang Liu, Li Lu, Wei-Hua Fu

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Abstract

BACKGROUND

Trastuzumab-targeted therapy is currently the standard of care for advanced human epidermal growth factor receptor 2 (HER2)-positive gastric cancer. However, the emergence of resistance to trastuzumab poses significant challenges.

AIM

To identify the key genes associated with trastuzumab resistance. These results provide a basis for the development of interventions to address drug resistance and improve patient outcomes.

METHODS

High-throughput sequencing and bioinformatics were used to identify the differentially expressed pivotal gene *BIRC3* and delineate its potential function and pathway regulation. Tumor samples were collected from patients with HER2-positive gastric cancer to evaluate the correlation between *BIRC3* expression and trastuzumab resistance. We established gastric cancer cell lines with both highly expressed and suppressed levels of *BIRC3*, followed by comprehensive *in vitro* and *in vivo* experiments to confirm the involvement of *BIRC3* in trastuzumab

resistance and to elucidate its underlying mechanisms.

RESULTS

In patients with HER2-positive gastric cancer, there is a significant correlation between elevated *BIRC3* expression in tumor tissues and higher T stage, tumor node metastasis stage, as well as poor overall survival and progression-free survival. *BIRC3* is highly expressed in trastuzumab-resistant gastric cancer cell lines, where it inhibits tumor cell apoptosis and enhances trastuzumab resistance by promoting the phosphorylation and activation of the phosphoinositide 3-kinase-Akt (PI3K-AKT) pathway in HER2-positive gastric cancer cells, both *in vivo* and *in vitro*.

CONCLUSION

This study revealed a robust association between high *BIRC3* expression and an unfavorable prognosis in patients with HER2-positive gastric cancer. Thus, the high expression of *BIRC3* stimulated PI3K-AKT phosphorylation and activation, stimulating the proliferation of HER2-positive tumor cells and suppressing apoptosis, ultimately leading to trastuzumab resistance.

Key Words: Gastric cancer; Human epidermal growth factor receptor 2; Trastuzumab; Drug-resistance; *BIRC3*

Core Tip: Our study discovered that the overexpression of *BIRC3* leads to the stimulation of phosphoinositide 3-kinase-Akt phosphorylation and activation. Consequently, this enhances the proliferation of human epidermal growth factor receptor 2-positive tumor cells and inhibits apoptosis, resulting in resistance to trastuzumab.

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INTRODUCTION

Gastric cancer is a highly prevalent and lethal form of cancer worldwide[1,2]. As our understanding of the molecular landscape of gastric cancer continues to improve, new therapeutic targets and drugs have been discovered and developed, leading to improved treatment outcomes. In the context of cancer, human epidermal growth factor receptor 2 (HER2) expression is considered to be significantly associated with cancer development and the escalation of resistance to chemotherapy drugs[3]. Trastuzumab is considered the standard and optimal treatment for patients with HER2-positive metastatic gastric cancer[4].

Trastuzumab inhibits the growth of HER2-positive tumor cells through immune-related mechanisms, such as antibody-dependent or complement-dependent cytotoxicity[5,6]. However, with the continued use of HER2-targeted therapies such as trastuzumab, most patients experience reduced therapeutic sensitivity resulting in tumors more tolerant to HER2-targeted therapies, leading to disease relapse and progression, which significantly affects patient prognosis. Several potential mechanisms of trastuzumab resistance have been proposed. These factors include HER2 heterogeneity, formation of HER2 heterodimers, and alterations in intracellular signaling, among others. Notably, the abnormal activation of the phosphoinositide 3-kinase (PI3K) pathway is recognized as a significant mechanism underlying resistance to HER2-targeted therapy[7].

Multiple studies have identified a new PI3K signaling pathway-related protein: *BIRC3*, also known as baculoviral inhibitor of apoptosis (IAP) repeat containing 3. *BIRC3* encodes cIAP2 (cellular IAP 2) and belongs to the IAP family[8,9]. These apoptosis-inhibitory proteins are highly conserved and inhibit apoptosis by suppressing caspase activity and regulating immune-related signaling pathways. Studies have shown that IAPs are frequently overexpressed in cancer, and that their expression levels are associated with tumorigenesis, chemotherapy resistance, disease progression, and survival differences[10].

Previous studies have demonstrated that high *BIRC3* expression is linked to clinicopathological characteristics and poor prognosis in colorectal cancer, bladder cancer, and glioblastoma[11,12]. Furthermore, *BIRC3* overexpression is associated with chemotherapy resistance in breast cancer and oral squamous cell carcinoma. However, its role in gastric cancer, particularly in relation to resistance to trastuzumab-targeted therapy in HER2-positive gastric cancer, remains unexplored. Hence, our study aimed to investigate the involvement of *BIRC3* in the development of trastuzumab-targeted therapy resistance and to unravel the underlying mechanism with the goal of overcoming trastuzumab resistance in HER2-positive gastric cancer.

MATERIALS AND METHODS

Patients and tissue samples

We examined 28 HER2-positive tumor tissue specimens from patients with gastric cancer who underwent curative gastrectomy in the Department of General Surgery at Tianjin Medical University General Hospital (Tianjin, China) between March 2018 and May 2021. The expression intensity of HER2 was assessed using immunohistochemistry (IHC), employing a four-grade scoring system (ranging from 0 to 3 +) to determine the proportion of stained tumor cells. HER2 overexpression was characterized by an IHC score of 3 +. In instances where HER2 staining was ambiguous (IHC 2 +), Fluorescence in situ hybridization was employed to validate the amplification status of the HER2. None of the enrolled patients underwent neoadjuvant therapy before gastrectomy. Follow-up evaluations were conducted every three months during the initial two years post-radical surgery and every six months thereafter.

High-throughput sequencing and subsequent bioinformatics analysis

Two-terminal sequencing was performed using an Illumina NovaSeq 6000 (LC Bio Technology Co., Ltd. Hangzhou, Zhejiang Province, China) according to standard protocols in the PE150 sequencing mode. Subsequent bioinformatics analyses were performed on the sequencing data, and R language was used to conduct correlation analyses of gene expression within the samples.

Plasmids and RNA oligonucleotide transfection

To identify the "*BIRC3*" gene in the gene bank of the national center for biotechnology information website, we selected the transcript-id "NM_001165.5". This enabled us to obtain the coding sequence. The BamHI and NotI restriction sites were selected for plasmid generation. The expression plasmid pLVX-IRES-puro-*BIRC3* was synthesized by Hongxun Biotechnology Co., Ltd (Suzhou, Jiangsu Province, China). For the siRNA, featuring the sequence hs-*BIRC3*-si: Forward 5'-CAGUUCGUACAUUUCUUUCAUdTdT-3,' design and synthesis was carried out by Hongxun Biotechnology Co., Ltd (Suzhou, Jiangsu Province, China). Verification of the sequencing results ensured alignment with the original sequence sequenced by Hongxun Biotechnology Co., Ltd (Suzhou, Jiangsu Province, China).

Cell culture

In this study, the NCI-N87 cell line, originating from human HER2-positive gastric carcinoma, was used. The cell line was procured from the Institute of General Surgery (Tianjin, China). To induce resistance to trastuzumab, the concentration of trastuzumab was systematically increased from 10 μg/mL to 1000 μg/mL, and viability was examined using the cell counting kit-8 (CCK8) assay. The resultant trastuzumab-resistant cell line was denoted as NCI-N87R and was sustained for approximately half a year. The cells were grown at 37 °C under a 5% carbon dioxide atmosphere in a humid setting. Transfection trials were performed in a the trastuzumab-resistant NCI-N87R cell line using *BIRC3* siRNA and a non-coding siRNA (siRNA-NC). Additionally, the trastuzumab-sensitive NCI-N87 cell line was transfected with a *BIRC3* overexpression plasmid pLVX-IRES-puro-*BIRC3* or the empty plasmid pLVX-IRES-puro-NC. The efficacy of transfection was confirmed through quantitative reverse transcription polymerase chain reaction (RT-qPCR) and western blot analyses, and alterations in the cellular proliferation capability were examined using CCK8 and 5-ethynyl-2'-deoxyuridine (EdU) assays.

CCK8 assay

To evaluate cell viability, a manual lauryl sodium sulfate cell counting kit-8 (CCK8) assay was performed. We plated 2500 cells per well in 96-well plates. Various concentrations of trastuzumab were introduced into the culture medium, and after four days, a CCK8 assay was carried out to quantify cell viability at optical density 450 nm. A total of nine trastuzumab concentration gradients in five replicate wells were established in the medium, ranging from 0 μg/mL to 1000 μg/mL: 0 μg/mL, 1 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 500 μg/mL, and 1000 μg/mL.

RT-qPCR

BIRC3 levels were analyzed using RT-qPCR. The ChamQ universal synergetic binding reagent qPCR master mix was used for RT-qPCR assessments, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) used as the reference gene. The primers utilized for *BIRC3* in the RT-qPCR analysis were as follows: Forward 5'-TTTCCGTGGCTCTTATTCAAACT-3' and reverse 5'-GCACAGTGGTAGGAACTTCTCAT-3c'; for *GAPDH*, forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-TGGTGAAGACGCCAGTGGA-3'. The RT-qPCR process was repeated three times, and the data were analyzed using GraphPad Prism 9.0 to determine the ^{2}Ct values. Statistical significance was set at $P \leq 0.05$.

IHC staining and analysis

IHC analyses were performed to assess *BIRC3* protein expression in paraffin-embedded sections of tissues obtained from patients with gastric cancer. Additionally, the expression levels of *BIRC3*, phosphorylated AKT (pAKT), and caspase 3 in paraffin sections of tumors formed from different groups of mice were analyzed. The IHC scoring formula was as follows: IHC score = staining intensity score (a) × percentage score of positive cells (b), where (a) represents staining intensity, with a negative score of 0, a weak positive score of 1, a positive score of 2, and a strong positive score of 3, and (b) represents the proportion of positive cells, with a score of 0 for 0% positive cells, a score of 1 for 1%-25% positive cells, a score of 2 for 26%-50% positive cells, a score of 3 for 51%-75% positive cells, and a score of 4 for 76%-100% positive cells.

Low expression was indicated by an IHC score of less than 4 points, whereas high expression was indicated by a score of 4 points or more.

In vivo tumor model

In the dynamic tumor model, the transgenic strain N87-LV-*BIRC3* with stable overexpression was developed by lentiviral transfection of the *BIRC3* gene. Subsequently, the transgenic strain N87R-LV-*BIRC3*-si with a stable knockdown of *BIRC3* was created by lentiviral transfection with *BIRC3* siRNA.

For the *in vivo* tumorigenesis experiment, specific pathogen free (SPF) BALB/c nude mice were divided into five groups. All appropriate measures were taken to minimize pain or discomfort, and comply with ARRIVE guidelines. The group that received NCI-N87 cell inoculation was designated with the N87-NC group. The group that received NCI-N87 cells transfected with the overexpressed LV-GFP-puro-*BIRC3* virus was labeled the N87-LV-*BIRC3* group. The group that received NCI-N87 cells transfected with the overexpressed LV-GFP-puro-*BIRC3* virus and treated with an allosteric AKT inhibitor MK-2206 was denoted as the N87-*BIRC3*-AKTi group. The NCI-N87R group, inoculated with drug-resistant cells, was designated as the N87R group. The group that received NCI-N87R cells transfected with the knockout LV-GFP-puro-*BIRC3*-si virus was named N87R-LV-*BIRC3*-si.

