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ABOUT COVER

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ORIGINAL ARTICLE

Clinical and Translational Research

High expression of autophagy-related gene *EIF4EBP1* could promote tamoxifen resistance and predict poor prognosis in breast cancer

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Abstract

BACKGROUND

Breast cancer (BC) remains a public health problem. Tamoxifen (TAM) resistance has caused great difficulties for treatment of BC patients. Eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) plays critical roles in the tumorigenesis and progression of BC. However, the expression and mechanism of EIF4EBP1 in determining the efficacy of TAM therapy in BC patients are still unclear.

AIM

To investigate the expression and functions of EIF4EBP1 in determining the efficacy of TAM therapy in BC patients.

METHODS

High-throughput sequencing data of breast tumors were downloaded from the Gene Expression Omnibus database. Differential gene expression analysis identified *EIF4EBP1* to be significantly upregulated in cancer tissues. Its prognostic value was analyzed. The biological function and related pathways of *EIF4EBP1* was analyzed. Subsequently, the expression of EIF4EBP1 was determined by real-time reverse transcription polymerase chain reaction and western blotting. Cell Counting Kit-8 assays, colony formation assay and wound healing assay were used to understand the phenotypes of function of EIF4EBP1.

RESULTS

EIF4EBP1 was upregulated in the TAM-resistant cells, and EIF4EBP1 was related to the prognosis of BC patients. Gene Set Enrichment Analysis showed that EIF4EBP1 might be involved in Hedgehog signaling pathways. Decreasing the expression of EIF4EBP1 could reverse TAM resistance, whereas overexpression of

EIF4EBP1 promoted TAM resistance.

CONCLUSION

This study indicated that EIF4EBP1 was overexpressed in the BC and TAM-resistant cell line, which increased cell proliferation, invasion, migration and TAM resistance in BC cells.

Key Words: Breast cancer; Eukaryotic translation initiation factor 4E binding protein 1; Tamoxifen; Resistance; Prognosis; Bioinformatics

Core Tip: Breast cancer is the most frequently diagnosed cancer in women and the leading cause of female deaths from cancer worldwide. Eukaryotic translation initiation factor 4E binding protein 1 was overexpressed in the breast cancer tamoxifen-resistant cell line, and high expression of eukaryotic translation initiation factor 4E binding protein 1 was associated with poor prognosis in tamoxifen-treated patients.

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INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed cancer in females and the leading cause of female deaths from cancer worldwide. BC is the most prevalent cancer worldwide. An estimated 3.9 million women have been diagnosed with BC in the past 5 years, and more than 350000 annual deaths worldwide are attributed to BC[1,2]. Estrogen receptor alpha (ERα) is expressed in more than 70% of all BC cases, playing key roles in the gene transcription of genes related to the development of BC cells[3,4]. Endocrine therapy is the major treatment strategy for both premenopausal and postmenopausal ER-positive patients. It functions by blocking the ERs or inhibiting estrogen production[5,6].

Tamoxifen (TAM), a selective estrogen modulator, is the most frequently prescribed antiestrogenic medication in the BC setting[7]. The introduction of TAM has significantly prolonged the overall survival (OS) and disease-free survival of BC patients[8,9]. However, approximately half of BC patients have intrinsic resistance to TAM or develop acquired drug resistance to the medication during treatment. TAM resistance remains one of the major causes of BC mortality today [10]. Therefore, it is necessary to identify biomarkers and therapeutic targets and to understand the molecular mechanisms of TAM resistance to improve patient survival.

Autophagy is a lysosomal degradation process that plays critical roles in cell survival and maintenance through the degradation of cytoplasmic organelles, proteins and macromolecules as well as the recovery of metabolites[11,12]. Studies have shown that defects in autophagy pathways can promote or inhibit drug resistance in many cancer types[13,14]. Eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) encodes one member of a family of translational repressor proteins that directly interacts with eIF4E. The Human Autophagy Database (http://www.autophagy.lu/) provides a complete list of human genes and proteins that are directly or indirectly involved in autophagy, and EIF4EBP1 is found in this database.

Rutkovsky *et al*[15] found that EIF4EBP1 was overexpressed in BC cells and that knockdown of EIF4EBP1 led to dramatic reductions in cell growth. Du *et al*[16] showed that EIF4EBP1 has significant prognostic value for BC. It has been reported that EIF4EBP1 is an independent prognostic factor for progesterone receptor-positive BC and that high expression of EIF4EBP1 was associated with drug resistance to endocrine treatment in the ER/progesterone receptor-positive patients[17]. Moreover, Hsieh *et al*[18] illustrated that EIF4EBP1 could enhance drug resistance in prostate cancer cells. However, the expression and molecular mechanism of EIF4EBP1 in TAM resistance in BC remains unknown.

In this study, we investigated the effects of EIF4EBP1 on TAM resistance and established it as a novel biomarker for TAM resistance. Bioinformatics analysis indicated that *EIF4EBP1* was overexpressed in TAM-resistant BC cells. Moreover, high expression of EIF4EBP1 was associated with poor prognosis in TAM-resistant BC patients with TAM resistance and with a higher probability of metastasis and endocrine therapy resistance. *In vitro* experiments indicated that the expression level of EIF4EBP1 was positively correlated with TAM resistance in TAM-resistant BC cells.