All experimental groups received a trastuzumab injection (200 μg/kg) into the abdominal cavity every two days. Furthermore, the N87-LV-*BIRC3*-AKTi group received 10 mg/kg AKT inhibitor (MK-2206) at the same intervals for seven administrations.

Statistical analysis

Each experiment was replicated at least three times, and the data presented reflect the average outcomes. Statistical analysis was performed using χ^2 test for IHC scores and patient pathological features. the statistical review of the study was performed by a biomedical statistician. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Construction of a trastuzumab-resistant tumor model

We created a trastuzumab-resistant variant HER2-positive gastric cancer cell line NCI-N87, designated NCI-N87R. The morphology of NCI-N87R cells closely mirrored that of its parental cell line, NCI-N87 (Figure 1A). As shown in Figure 1B, NCI-N87R cells exhibited remarkable resistance to trastuzumab when subjected to a concentration of 1000 μg/mL, as determined by a CCK-8 assay. The proliferative activity of NCI-N87R was (105.4 ± 8.240), whereas that of NCI-N87 cells was (64.34 ± 6.371, $P < 0.0001$). Consequently, we successfully generated a trastuzumab-resistant cell line, NCI-N87R, by using a low-concentration stepwise addition method.

High-throughput sequencing and bioinformatics analyses for hub gene screening

Following the successful generation of a trastuzumab-resistant cell line, we conducted high-throughput sequencing and bioinformatics analyses. By comparing the NCI-N87R cells with normal NCI-N87 cells and applying a filter with $|\log_2$ (fold change)$| \geq 2$ and $P \leq 0.05$, we identified a total of 506 up-regulated genes and 501 down-regulated genes. A volcano map depicting these differentially expressed genes is shown in Figure 2A. Subsequently, we performed gene ontology and protein interaction network analyses of these differentially expressed genes, as shown in Figure 2B and C, respectively. We then utilized the maximum neighborhood component centrality (DMNC) and MNC algorithms in Cytoscape 3.9.1 software to identify the top ten differentially expressed genes. The molecular complex detection algorithm was employed to screen genes, resulting in the identification of the two modules with the highest scores (score = 3.33), as illustrated in Figure 2D, Table 1 and Table 2. After consolidating the genes, we visualized the top ten genes selected using different algorithms from Cyto Hubba using a Venn plot (Figure 2E). We identified *BIRC3* as a pivotal gene that may contribute to trastuzumab resistance in patients with HER2-positive gastric cancer.

Expression of BIRC3 in human gastric cancer cell lines and trastuzumab-resistant cell lines

RT-qPCR analysis revealed a significant upregulation in the mRNA levels of *BIRC3* in NCI-N87R cells compared to that in control parental NCI-N87 cells ($P < 0.0001$) (Figure 3A). Western blotting confirmed that the protein levels of *BIRC3* were significantly higher in NCI-N87R cells than in NCI-N87 cells ($P < 0.001$) (Figure 3B). These findings suggest that trastuzumab-resistant gastric cancer cells exhibit increased expression of *BIRC3* compared to trastuzumab-sensitive cells.

BIRC3 facilitates trastuzumab resistance in HER2-positive gastric cancer by promoting tumor cell proliferation

To investigate the role of *BIRC3* in the response of human HER2-positive gastric cancer cells to trastuzumab, we used *BIRC3*-siRNA to downregulate the levels of *BIRC3* in NCI-N87R cells. Cell viability was evaluated using CCK8 assays, revealing significant reductions in cell survival (66.38 ± 6.93 *vs* 98.78 ± 14.54, $P = 0.0047$) ($P < 0.0001$). Notably, the degradation of *BIRC3* markedly decreased cell growth in NCI-N87R cells when exposed to 1000 μg/mL of trastuzumab compared to the control group (Figure 4A). Consequently, the decreased expression of *BIRC3* results in diminished trastuzumab resistance in trastuzumab-resistant gastric cancer cells.

Subsequently, we introduced the *BIRC3* overexpressing plasmid pLVX-IRES-puro-*BIRC3* or control empty plasmid pLVX-IRES-puro-NC into the HER2-positive gastric cancer cell line NCI-N87, designated N87-*BIRC3*-OE and N87-*BIRC3*-NC, respectively. The mRNA and protein levels of *BIRC3* were significantly higher in N87-*BIRC3*-OE cells than in N87-

Table 1 Screening TOP 10 gene by different algorithms of cytohubba

DMNC	MNC
HLA-DRB5	*HLA-DRB1*
PTPN22	*HLA-DRA*
CD74	*MUC6*
CD86	*MUC5B*
HLA-A	*HLA-DRB5*
HLA-DRB1	*PER1*
HLA-DRA	*CSNK1E*
PER1	*BIRC3*
CSNK1E	*NOD2*
BIRC3	*PTPN22*

DMNC: Density of maximum neighborhood component; MNC: Maximum neighborhood component.

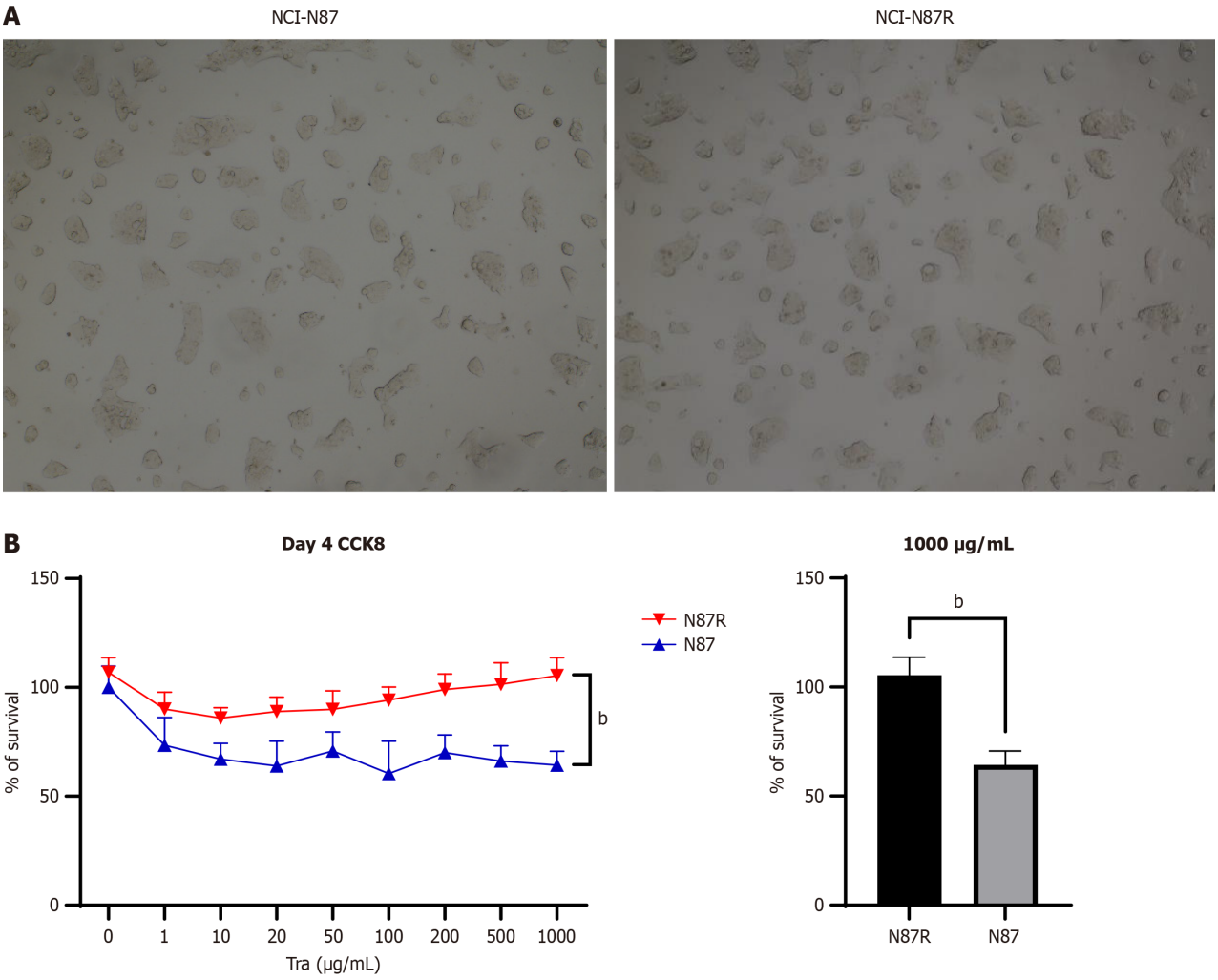
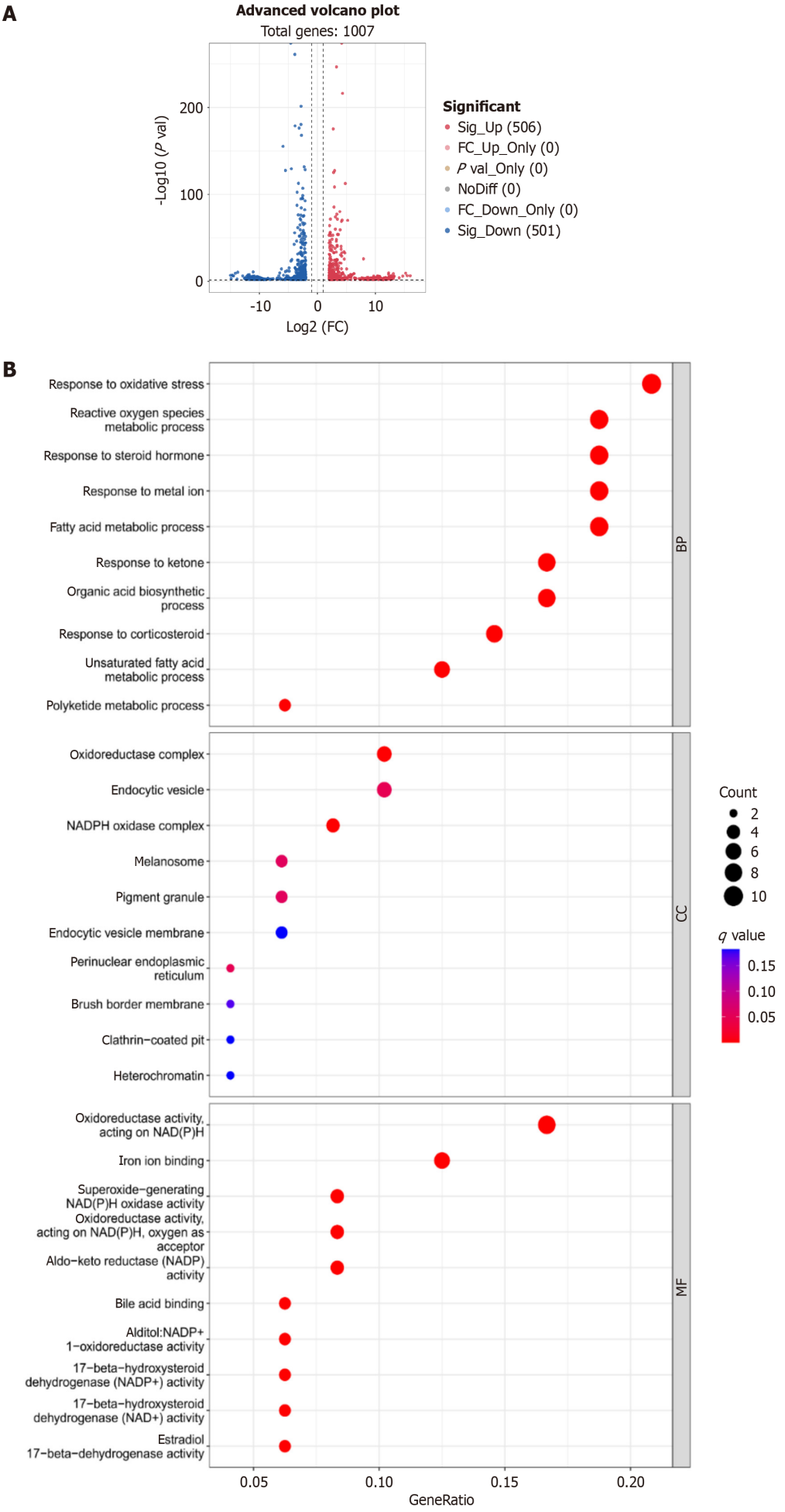