MATERIALS AND METHODS

Datasets of TAM-resistant BC tissues

In this study, high-throughput sequencing data of TAM-resistant BC were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database. The GSE21648 dataset contains one TAM-sensitive sample and six TAM-resistant samples, and the GSE26459 dataset contains three TAM-sensitive samples and three TAM-resistant samples. Then, the data in these datasets were normalized and summarized by the R software packages "limma" and "affy." The R software package "limma" was used to identify differentially expressed genes (DEGs) in these datasets. The Benjamini-Hochberg method was used to adjust the fold change and *P* values. For DEGs, the cut-off criteria were |log fold change| > 1 and *P* value < 0.05.

Bioinformatics analysis of EIF4EBP1

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) is a web-based tool that can be used to conduct patient survival analysis based on The Cancer Genome Atlas database[19]. Kaplan-Meier plotter (https://kmplot.com/analysis/) is a web-based tool that can be used to conduct patient survival analysis based on the Gene Expression Omnibus, EGA, and The Cancer Genome Atlas databases[20]. In this study, survival was compared between the high and low *EIF4EBP1* expression groups based on GEPIA and Kaplan-Meier plotter. The cutoff criterion was $P < 0.05$. GSEA is a powerful method to explore biological insights and potential pathways related to a gene list by determining the genes that were in previously identified gene sets[21]. In this study, GSEA was used to explore the potential functions and molecular mechanisms of EIF4EBP1 in TAM-resistant BC cells. The six TAM-resistant BC samples in GSE21648 were classified into two groups according to the median expression level of *EIF4EBP1*. Then, GSEA was conducted by the R package "clusterProfiler". The reference gene set used for GSEA was h.all.v6.2.sytmbols.gmt obtained from the Molecular Signatures Database.

BC samples and cell culture

There were 71 BC tissue specimens acquired from patients at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). The diagnosis of BC was made by two pathologists, and none of the patients had received any treatment (radiotherapy or endocrine therapy). This study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. All individuals were informed of the purpose of the study and had given written informed consent.

The BC cell line T47D (ER-positive) was purchased from the American Type Culture Collection (Manassas, VA, United States). 4-hydroxytamoxifen was purchased from Sigma-Aldrich (Shanghai, China). The TAM-resistant T47D-R cell line was obtained by continuous exposure to 4-hydroxytamoxifen (6 μM). Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

Quantitative reverse transcription-polymerase chain reaction assay

The expression of *EIF4EBP1* in BC samples and cell culture was measured by real-time reverse transcription polymerase chain reaction (RT-qPCR). RNAiso Plus (TaKaRa, Otsu, Japan) was used to extract total RNA from BC cells. The PrimeScriptTM RT Reagent Kit (TaKaRa) was used to generate complementary DNA. RT-qPCR was performed using the TB Green Premix Ex Taq™ II kit on a MasterCycler5333 instrument (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression of genes. *GAPDH* was used as an internal control.

Immunohistochemistry assay

In this study, the expression of EIF4EBP1 in BC tissues from our hospital was determined by immunohistochemistry assay. Immunohistochemical analysis of BC samples were performed according to standard protocols. In brief, paraffin sections were dewaxed using xylene and rehydrated in a graded ethanol series. Hydrogen peroxide (0.3%) was used to block endogenous peroxidase activity. The Ventana Discovery XT automated stainer was used for immunohistochemistry, and ImageJ software was used for visualization.

Cell transfection

Short interference RNAs (siRNAs) for *EIF4EBP1* and corresponding scrambled siRNA negative controls were synthesized by GenePharma (Shanghai, China). The *EIF4EBP1* plasmid and negative control vector were purchased from GenePharma (Shanghai, China). Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) was used to transiently transfect cells according to the manufacturer's instructions. The transfection efficiency was evaluated by RT-qPCR after 24 h. Then, the slides with tissue samples were heated in sodium citrate buffer (95 °C, 10 min) and sealed in normal goat serum (37 °C, 1 h). The tissue samples were incubated with an anti-EIF4EBP1 antibody (Abcam, Shanghai, China) for 1 h at 37 °C. Then, the tissue samples were incubated with a secondary antibody conjugated with horseradish peroxidase (Abcam). The experimental results were independently assessed by three blinded pathologists and analyzed using ImageProPlus software (version 6; MediaCybernetics, Rockville, MD, United States).

Cell Counting Kit-8 assay

A Cell Counting Kit-8 (CCK-8) assay was performed according to the manufacturer's instructions to evaluate the effect of EIF4EBP1 knockdown or overexpression of EIF4EBP1 on the sensitivity of T47D-R cells to TAM treatment. T47D-R cells

were seeded into 96-well plates (5000 cells/well) and incubated with TAM at different concentrations for 48 h. Subsequently, 10 μL of CCK-8 solution (Abcam) was added to each well. The OD450 values were measured with a microplate reader. Experiments were performed in triplicate.

Colony formation assay

To evaluate the effect of EIF4EBP1 knockdown or overexpression of EIF4EBP1 on the proliferation of the T47D-R cells after TAM treatment, a colony formation assay was performed. Cells in the logarithmic growth phase were seeded in 6-well plates at a density of 400 cells/well. T47D-R cells were cultured in a medium supplemented with 5 μM TAM. After 2 wk, cell colonies were fixed with 4% paraformaldehyde and visualized by staining with 0.1% crystal violet. Then cell colonies were counted and photographed. Experiments were performed in triplicate.

Transwell assay

The invasion and migration abilities of T47D-R cells with knockdown or overexpression of EIF4EBP1 were analyzed *via* a transwell assay. T47D-R cells were seeded into 6-well plates at 1×10^5 cells/well for 24 h. Then T47D-R cells (200 mL/well) were seeded in a transwell chamber with 10% TBS and culture medium. The cells were cultured at 37 °C with 5% CO_2 for 24 h. Then the liquid in the transwell chamber was removed. Cells in the lower chamber were fixed with 100% methanol, stained with 0.1% crystal violet and observed under a microscope. Experiments were performed in triplicate.

Wound healing assay

The invasion and migration abilities of T47D-R cells with EIF4EBP1 knockdown or overexpression of EIF4EBP1 were analyzed by wound healing assay. T47D-R cells were seeded in 6-well culture plates at 1×10^5 cells/well. After overnight incubation with 5 μM TAM, a wound was created with a sterile pipette tip. Then, the cells were imaged at 0 h and 48 h after the wound was created. Experiments were performed in triplicate.

Statistical analysis

In this study, all statistical analyses were performed by R software (version 3.5.3), SPSS 22.0 (SPSS Inc., Chicago, IL, United States) and GraphPad Prism 7.0. The R software package "limma" was used to identify DEGs. The OS of patients was analyzed by the R software package "survival ROC". The experimental data were presented as the mean ± standard deviation values. The differences between the low and high EIF4EBP1 expression groups were compared by Pearson's χ^2 test. The Kaplan-Meier method was employed to analyze the OS of BC patients, and the log-rank test was used to estimate the differences between groups. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Differential expression and bioinformatics analysis of EIF4EBP1

In this study, high-throughput sequencing data of TAM-resistant BC cells were downloaded and re-analyzed. As shown in Figures 1A and B, *EIF4EBP1* was upregulated in TAM-resistant BC cells in GSE21618 and GSE26459. Survival analysis based on the GEPIA database suggested that high expression of *EIF4EBP1* was significantly associated with worse OS (*P* = 0.023, Figure 1C) and disease-free survival (*P* = 0.01, Figure 1D). Moreover, survival analysis based on the Kaplan-Meier plotter database showed that high expression of *EIF4EBP1* was significantly associated with worse OS (*P* = 6.5e-08, Figure 1E), disease-free survival (*P* < 1E-16, Figure 1F) and post-progression survival (*P* = 6.5e-08, Figure 1G). These results suggested that EIF4EBP1 could be a prognostic marker for BC patients.

The potential functions and molecular mechanisms of EIF4EBP1 in TAM-resistant BC cells were explored by GSEA. The TAM-resistant BC samples in GSE21618 were divided into two groups according to the median expression level of *EIF4EBP1*. Then, the potential functions and molecular mechanisms were explored by GSEA. Gene Ontology analysis based on the GSEA results suggested that cyclic nucleotide biosynthetic processes, negative regulation of cell substrate adhesion and nucleobase biosynthetic processes were upregulated, whereas antigen processing and presentation of endogenous peptide antigen, autophagosome maturation and intrinsic component of endoplasmic reticulum membrane were downregulated (Figure 1H). Kyoto Encyclopedia of Genes and Genomes analysis indicated that aminoacyl tRNA biosynthesis, the Hedgehog (Hh) signaling pathway and the peroxisome proliferator-activated receptor (PPAR) signaling pathway were upregulated, while the cell adhesion molecules cams, cytosolic DNA sensing pathways and ErbB signaling pathways were downregulated (Figure 1H).

The expression of EIF4EBP1 in TAM-resistant cells and clinical BC samples

In this study, RT-qPCR and immunohistochemistry were used to explore the expression of EIF4EBP1. As shown in Figures 1I, 1J and 2, EIF4EBP1 was significantly upregulated in BC tissues and TAM-resistant T47D cells. Moreover, the expression of EIF4EBP1 in BC tissues obtained at our hospital was determined, and the correlations of its expression with clinicopathological data (age, lymph node metastasis, radiotherapy status, endocrine therapy status, tumor stage, histological grade and metastasis stage) were calculated (Table 1). As shown in Table 1, the expression of EIF4EBP1 was significantly associated with lymph node metastasis (*P* < 0.0001), endocrine therapy status (*P* = 0.0005) and metastasis stage (*P* < 0.0001).