Figure 1 Construction of trastuzul tumor resistance model. A: The morphology of the resistant strain NCI-N87R is similar to that of the parent cell line NCI-N87; B: Cells were treated with 1000 μg/mL trastuzumab for 4 days, and cell viabilities were detected by cell counting kit-8 assay. $^{b}P < 0.0001$. CCK8: Cell counting kit-8; Tra: Trastuzumab.


A
Advanced volcano plot
Total genes: 1007
-Log10 (P val)
200
100
0
-10
0
10
Log2 (FC)
Significant
Sig_Up (506)
FC_Up_Only (0)
P val_Only (0)
NoDiff (0)
FC_Down_Only (0)
Sig_Down (501)
B
Response to oxidative stress
Reactive oxygen species metabolic process
Response to steroid hormone
Response to metal ion
Fatty acid metabolic process
Response to ketone
Organic acid biosynthetic process
Response to corticosteroid
Unsaturated fatty acid metabolic process
Polyketide metabolic process
BP
Oxidoreductase complex
Endocytic vesicle
NADPH oxidase complex
Melanosome
Pigment granule
Endocytic vesicle membrane
Perinuclear endoplasmic reticulum
Brush border membrane
Clathrin-coated pit
Heterochromatin
CC
Oxidoreductase activity, acting on NAD(P)H
Iron ion binding
Superoxide-generating NAD(P)H oxidase activity
Oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor
Aldo-keto reductase (NADP) activity
Bile acid binding
Alditol:NADP+ 1-oxidoreductase activity
17-beta-hydroxysteroid dehydrogenase (NADP+) activity
17-beta-hydroxysteroid dehydrogenase (NAD+) activity
Estradiol 17-beta-dehydrogenase activity
MF
0.05
0.10
0.15
0.20
GeneRatio
Count
2
4
6
8
10
q value
0.15
0.10
0.05

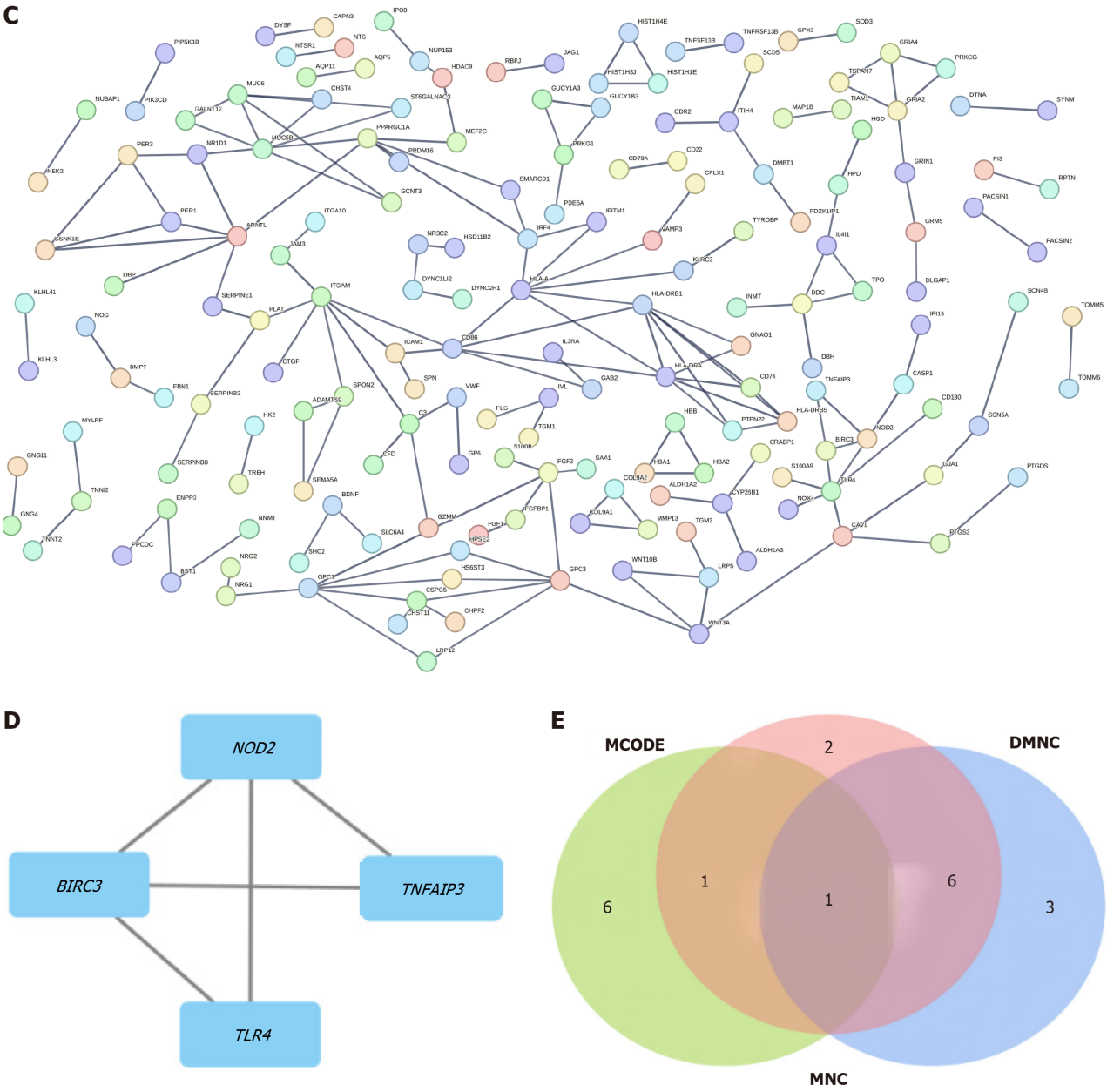


Figure 2 High throughput sequencing and bioinformatics analysis screening Hub genes. A: The volcano plot was generated from mRNA sequencing analysis of NCI-N87 and NCI-N87R; B: Gene pathway analysis was performed; C: Protein interaction network analysis for *BIRC3* was performed using the STRING online tools; D: the top differentially expressed genes were identified by the maximum neighborhood component centrality and neighborhood component centrality algorithms; E: Venn diagram was presented to show the top 10 genes selected using different algorithms, based on the CytoHubba online tools. FC: Fold change; DMNC: Density of maximum neighborhood component; MNC: Maximum neighborhood component.

BIRC3-NC cells, as shown in Figure 4B and C. Upon exposure to trastuzumab, both cell types exhibited decreased cell viability. However, the decline in cell viability was less pronounced in N87-*BIRC3*-OE cells compared to N87-*BIRC3*-NC cells, as demonstrated by the CCK8 assay (104.2 ± 14.59 *vs* 72.06 ± 6.859, $P = 0.0021$) and EdU assay ($P < 0.0001$) (Figure 4D and E). Therefore, the overexpression of *BIRC3* promoted trastuzumab resistance in the HER2-positive gastric cancer cell line NCI-N87.

BIRC3 is correlated with trastuzumab resistance and poor prognosis in patients with HER2-positive gastric cancer

To further investigate this correlation, we procured 28 paraffin-embedded tissue samples from individuals diagnosed with HER2-positive gastric cancer for IHC analysis of *BIRC3* protein levels. Notably, *BIRC3* was predominantly localized to the cytoplasm, characterized by discernible brown particles (Figure 5A). Subsequently, these gastric cancer specimens were stratified into two distinct cohorts based on their *BIRC3* expression profiles: The *BIRC3* high and *BIRC3* Low groups. To further elucidate the relationship between *BIRC3* expression and prognosis among patients with HER2-positive gastric cancer, sophisticated analytical tools, such as the gene expression omnibus and Kaplan-Meier plotter, were employed. The results are presented in Table 3 and underscore a significant association, revealing that elevated *BIRC3* levels were statistically associated with advanced T stage ($P = 0.030$), elevated tumor node metastasis stage ($P = 0.016$), increased risk

Table 2 Genes screened by molecular complex detection

Cluster	Score	Nodes	Edges	Node IDs
1	3.333	4	5	*GRIA2, GRIA4, TSPAN7, PRKCG*
2	3.333	4	5	*NOD2, TNFAIP3, BIRC3, TLR4*
3	3	3	3	*WNT10B, LRP5, WNT3A*
4	3	3	3	*HIST1H1E, HIST1H4E, HIST1H3J*
5	3	3	3	*HBB, HBA2, HBA1*
6	3	3	3	*GUCY1B3, GUCY1A3, PRKG1*
7	3	3	3	*HLA-DRA, GNAO1, HLA-DRB1*
8	3	3	3	*TPO, DDC, IL4I1*
9	3	3	3	*PER3, CSNK1E, PER1*
10	3	3	3	*MMP13, COL9A1, COL9A2*
11	3	3	3	*SPON2, ADAMTS9, SEMA5A*

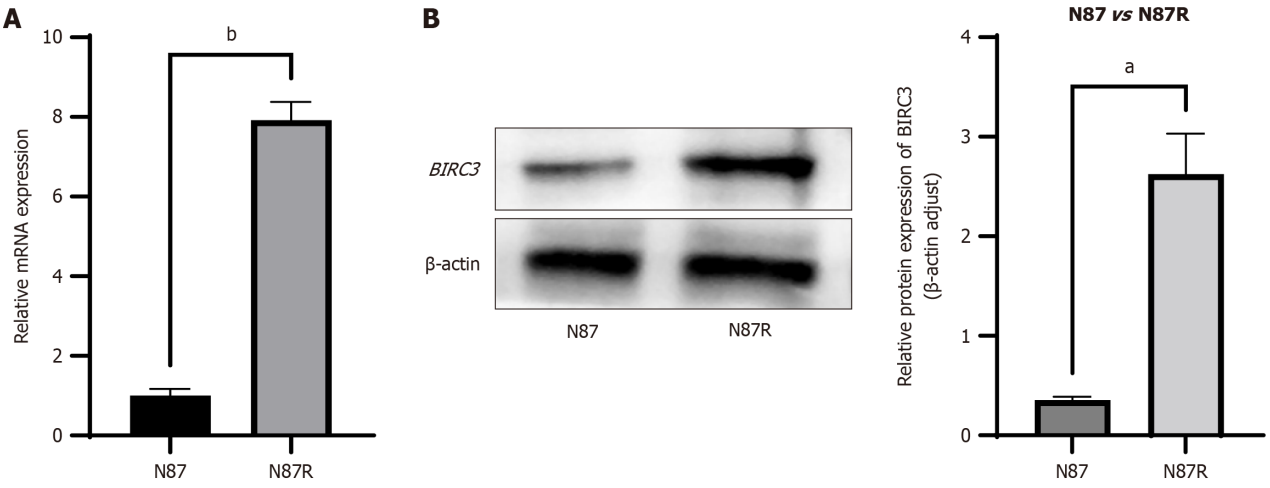


Figure 3 Expression of *BIRC3* in human gastric cancer cell lines and trastuzul resistant cell lines. A: mRNA levels of *BIRC3* in NCI-N87, and NCI-N87R cells were examined by reverse transcription polymerase chain reaction; *GAPDH* was used as a control; B: Protein levels of *BIRC3* in NCI-N87, and NCI-N87R cells were examined by western blotting, β-actin was a negative control. $^{a}P < 0.001$; $^{b}P < 0.0001$.

of overall survival ($P = 0.038$), and decreased disease-free survival rate ($P = 0.046$) within the cohort of 28 HER2-positive gastric cancer patients (Figure 5B). To verify the robustness of our findings, a parallel analysis was conducted on 32 HER2-negative patients, yielding results that were not statistically different (Table 4).

Within the scope of this study, 20 of 28 patients received a treatment regimen that combined trastuzumab with chemotherapy (Figure 5C). Among these, 10 of 20 patients received capecitabine and oxaliplatin chemotherapy, 5 of 20 received tegafur gimeracil oteracil potassium capsule and oxaliplatin chemotherapy, and 5 of 20 received treatment with Tegio or capecitabine chemotherapy. Following trastuzumab therapy, four patients displayed disease progression within a 3-month timeframe, while the remaining 13 patients exhibited sensitivity to trastuzumab during the same period. Additionally, at the time of analysis, three patients had not yet completed the full 3-month treatment protocol. IHC results indicated markedly elevated expression levels of *BIRC3* in the trastuzumab-resistant group compared to the trastuzumab-sensitive group.

These findings underscore the correlation between *BIRC3* expression in tumor cells, resistance to trastuzumab treatment, and adverse prognostic implications in patients diagnosed with gastric cancer.

Tumor cell-derived BIRC3 enhances the proliferation of HER2-positive gastric cancer cells treated with trastuzumab by activating AKT pathway

In this study, we explored the mechanism underlying *BIRC3* action in HER2-positive gastric cancer cells. Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis revealed that the differentially expressed genes were predominantly enriched in the PI3K-AKT and Ras pathways (Figure 6A). Existing literature has documented that aberrant activation of the PI3K-AKT pathway in HER2-positive tumor cells can trigger resistance to targeted trastuzumab therapy. Moreover, it has been observed that anomalous expression of *BIRC3* can stimulate the activation of the AKT

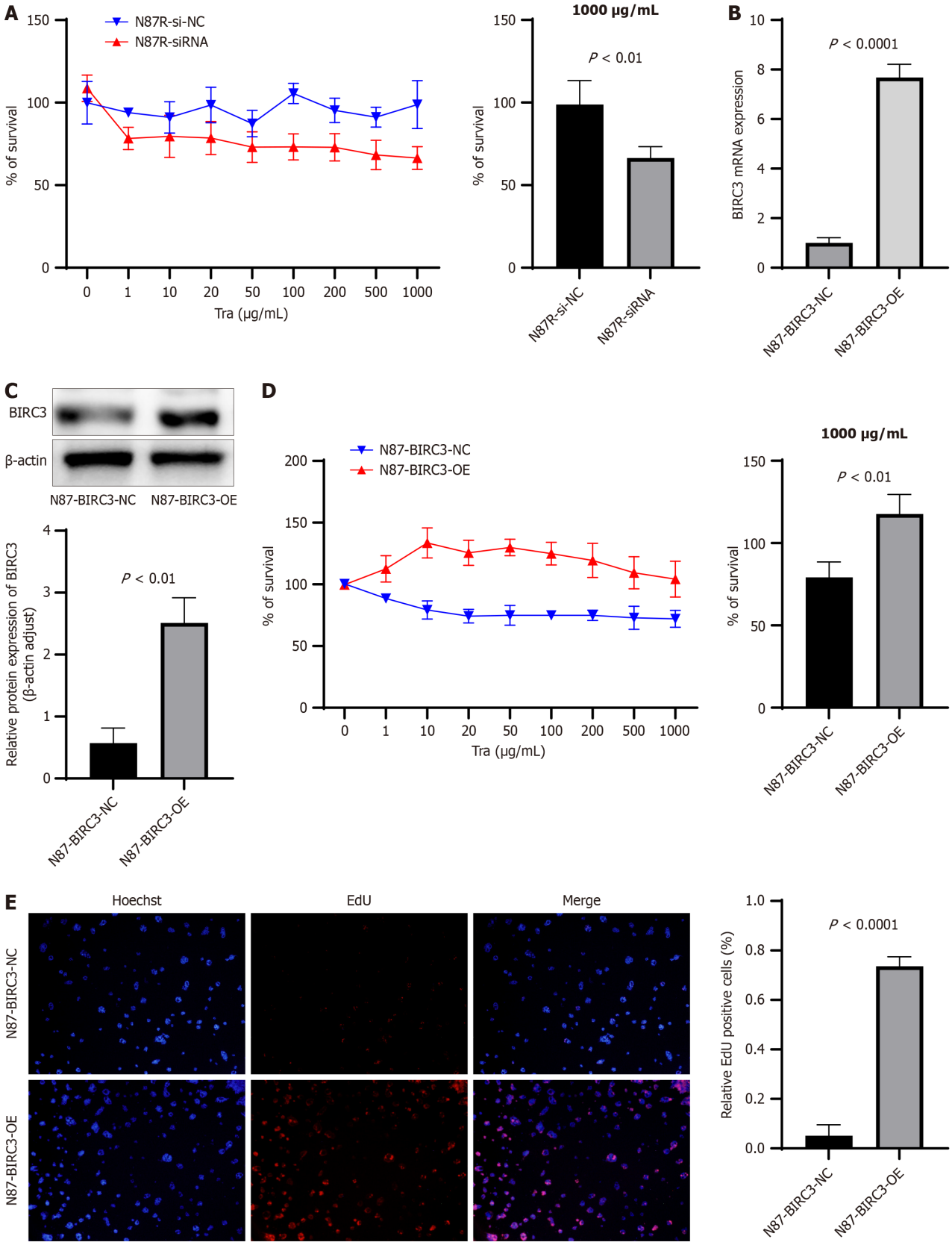


Figure 4 *BIRC3* promotes the resistance of human epidermal growth factor receptor 2-position gastric cancer to trastuzumab by promote the proliferation of tumor cell. A: *BIRC3* was depleted using *BIRC3*-siRNA in NCI-N87R cells. Cell viabilities of N87R-siRNA cells and N87R-siNC 1000 μg/mL trastuzumab were examined by cell counting kit-8 assay; B: mRNA levels of *BIRC3* in N87 cells after transfection of *BIRC3* overexpressing plasmids or control empty plasmids (designated as N87-*BIRC3*-OE and N87-*BIRC3*-NC) were examined by reverse transcription polymerase chain reaction; C: Protein levels of *BIRC3* in N87-*BIRC3*-OE and N87-*BIRC3*-NC were examined by western blotting. β-actin was a negative control; D: Cell viabilities of N87-*BIRC3*-OE and N87-*BIRC3*-NC cells 1000 μg/mL trastuzumab were examined by cell counting kit-8 assay; E: Cell viabilities of N87-*BIRC3*-OE and N87-*BIRC3*-NC cells 1000 μg/mL trastuzumab were examined by 5-ethynyl-2'-deoxyuridine assay. Tra: Trastuzumab; EdU: 5-ethynyl-2'-deoxyuridine.