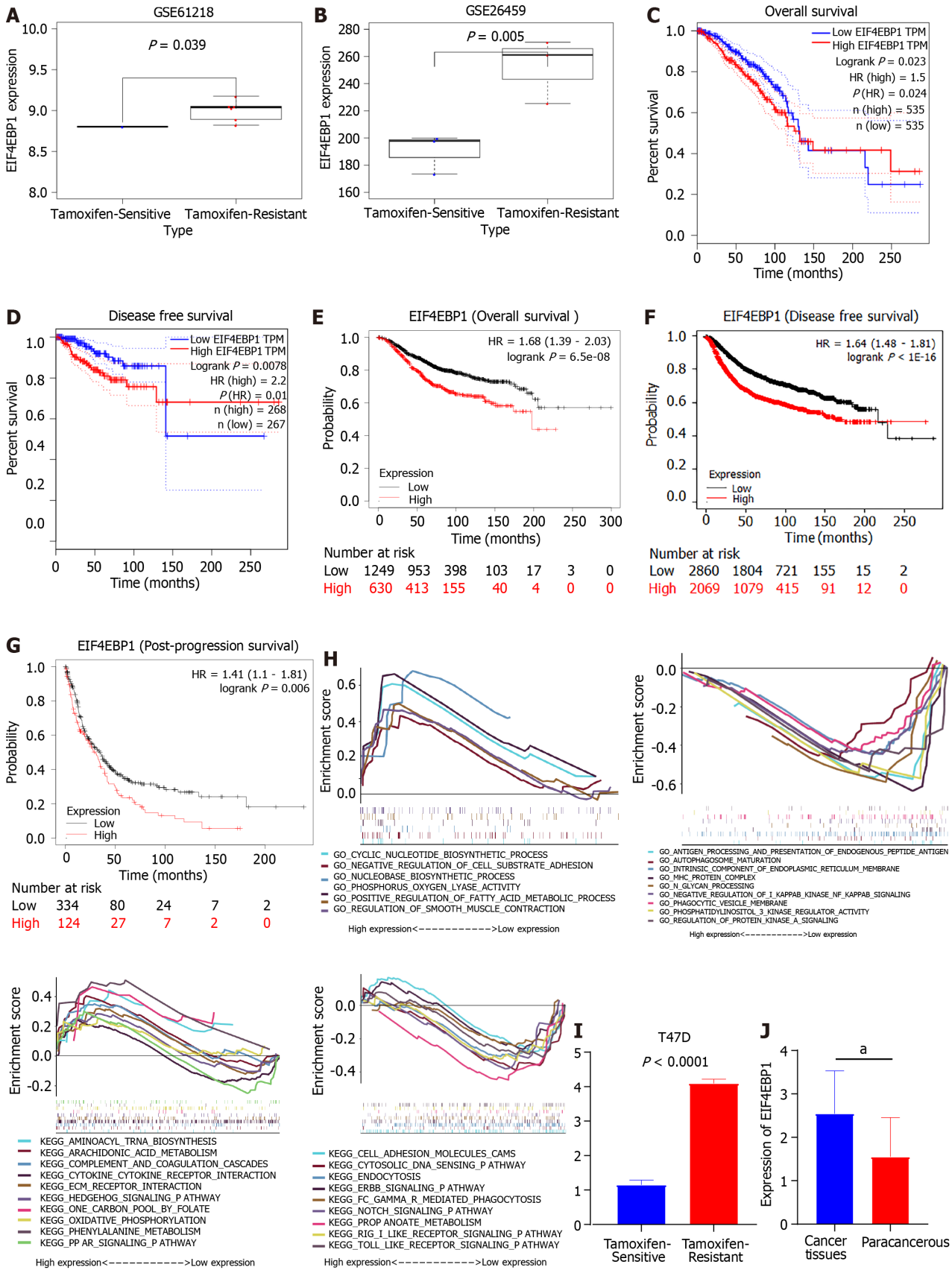

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Figure 1 Eukaryotic translation initiation factor 4E binding protein 1 was upregulated, which correlated with poor survival in tamoxifen-resistant breast cancer cells. A and B: Box diagram showed that eukaryotic translation initiation factor 4E binding protein 1 (*EIF4EBP1*) was upregulated in tamoxifen-resistant breast cancer cells in GSE21618 and GSE26459; C: Overall survival of *EIF4EBP1* analysis by Gene Expression Profiling Interactive Analysis; D: Disease-free survival of *EIF4EBP1* by Gene Expression Profiling Interactive Analysis; E: Overall survival of *EIF4EBP1* by Kaplan-Meier plotter; F: Disease-free

survival of *EIF4EBP1* by Kaplan-Meier plotter; G: Post-progression survival of *EIF4EBP1* by Kaplan-Meier plotter; H: Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of *EIF4EBP1* by GSEA; I: *EIF4EBP1* was upregulated in tamoxifen-resistant breast cancer cells and confirmed by real-time reverse transcription polymerase chain reaction; J: *EIF4EBP1* was upregulated in breast cancer tissues and confirmed by real-time reverse transcription polymerase chain reaction. Error bars represent means ± standard deviations of triplicate analyses. $^{a}P < 0.05$. EIF4EBP1: Eukaryotic translation initiation factor 4E binding protein 1; HR: Hazard ratio.

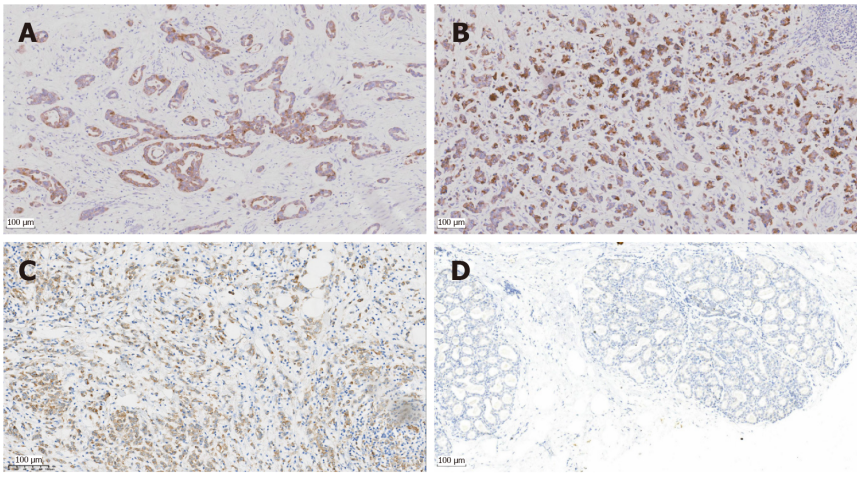

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Figure 2 Immunohistochemical staining for eukaryotic translation initiation factor 4E binding protein 1 in breast cancer tissues. A: Invasive ductal carcinoma; B: Invasive micropapillary carcinoma; C: Invasive lobular carcinoma; D: Normal breast cancer tissues.

Knockdown of EIF4EBP1 in TAM-resistant cells

To explore the functions of EIF4EBP1 in TAM-resistant cells, the expression of EIF4EBP1 was reduced by transfecting siRNAs targeting *EIF4EBP1*. RT-qPCR showed that transient transfection of siRNA significantly decreased the expression of *EIF4EBP1* (Figure 3A). The results of a CCK-8 assay suggested that downregulation of EIF4EBP1 significantly decreased the degree of resensitization to TAM in T47D-R cells (Figure 3B). Colony formation experiments indicated that the number of colonies consisting of T47D-R cells was significantly decreased after siRNA transfection (Figure 3C). The results of the transwell and wound healing assays indicated that EIF4EBP1 knockdown could reduce the invasion and migration of T47D-R cells treated with TAM. These results indicated that knockdown of EIF4EBP1 caused T47D-R cells to be resensitized to TAM.

Overexpression of EIF4EBP1 in TAM-resistant cells

To further understand the functions of EIF4EBP1 in TAM-resistant cells, the expression of EIF4EBP1 was upregulated by the transient transfection of a plasmid expressing *EIF4EBP1*. The overexpression of *EIF4EBP1* was confirmed by RT-qPCR (Figure 4A). The cell viability of T47D-R cells treated with TAM was assessed by CCK-8 and colony formation assays. The results indicated that the resistance of T47D-R cells was further enhanced after *EIF4EBP1* plasmid transfection (Figures 4B and C). The invasion and migration of T47D-R cells treated with TAM were explored by transwell and wound healing assays. The results suggested that cell invasion and migration were increased by EIF4EBP1 overexpression. These results indicate that the overexpression of EIF4EBP1 could increase the resistance of T47D-R cells to TAM.

DISCUSSION

BC is one of the most common cancers in the world, and ER-positive BC is the most common subtype of BC. Endocrine therapy, which targets the ER directly and/or suppresses estrogen production, is the main treatment strategy for ER-positive BC[21]. TAM is a nonsteroidal antiestrogen drug and has historically been the most widely used antiestrogen drug for the treatment of ER-positive BC patients[19,20]. Although TAM has greatly reduced the recurrence and mortality of BC, the emerging and acquired resistance to TAM has been a major obstacle for the successful treatment of patients[22,23]. Many studies have been conducted on the potential mechanism of TAM resistance, and several mechanisms have been shown to be related to TAM resistance. These mechanisms include autophagy, mutations of the ER and endoplasmic reticulum stress[10,22,24,25].

Autophagy is a "self-degradative" process in which cellular materials are sent to lysosomes for degradation. Autophagy plays a critical role in the turnover of cell components and provides energy and macromolecules[26,27]. Studies have shown that autophagy plays dual, context-dependent roles in drug resistance: It can kill drug-resistant cancer cells with inactive apoptotic pathways, but it can also participate in the development of drug resistance and

Table 1 Relationships between the expression of eukaryotic translation initiation factor 4E binding protein 1 and the clinical-pathological features in breast cancer tissues

Groups	*n*	Expression of EIF4EBP1		*P* value
		Low expression	High expression	
Age in yr				0.5273
< 50	34	20	14	
≥ 50	37	19	18	
Metastasis of lymph nodes				0.0000
Negative	23	20	3	
1 ≤ N+ ≤ 3	20	12	8	
N+ > 3	28	7	21	
Radiotherapy				0.7274
No	36	20	16	
Yes	35	18	17	
Chemotherapy				0.0005
TAM	52	35	17	
TAM + OFS	19	4	15	
Tumor stage				0.8371
I	17	9	8	
II	44	24	20	
III	9	5	4	
IV	1	1	0	
Histological grade				0.3111
I-II	59	34	25	
III	12	5	7	
Metastasis stage				0.0000
M0	48	35	13	
M1	23	4	19	

EIF4EBP1: Eukaryotic translation initiation factor 4E binding protein 1; OFS: Ovarian function suppression; TAM: Tamoxifen.

protect cancer cells from endocrine therapy drugs[13,14].

EIF4EBP1 directly interacts with a limiting component of the multisubunit complex that recruits 40S ribosomal subunits to the 5′ end of mRNAs. Further studies showed that EIF4EBP1 was upregulated in many cancer types including BC, hepatocellular carcinoma, lung squamous cell carcinoma and glioblastoma[16,28-30], suggesting that EIF4EBP1 plays an important role in tumorigenesis. Ito *et al*[31] showed that EIF4EBP1 was overexpressed and phosphorylated in renal cell carcinoma and was involved in the clinical chemoresistance of renal cell carcinoma cells to mechanistic target of rapamycin complex 1 inhibitors. Tsai *et al*[32] found that EIF4EBP1 could induce glioma stem-like cells through the epidermal growth factor receptor/protein kinase B cascade, making a major contribution to drug resistance. However, the expression and molecular mechanisms of EIF4EBP1 in TAM resistance in BC remained unrevealed.

In this study, we investigated the role of EIF4EBP1 in TAM resistance. Scholars have performed studies on the role of EIF4EBP1 in TAM resistance, and their conclusions of these studies were similar with no controversy. Du *et al*[16] pointed out in a 2020 study that EIF4EBP1 showed significant prognostic value as a prognostic indicator in BC, specifically indicating poor prognosis. A 2019 study reported that EIF4EBP1 was located within the 8p11-p12 genomic locus, frequently highly amplified in BC and predicted poor prognosis and resistance to endocrine therapy. Another study, from 2022, indicated that the addition of EIF4EBP1 to cultures significantly reduced the proliferation and metastasis of TNBC cells[33]. These studies demonstrated that EIF4EBP1 plays an oncogenic role in BC.