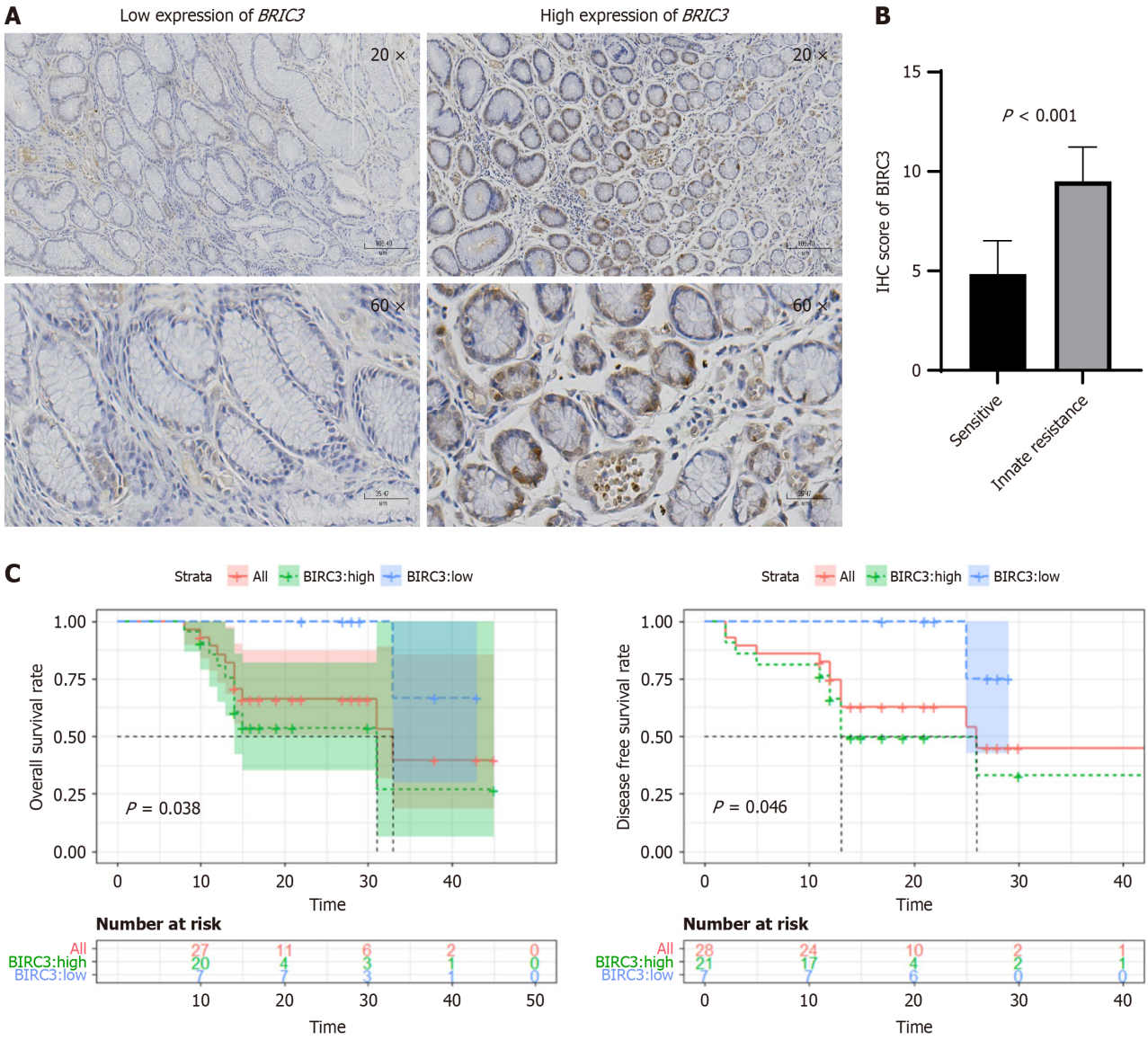


Figure 5 *BIRC3* is correlated with trastuzumab resistance and poor prognosis and the in human epidermal growth factor receptor 2-positive gastric cancer patients. A: Immunohistochemistry (IHC) staining for *BIRC3* in gastric cancer (GC) tissues were shown; B: 28 human epidermal growth factor receptor 2 (HER2)-positive patients were stratified into negative and positive groups according to IHC staining for *BIRC3*; C: The IHC results demonstrated significantly higher expression levels of *BIRC3* in the trastuzumab resistant group compared to the sensitive group. Kaplan-Meier analysis of overall survival in HER2-positive GC patients was performed based on *BIRC3* levels. IHC: Immunohistochemistry.

signaling pathway.

BIRC3 siRNA or siRNA-NC was transfected into NCI-N87R cells, resulting in two distinct cohorts: N87-siRNA and N87-si-NC. We conducted qPCR to assess the expression of *BIRC3*. As illustrated in Figure 6B, the N87-siRNA group displayed a notable decrease in *BIRC3* protein expression compared to the N87-si-NC group (0.6134 ± 0.06462 *vs* 0.9811 ± 0.03973, $P = 0.0011$). Furthermore, western blotting indicated that the downregulation of *BIRC3* significantly diminished the expression of AKT and pAKT in NCI-N87R cells (0.4776 ± 0.01184 *vs* 1.282 ± 0.1165, $P = 0.0003$). Additionally, Figure 6B shows that the expression levels of *BIRC3* (1.037 ± 0.1829 *vs* 0.4307 ± 0.09833, $P = 0.0072$), AKT, and pAKT proteins (0.9639 ± 0.04096 *vs* 0.3095 ± 0.01921, $P < 0.0001$) were markedly elevated in N87-*BIRC3*-OE cells compared to those in N87-*BIRC3*-NC cells.

To investigate the activation of the AKT pathway by *BIRC3* and its role in trastuzumab resistance, we conducted an AKT pathway blockade experiment. NCI-N87 cells were pretreated with MK-2206, an AKT inhibitor, and transfected with the overexpression plasmid pLVX-IRES-puro-*BIRC3*, to generate N87-AKTi-OE cells. As illustrated in Figure 6C and D, the CCK8 and EdU detection assays demonstrated a significantly higher cell proliferation activity in the N87-*BIRC3*-OE group compared to the N87-NC and N87-AKTi-OE groups, with statistically significant differences ($P < 0.001$). However, there was no discernible difference in cell proliferation between the N87-NC and N87-AKTi-OE groups. These results indicated that transfection with *BIRC3* significantly enhanced the proliferative capacity of N87-NC cells, which was attenuated by AKT inhibitors ($P < 0.001$). Furthermore, western blot analysis revealed a substantial increase in AKT phosphorylation in the sensitive cell line NCI-N87 upon *BIRC3* overexpression, whereas AKT inhibitors reduced AKT phosphorylation (Figure 6E). This suggested that *BIRC3* augmented the proliferative ability of NCI-N87 cells by

Table 3 Relationship between *BIRC3* and clinical pathology in human epidermal growth factor receptor 2 (+) group, *n* (%)

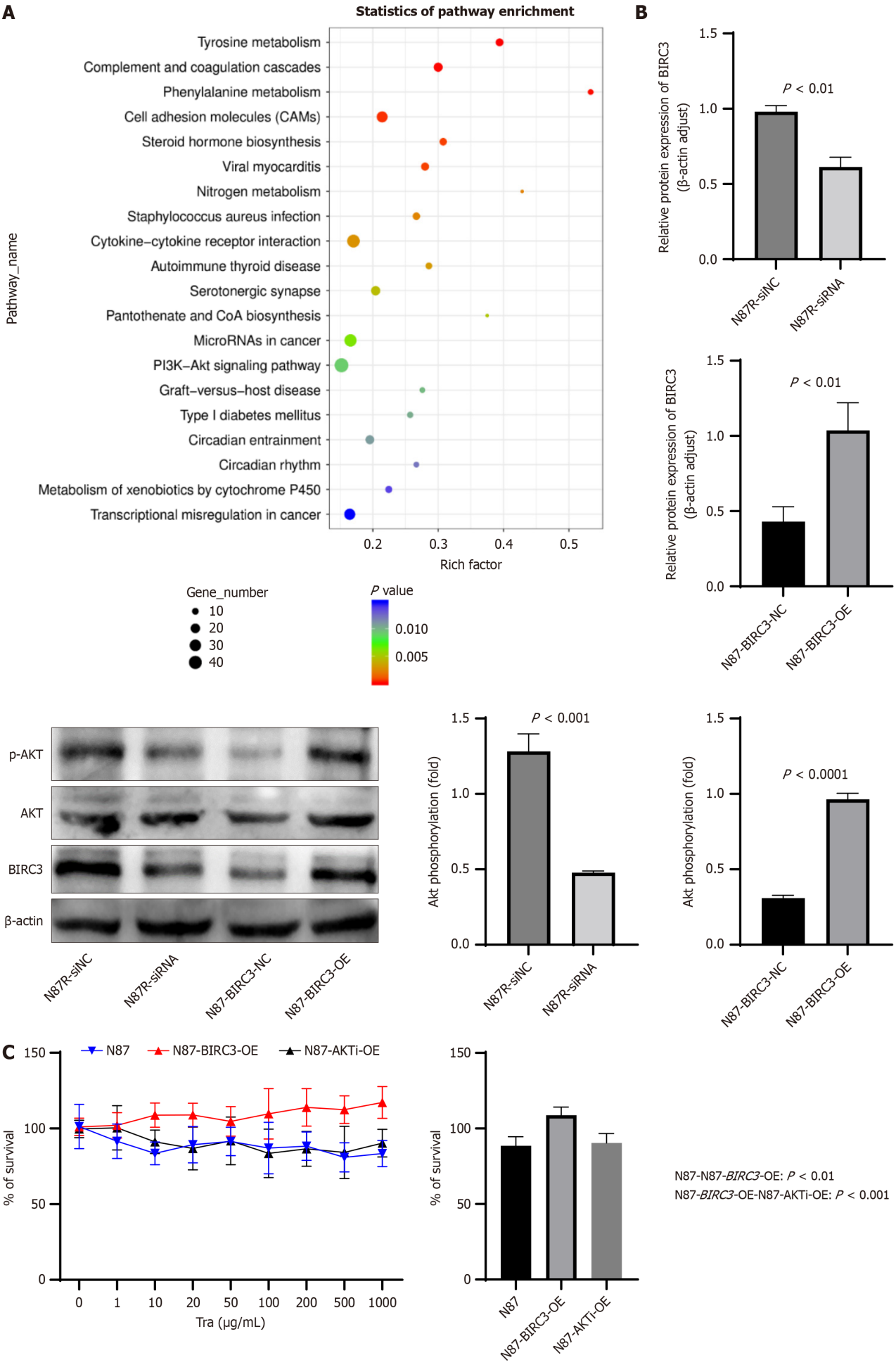
	BIRC^{low} group (*n* = 7)	*BIRC*^{high} group (*n* = 21)	*P* value
Sex (male)	6 (85.7)	19 (90.5)	1.000
Age (year)	63.71 ± 8.45	63.95 ± 8.52	0.949[2]
BMI (kg/m^2)	22.94 ± 2.81	24.56 ± 3.40	0.270[2]
Tumor size (cm)	3.64 ± 2.34	4.90 ± 1.78	0.146[2]
Differentiation			0.429[1]
Poor	3 (42.9)	14 (66.7)	
Moderate	4 (50.0)	6 (28.6)	
High	0 (0.0)	1 (4.8)	
T stage			0.030
1	1 (14.3)	0 (0)	
2	2 (28.6)	2 (9.5)	
3	4 (57.1)	2 (9.5)	
4	0 (0.0)	17 (81.0)	
N stage			0.245
0	3 (42.9)	5 (23.8)	
1	2 (28.6)	9 (42.9)	
2	2 (28.6)	3 (14.3)	
3	0 (0.0)	4 (19.0)	
M stage			-
0	7 (100.0)	19 (90.5)	
1	0 (0.0)	2 (9.5)	
TNM stage			0.016
I	1 (4.8)	0 (0.0)	
II	2 (9.5)	0 (0.0)	
III	4 (19)	19 (90.5)	
IV	0 (0.0)	2 (9.5)	
Survival			0.571[1]
Live	6 (85.7)	11 (52.4)	
Dead	1 (14.3)	10 (47.6)	
OS (month)			
mean ± SD	31.43 ± 7.138	17.0 ± 8.63	0.001[2]
Median (range)	29 (22-43)	14 (8-45)	-
Recurrence			0.571[1]
No	6 (85.7)	10 (47.6)	
Recurrence	1 (14.3)	11 (52.4)	
DFS (month)			
mean ± SD	24.14 ± 4.34	14.76 ± 9.89	0.002[2]
Median (range)	25 (17-29)	13 (2-45)	-

[1]*P* calculated by fisher test.
[2]*P* calculated by *t* text.
BMI: Body mass index; TNM: Tumor node metastasis; OS: Overall survival; DFS: Disease-free survival.