However, most of these conclusions are based on bioinformatics analysis. The role of EIF4EBP1 in TAM resistance has not been experimentally demonstrated. This study indicated that EIF4EBP1 enhanced the resistance of T47D-R cells to TAM. EIF4EBP1 is an autophagy-related gene; some studies have demonstrated a role for EIF4EBP1 in autophagy. For example, it has been reported that in CACO-2 cells exposed to cetuximab, EIF4EBP1 expression and autophagosome

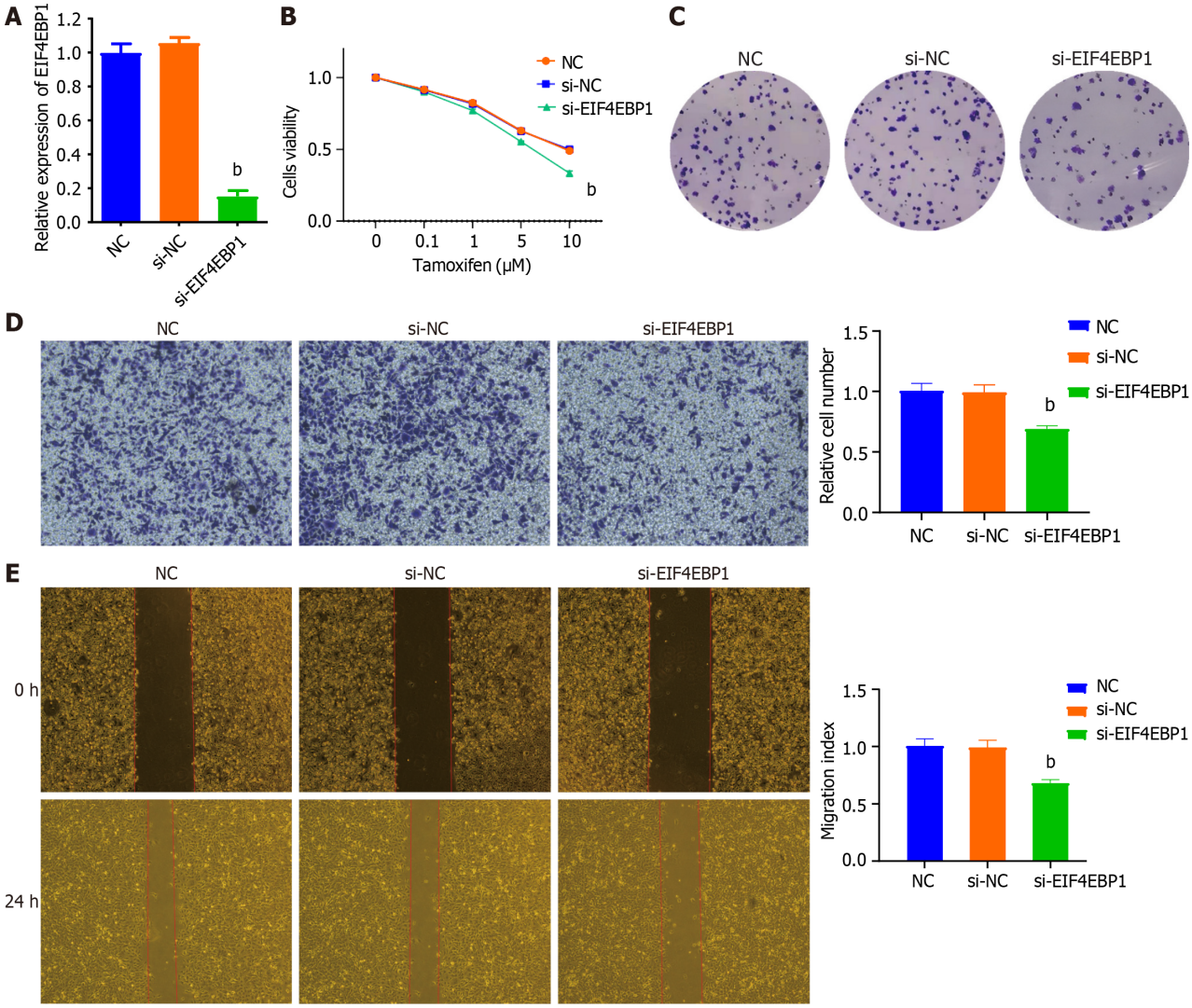

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Figure 3 Knockdown of eukaryotic translation initiation factor 4E binding protein 1 in T47DR cells reduced the resistance to tamoxifen. A: In T47D-R cells that downregulated the expression of eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), the expression of *EIF4EBP1* was detected by real-time reverse transcription PCR; B:Cell Counting Kit-8 assay of EIF4EBP1 knockdown in T47DR cells treated with tamoxifen; C: Colony formation experiments of EIF4EBP1 knockdown in T47DR cells treated with tamoxifen; D: Wound healing assay showed that downregulated expression of EIF4EBP1 can more effectively decrease migration in the T47D-R cell line; E: The Transwell assay showed that downregulated expression of EIF4EBP1 can inhibit the cell invasion in the T47D-R cell line. Data are expressed as the mean ± standard deviation from three independent experiments. $^{b}P < 0.01$. EIF4EBP1: Eukaryotic translation initiation factor 4E binding protein 1; NC: Negative control; si: Silencing RNA.

formation increased, and autophagy increased the efficacy of cetuximab in colorectal cancer[34]. Moreover, Lai *et al*[35] indicated that YXM110 is a new synthetic drugs that exhibits excellent anti-tumor activity in many cancer cells by mediating EIF4EBP1 depletion and regulating autophagy. In this study, we suggested that EIF4EBP1 may increase the resistance of T47D-R cells to TAM by regulating autophagy.