Table 4 Relationship between *BIRC3* and clinical pathology in human epidermal growth factor receptor 2 (-) group, *n* (%)

	BIRC^{low} group (*n* = 23)	BIRC^{high} group (*n* = 37)	*P* value
Sex (male)	19 (82.6)	24 (81.1)	0.270[1]
Age (year)	65.43 ± 10.77	64.97 ± 7.58	0.110[2]
BMI (kg/m^2)	23.46 ± 3.43	24.84 ± 3.77	0.892[2]
HER2			0.047
HER2 (-)	16 (69.6)	16 (43.2)	
HER2 (+)	7 (30.4)	21 (56.8)	
Tumor size (cm)	3.43 ± 2.02	4.59 ± 1.84	0.500[2]
Differentiation			0.489
Poor	9 (39.1)	24 (64.9)	
Moderate	14 (60.9)	10 (27.0)	
High	0 (0.0)	3 (8.1)	
T stage			0.001
1	5 (13.5)	1 (2.7)	
2	7 (18.9)	6 (16.2)	
3	10 (27.0)	11 (29.7)	
4	1 (2.7)	19 (51.4)	
N stage			0.208
0	11 (29.7)	8 (21.6)	
1	10 (27.0)	20 (54.1)	
2	2 (5.4)	5 (13.5)	
3	0 (0.0)	4 (10.8)	
M stage			0.69[1]
0	23 (100.0)	35 (94.6)	
1	0 (0.0)	2 (5.4)	
TNM stage			0.007
I	4 (10.8)	1 (2.7)	
II	5 (13.5)	1 (2.7)	
III	12 (32.4)	24 (64.9)	
IV	2 (5.4)	11 (29.7)	
OS (month)			
mean ± SD	32.2 ± 16.33	21.81 ± 15.17	0.015[2]
Median (range)	33 (3-53)	15 (5-53)	-
DFS (month)			
mean ± SD	26.74 ± 14.80	18.57 ± 14.21	0.040[2]
Median (range)	26 (3-52)	15 (2-53)	-

[1]*P* calculated by fisher test.
[2]*P* calculated by *t* text.
BMI: Body mass index; TNM: Tumor node metastasis; OS: Overall survival; DFS: Disease-free survival.


A
Statistics of pathway enrichment
Tyrosine metabolism
Complement and coagulation cascades
Phenylalanine metabolism
Cell adhesion molecules (CAMs)
Steroid hormone biosynthesis
Viral myocarditis
Nitrogen metabolism
Staphylococcus aureus infection
Cytokine–cytokine receptor interaction
Autoimmune thyroid disease
Serotonergic synapse
Pantothenate and CoA biosynthesis
MicroRNAs in cancer
PI3K–Akt signaling pathway
Graft–versus–host disease
Type I diabetes mellitus
Circadian entrainment
Circadian rhythm
Metabolism of xenobiotics by cytochrome P450
Transcriptional misregulation in cancer
Pathway_name
0.2
0.3
0.4
0.5
Rich factor
Gene_number
10
20
30
40
P value
0.010
0.005
B
Relative protein expression of BIRC3 (β-actin adjust)
P < 0.01
N87R-siNC
N87R-siRNA
Relative protein expression of BIRC3 (β-actin adjust)
P < 0.01
N87-BIRC3-NC
N87-BIRC3-OE
p-AKT
AKT
BIRC3
β-actin
N87R-siNC
N87R-siRNA
N87-BIRC3-NC
N87-BIRC3-OE
Akt phosphorylation (fold)
P < 0.001
N87R-siNC
N87R-siRNA
Akt phosphorylation (fold)
P < 0.0001
N87-BIRC3-NC
N87-BIRC3-OE
C
N87
N87-BIRC3-OE
N87-AKTi-OE
% of survival
Tra (μg/mL)
0
1
10
20
50
100
200
500
1000
% of survival
N87
N87-BIRC3-OE
N87-AKTi-OE
N87-N87-*BIRC3*-OE: P < 0.01
N87-*BIRC3*-OE-N87-AKTi-OE: P < 0.001

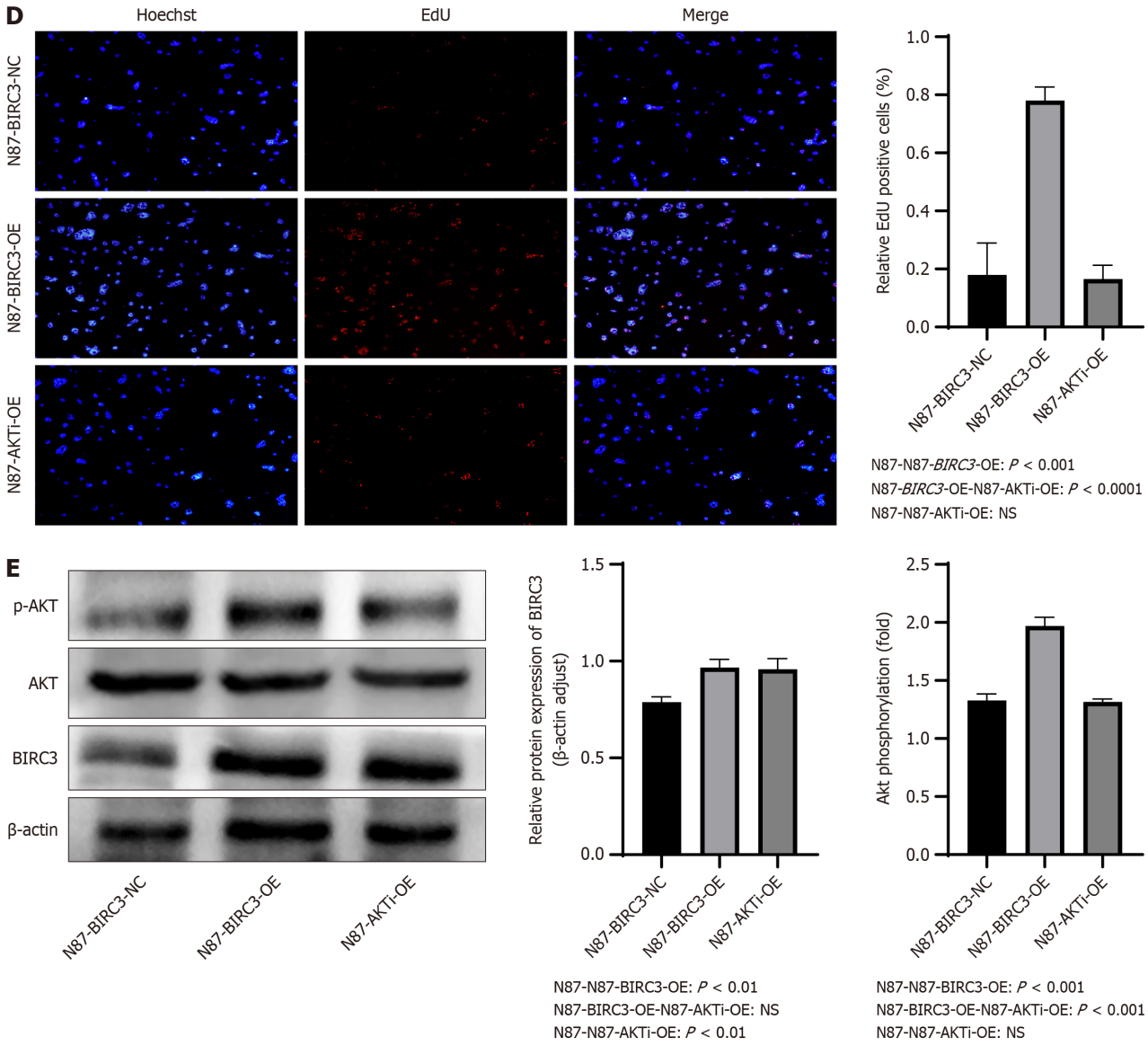


Figure 6 Tumor cell-derived *BIRC3* enhancing the proliferation of human epidermal growth factor receptor 2-positive gastric cancer cell treated with trastuzumab activating AKT pathway. *BIRC3* siRNA or siRNA-NC was transfected into NCI-N87R cells (designated as N87R-siRNA and N87R-siNC). NCI-N87 cells were pretreated with an Akt inhibitor (MK-2206) and transfected with the overexpression plasmid pLVX-IRES-puro-*BIRC3* (designated as N87-AKTi-OE). A: Revealed that the differential genes were mainly enriched in pathways by the Kyoto encyclopedia of genes and genomes enrichment analysis; B: Protein levels of *BIRC3* and AKT, p-AKT were examined by western blotting. β-actin was a negative control; C: Cell viabilities of N87, N87-*BIRC3*-OE and N87-*BIRC3* -NC cells 1000μg/mL trastuzumab were examined by cell counting kit-8 assay; D: Cell viabilities of N87, N87-*BIRC3*-OE and N87-*BIRC3*-NC cells 1000μg/mL trastuzumab were examined by 5-ethynyl-2'-deoxyuridine assay; E: Protein levels of *BIRC3* and AKT, p-AKT in N87,N87-*BIRC3*-OE, and N87-AKTi-OE cells were examined by western blotting. β-actin was a negative control. Tra: Trastuzumab; EdU: 5-ethynyl-2'-deoxyuridine; NS: No significance.

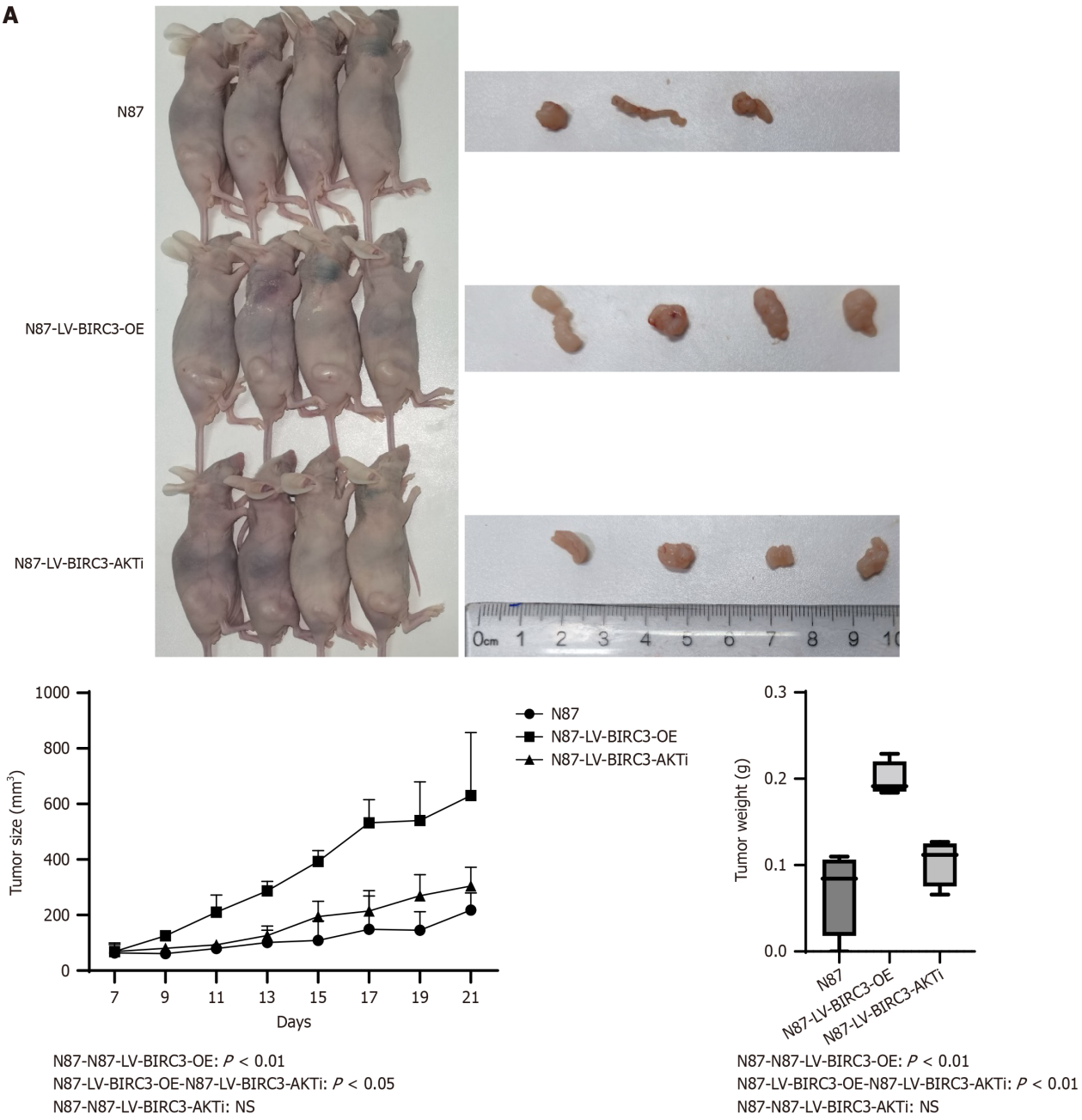
activating the AKT pathway.