Given the critical role played by EIF4EBP1 in the development of drug resistance and BC, we explored whether EIF4EBP1 is involved in TAM resistance. In this study, the gene expression profiles of TAM-resistant or TAM-sensitive BC cells were reanalyzed. *EIF4EBP1* was overexpressed in TAM-resistant cells, and high expression of *EIF4EBP1* was associated with poor prognosis in BC patients. Based on the clinical specimens from our hospital, we found that high expression of EIF4EBP1 was associated with metastasis and endocrine therapy in BC patients. Moreover, cell experiments suggested that EIF4EBP1 deficiency could reverse TAM resistance, whereas overexpression of EIF4EBP1 increased TAM resistance.

In this study, the potential functions and molecular mechanisms of EIF4EBP1 in TAM-resistant BC cells were identified by GSEA. Notably, the Hh signaling pathway was significantly enriched in the high EIF4EBP1 expression group. It has been reported that Hh signaling pathway is involved in developmental processes in vertebrates and that abnormal activation of this pathway plays an important role in tumorigenesis and maintenance of multiple cancers[36]. Ren *et al*[37] indicated that tumor suppressor candidate 3 may improve the expression of CD133 and ABCC1 by activating the Hh signaling pathway, and inhibition of the Hh signaling pathway could reduce drug resistance of colorectal cancer cells. Zeng *et al*[36] demonstrated that the inhibition of the Hh signaling pathway could induce autophagy in chronic myeloid