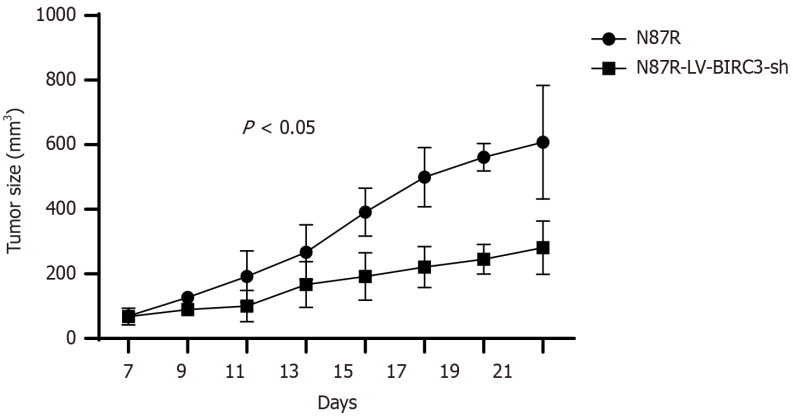
Tumor cell-derived BIRC3 inhibits apoptosis by activating the AKT pathway, leading to trastuzumab resistance

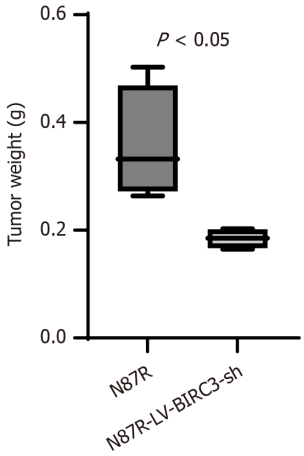
To examine tumorigenesis, SPF BALB/c nude mice were inoculated with the corresponding cells according to the described grouping plan. Tumor growth was monitored in nude mouse on the 7th day. Following the experimental design, the treatments were intraperitoneally administered every two days for two weeks.

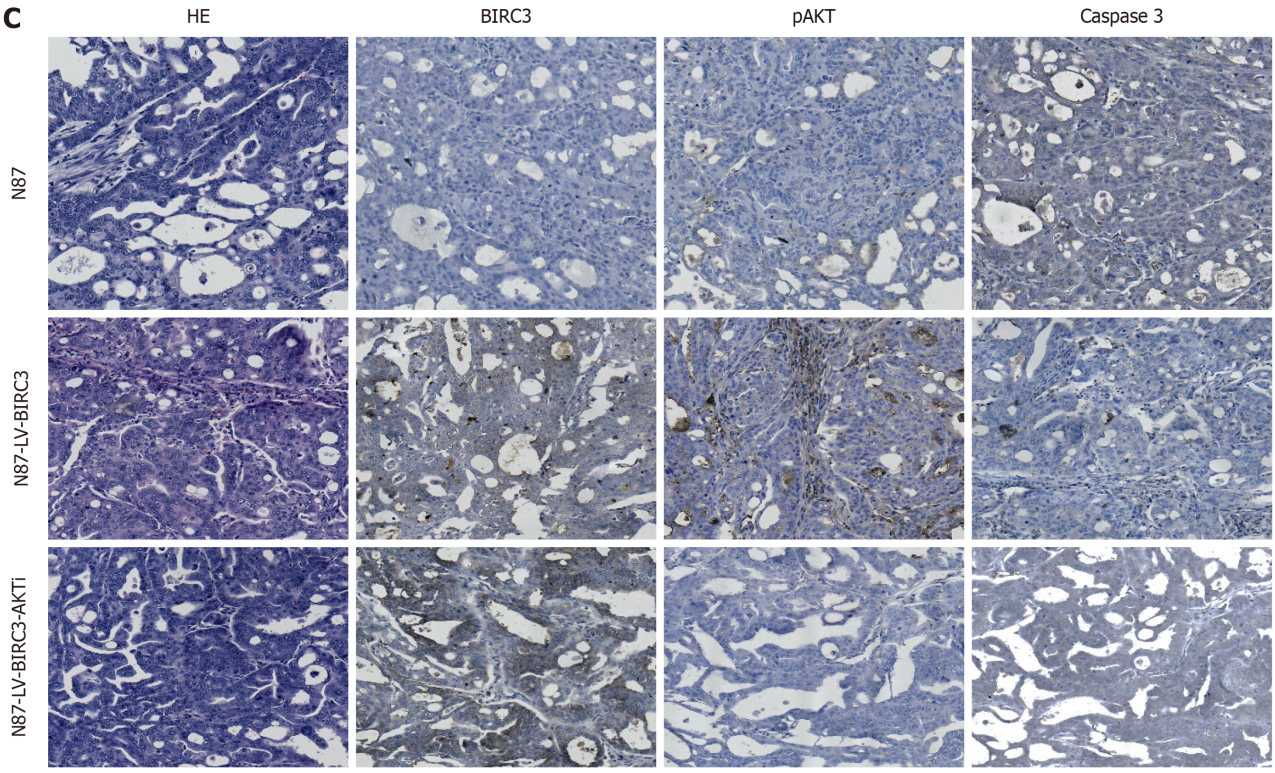
As shown in Figure 7A, one nude mouse in the N87-NC group was administered trastuzumab for two weeks after tumor formation, resulting in complete tumor regression. The N87-LV-*BIRC3* group displayed a significantly accelerated tumor growth rate and increased weight compared with both the N87-NC (P = 0.0084) and N87-LV-*BIRC3*-AKTi groups (P = 0.0044). However, no significant differences were observed between the N87-NC and N87-LV-*BIRC3*-AKTi groups. Besides, as depicted in Figure 7B, the NCI-N87R group exhibited accelerated tumor growth and greater weight relative to the N87R-LV-*BIRC3*-si group (0.3580 ± 0.1057 *vs* 0.1848 ± 0.01859, P = 0.0018).

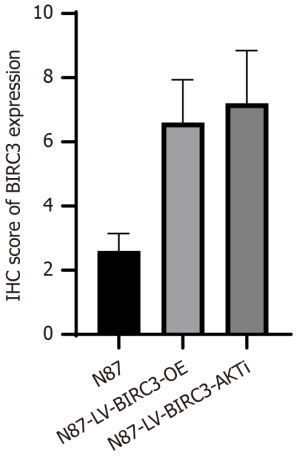
According to the IHC results, the expression of *BIRC3* was substantially enhanced in the N87R-LV-*BIRC3*-si (2.600 ± 0.5477 *vs* 6.7 ± 1.342, P = 0.0003) and N87-LV-*BIRC3*-AKTi (2.600 ± 0.5477 *vs* 7.20 ± 1.643, P = 0.0003) groups compared to the N87-NC group. Conversely, caspase 3 staining exhibited the opposite trend (P < 0.001). Additionally, the expression


A
N87
N87-LV-BIRC3-OE
N87-LV-BIRC3-AKTi
Tumor size (mm³)
Days
N87
N87-LV-BIRC3-OE
N87-LV-BIRC3-AKTi
N87-N87-LV-BIRC3-OE: P < 0.01
N87-LV-BIRC3-OE-N87-LV-BIRC3-AKTi: P < 0.05
N87-N87-LV-BIRC3-AKTi: NS
Tumor weight (g)
N87-N87-LV-BIRC3-OE: P < 0.01
N87-LV-BIRC3-OE-N87-LV-BIRC3-AKTi: P < 0.01
N87-N87-LV-BIRC3-AKTi: NS
B
N87R
N87R-LV-BIRC3-sh

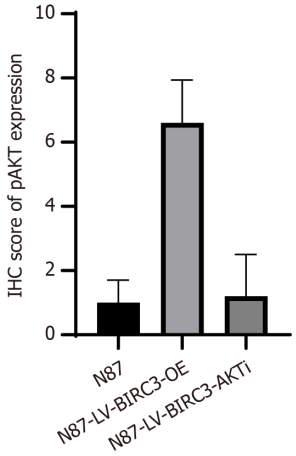




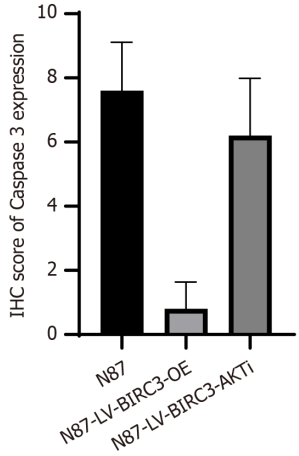




N87-N87-LV-BIRC3-OE: $P < 0.001$
N87-LV-BIRC3-OE-N87-LV-BIRC3-AKTi: NS
N87-N87-LV-BIRC3-AKTi: $P < 0.001$



N87-N87-LV-BIRC3-OE: $P < 0.0001$
N87-LV-BIRC3-OE-N87-LV-BIRC3-AKTi: $P < 0.001$
N87-N87-LV-BIRC3-AKTi: NS



N87-N87-LV-BIRC3-OE: $P < 0.0001$
N87-LV-BIRC3-OE-N87-LV-BIRC3-AKTi: $P < 0.001$
N87-N87-LV-BIRC3-AKTi: NS

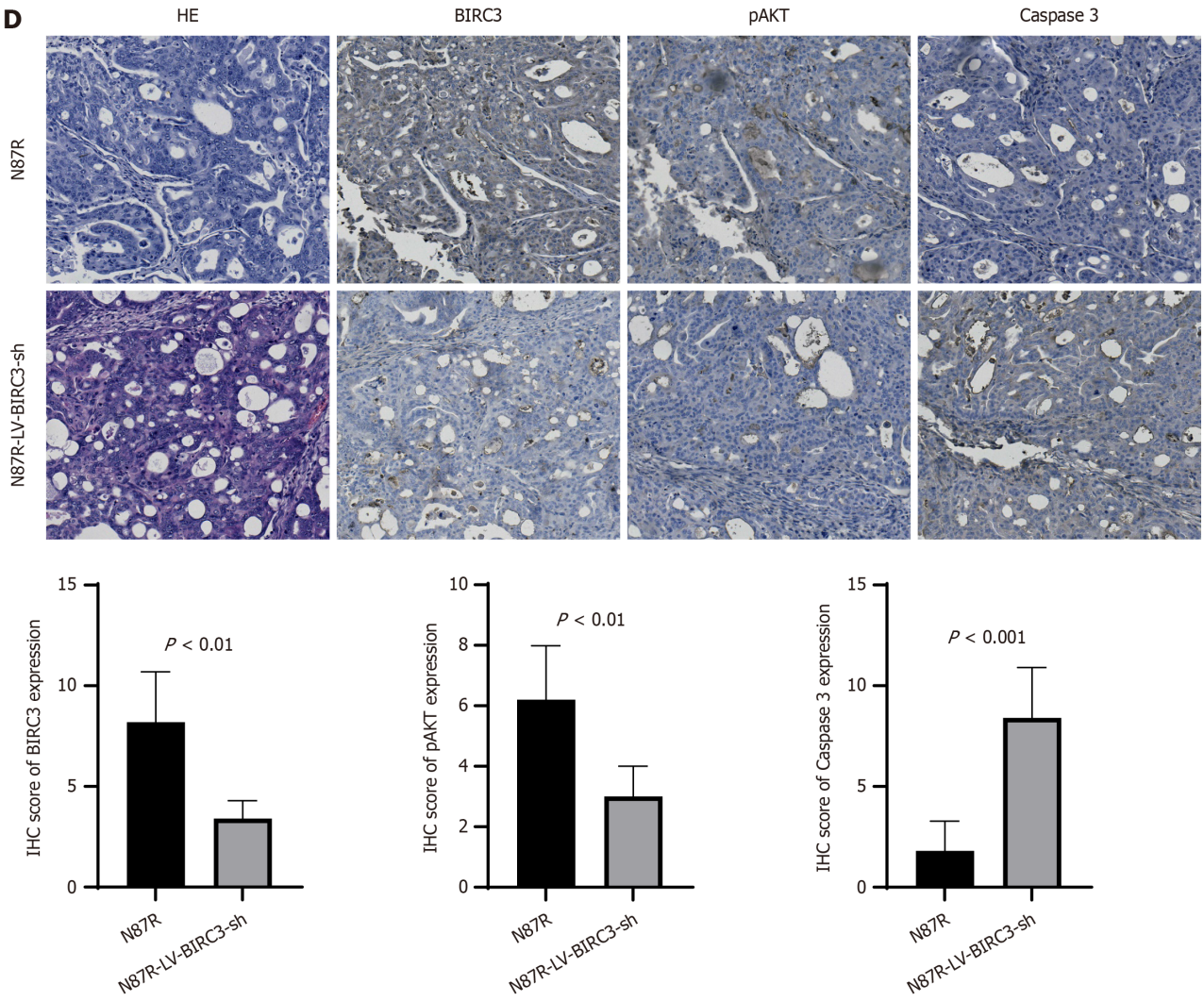


Figure 7 Tumor cell-derived *BIRC3* inhibits apoptosis by activating AKT pathway, lead to drug resistance to trastuzumab. A: Experimental in Balb/c nude mice. Tumor volume images, tumor weight and tumor growth curves of each group were shown. The different treatment groups included: (1) The stable control NCI-N87 cells (N87 group); (2) The stable *BIRC3* overexpressing NCI-N87 cells with the trastuzumab treatment (N87-LV-*BIRC3* group); and (3) The NCI-N87 transfected with the overexpressed virus LV-GFP-puro-*BIRC3* and treated with AKT inhibitor (MK-2206) (N87-LV-*BIRC3*-AKTi group); B: (1) The NCI-N87 was developed into a trastuzumab-resistant cell line(N87R group); (2) The group inoculated with NCI-N87R cells transfected with the knockout virus LV-GFP-puro-*BIRC3*-si (N87-LV-*BIRC3* group); C and D: Immunohistochemistry staining for *BIRC3*, pAKT and caspase 3 was presented, and the immunohistochemistry scores for *BIRC3*, pAKT and caspase 3 were statistically analyzed. IHC: Immunohistochemistry; HE: Hematoxylin-eosin; NS: No significance.

of pAKT in the N87-LV-*BIRC3* group was notably higher than that in the N87-NC (6.600 ± 1.342 *vs* 1.000 ± 0.7071, $P < 0.0001$) and N87-LV-*BIRC3*-AKTi groups (6.600 ± 1.342 *vs* 1.200 ± 1.304, $P = 0.0002$) (Figure 7C). Furthermore, when compared to the N87R group, the expression of *BIRC3* (8.200 ± 2.490 *vs* 3.400 ± 0.8944, $P = 0.0036$) and pAKT (6.200 ± 1.789 *vs* 3.000 ± 1.000, $P = 0.0082$) in the N87R-LV-*BIRC3*-si group significantly decreased, whereas the caspase 3 staining exhibited an opposite trend (1.800 ± 1.483 *vs* 8.400 ± 2.510, $P = 0.0010$) (Figure 7D).

These findings suggest that *BIRC3* potentiates the inhibition of apoptosis and stimulates tumor cell proliferation through activation of the pAKT pathway, ultimately leading to the resistance of HER2-positive gastric cancer cells to trastuzumab.

DISCUSSION

In this study, we established a trastuzumab-resistant cell line, NCI-N87R, using advanced sequencing and bioinformatics analyses. Our screening process identified the key gene, *BIRC3*, which is responsible for resistance to targeted therapy with trastuzumab.

We investigated the functional role of *BIRC3* in human gastric cancer, by focusing on its relationship with HER2. HER2 plays a crucial role in the development of gastric cancer, with approximately 5%-25% of gastric cancer cases exhibiting overexpression of the HER2. Studies have indicated that HER2 overexpression is an independent risk factor for advanced gastric cancer, leading to poor outcomes and shorter survival times[13,14]. The specific ligand of HER2 is unknown, but it is known to form hetero- or homodimers with itself or with other members of its family (*EGFR*, *HER3*, and *HER4*). This

dimerization leads to the phosphorylation of the tyrosine kinase domain, activating downstream signaling pathways such as the PI3K-AKT and *MAPK* pathways. These pathways play a crucial role in promoting cell proliferation, preventing cell apoptosis, and influencing cell growth, survival, and differentiation[15-17].

BIRC3 has been shown to have an anti-apoptotic role, as it directly binds and inhibits caspases 3, 7, and 9. Additionally, *BIRC3* can bind to polyubiquitin linked with lysine through its ubiquitin-related domain and activate the NF-κB signal pathway. This activation helps protect cells from tumor necrosis factor (TNF)-α induced cell apoptosis and maintain the survival of tumor cells[18]. Several studies have reported that *BIRC3* is overexpressed in more than 70% of human gastric cancers[19]. *BIRC3* knockout in gastric cancer cell lines has been shown to increase apoptosis, decrease proliferation, and delay cell migration[20]. Yoon *et al*[21] discovered that high *BIRC3* expression plays a crucial role in gastric cancer development after *Helicobacter pylori* infection. Furthermore, various studies have demonstrated that *BIRC3* exhibits similar effects in different tumor types. For example, high *BIRC3* expression is associated with clinicopathological characteristics and poor prognosis in colon cancer, pancreatic cancer, bladder cancer, and glioblastoma[9,22-25]. *BIRC3* overexpression plays a significant role in chemotherapy resistance in breast cancer, temozolomide resistance in glioblastoma multiforme, and cisplatin resistance induced by *COL11A1* in ovarian cancer[26-29]. These findings strongly suggest that *BIRC3* acts as a tumor promoter and has the potential to be a prognostic indicator in patients with tumors. Moreover, it may serve as a novel target for tumor treatment.

According to current literature, resistance to trastuzumab treatment in HER2-positive tumors can be attributed to various mechanisms. One important mechanism is abnormal activation of the downstream PI3K-AKT pathway[30,31]. Once AKT is phosphorylated and activated, it regulates several cellular processes including cell proliferation, differentiation, apoptosis, and angiogenesis through the involvement of Iκ-B kinase, procaspase 9, and mammalian target of rapamycin phosphorylation at the serine/threonine site[27-29]. Previous studies have demonstrated that AKT activation is associated with progressive disease and poor prognosis in certain tumor types[32]. In triple-negative breast cancer, overexpression of *BIRC3* activates the AKT signaling pathway, leading to tumor cell proliferation, metastasis, and poor prognosis[33]. Furthermore, research on resistance to HER2-positive tumor-targeted drugs has revealed that various molecular abnormalities, such as changes in receptor structure, co-expression with other transmembrane receptors, and abnormal activation of downstream pathways, can contribute to trastuzumab resistance[34-37].

The experimental results convincingly demonstrated that overexpression of *BIRC3* and AKT in N87 cells elicited a notable increase in cell proliferation and concurrent resistance to trastuzumab. Conversely, upon silencing *BIRC3* expression, the inverse effect was observed. Furthermore, inhibition of the AKT pathway resulted in a discernible decline in N87 cell proliferation, accompanied by increased susceptibility to trastuzumab. These findings suggest that *BIRC3* amplifies the proliferative potential of HER2-positive gastric cancer cells by activating the AKT pathway, thereby inducing resistance to targeted therapy with trastuzumab. Moreover, *in vitro* cytological experiments substantiated these observations, affirming that increased *BIRC3* expression activates the AKT pathway, resulting in enhanced proliferation of previously responsive cells after trastuzumab treatment, consequently fostering drug resistance. To confirm these conclusions, tumorigenesis experiments were conducted in nude mice. Additionally, a responsive experimental group was designed to provide further compelling evidence supporting the assertion that *BIRC3* induces trastuzumab-targeted drug resistance through the activation of the AKT pathway.

In the clinical data analysis section, several limitations are evident. This study is a single-center investigation and lacks data from larger multicenter samples. Consequently, a greater number of gastric cancer patients must be evaluated to enhance the robustness of the results. Although we conducted multiple repeated experiments to ensure the accuracy of our findings, additional results from diverse experimental methods are necessary for further improvement. Ultimately, more work is required to provide tangible benefits to clinical patients.

CONCLUSION

In conclusion, our findings illustrate that the knockdown of *BIRC3* diminishes the resistance of the trastuzumab-resistant cell line NCI-N87R. Conversely, high *BIRC3* expression induced resistance to trastuzumab. Moreover, upon transfection with the *BIRC3* overexpression vector, N87 cells exhibited resistance to trastuzumab. In addition, resistance to trastuzumab could be counteracted by AKT inhibitors, suggesting that *BIRC3* may trigger resistance to trastuzumab therapy by activating the AKT pathway.

ACKNOWLEDGEMENTS

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FOOTNOTES

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