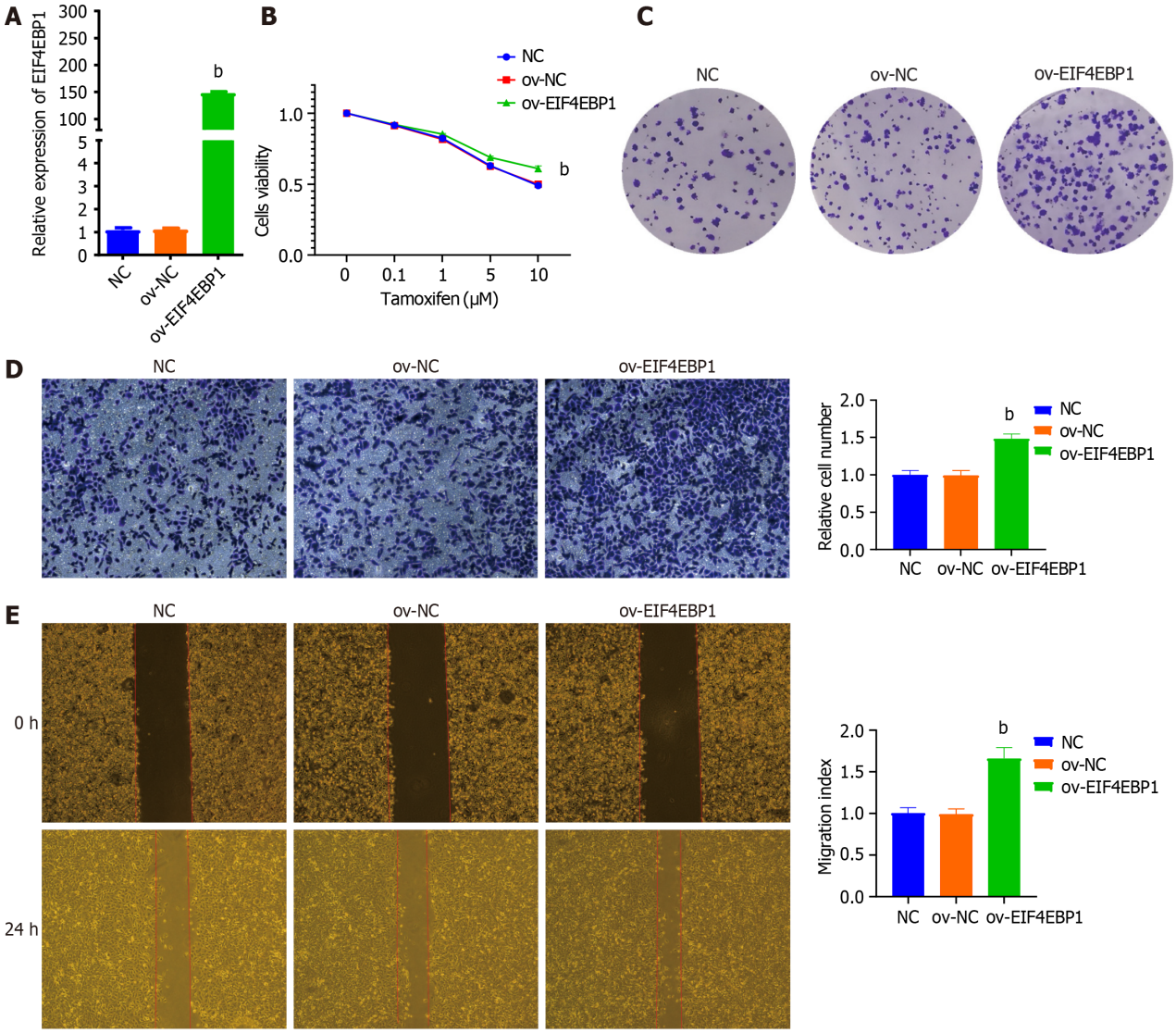

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Figure 4 Overexpression of eukaryotic translation initiation factor 4E binding protein 1 in T47DR cells increased the resistance to tamoxifen. A: In T47D-R cells that upregulated the expression of eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), the expression of *EIF4EBP1* was detected by real-time reverse transcription polymerase chain reaction; B: Cell Counting Kit-8 assay of EIF4EBP1 overexpression in T47D-R cells treated with tamoxifen; C: Colony formation experiments of EIF4EBP1 overexpression in T47D-R cells treated with tamoxifen; D: The wound healing assay showed that upregulated expression of EIF4EBP1 can more effectively increase migration in the T47D-R cell line; E: The Transwell assay showed that upregulated expression of EIF4EBP1 can increase cell invasion in the T47D-R cell line. Data are expressed as the mean ± SD from three independent experiments. $^{b}P < 0.01$. EIF4EBP1: Eukaryotic translation initiation factor 4E binding protein 1; NC: Negative control; ov: Overexpression.

leukemia cells, and inhibiting autophagy and the Hh signaling pathway could reduce cell viability and induce apoptosis of imatinib-resistant chronic myeloid leukemia cells. Thus, we hypothesize that EIF4EBP1 could induce TAM resistance through Hh signaling pathway and autophagy. However, the results need to be examined by further investigations.

We also found that components of the PPAR signaling pathway were significantly enriched in the high EIF4EBP1 expression group. The PPAR signaling pathway has been linked to glucose and lipid metabolic disorders, endothelial function and inflammation. It has been reported that PPAR-γ is upregulated in glioma cells, which could regulate genes associated with apoptosis and multidrug resistance and increase intracellular accumulation of drugs[38]. Moreover, Bräutigam *et al*[39] showed that the death ligand TRAIL (TNF superfamily member 10) could sensitize tumor cells to cytostatic drugs without affecting normal tissues. The combinatorial treatment with PPAR-γ ligands and TRAIL has been shown to synergistically induce apoptosis in ovarian cancer cell lines. Thus, we hypothesize that the PPAR-γ agonists may be promising drugs for targeting drug-resistant cells. Moreover, proteins in the ErbB signaling pathway were significantly enriched in the low EIF4EBP1 expression group. Studies have shown that the abnormal activation of ErbB family members is involved in tumorigenesis and in the escape from anti-tumor immunity in many types of cancers[40].

Song *et al*[41] indicated that the host genes of the identified circular RNAs in platinum-based drug-resistant non-small cell lung cancer cells were involved in the ErbB signaling pathway. Moreover, Macleod *et al*[42] indicated that ErbB receptor signaling was altered in cisplatin-resistant ovarian cancer cells, suggesting that downregulation of the ErbB signaling pathway could play an important role in the development of drug resistance. Thus, we hypothesize that

EIF4EBP1 could induce TAM resistance by regulating the ErbB signaling pathway.

This study had some limitations. First, as with many previous studies[43,44], only T47D cells were used to establish TAM-resistant cell lines. This may make our conclusions less generalizable. Moreover, gene set enrichment analysis was used to explore the potential functions and molecular mechanisms of EIF4EBP1 in TAM-resistant BC cells. However, these pathways have not been verified by *in vitro* and *in vivo* experiments.

CONCLUSION

In conclusion, our study showed that the overexpression of EIF4EBP1 was significantly associated with poor prognosis and metastasis in BC patients. Moreover, EIF4EBP1 plays important roles in the development of TAM resistance. EIF4EBP1 knockdown could reverse TAM resistance, whereas overexpression of EIF4EBP1 increased TAM resistance in BC cells. In addition, our GSEA results may provide new insights into the molecular mechanism of TAM resistance. In brief, EIF4EBP1 could be a marker for the early diagnosis and a therapeutic target for the therapy of TAM resistance.

ARTICLE HIGHLIGHTS

Research background

Tamoxifen (TAM) resistance is a major obstacle in the treatment of breast cancer (BC) patients. It has been reported that eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) plays critical roles in the tumorigenesis and development of BC.

Research motivation

TAM resistance remains one of the major causes of BC mortality today. Therefore, it is necessary to identify biomarkers and therapeutic targets and understand molecular mechanisms of TAM resistance to help patients.

Research objectives

The objective was to investigate the expression and functions of EIF4EBP1 in the efficacy of TAM therapy in BC patients.

Research methods

Gene Set Enrichment Analysis (GSEA) was performed to explore the biological functions and related pathways of EIF4EBP1. Real-time reverse transcription polymerase chain reaction were employed to explore the expression of *EIF4EBP1* in TAM-resistant and TAM-sensitive BC cell lines. Cell count kit-8 assay, colony formation experiments and the wound healing assay were used to understand the phenotypes of loss- and gain-of-function of EIF4EBP1 in a TAM-resistant cell line.

Research results

EIF4EBP1 was upregulated in TAM resistant cells, and EIF4EBP1 was associated with the prognosis of BC patients. GSEA suggested that EIF4EBP1 may be involved in the Hedgehog signaling pathway. Reducing the expression of EIF4EBP1 can reverse TAM resistance, while overexpression of EIF4EBP2 can promote TAM resistance.

Research conclusions

In this study, we investigated the role of EIF4EBP1 in TAM resistance. Scholars have performed studies on the role of EIF4EBP1 in TAM resistance, and their conclusions of these studies were similar with no controversy. However, most of these conclusions were based on bioinformatics analysis. The role of EIF4EBP1 in TAM resistance has not been experimentally demonstrated. This study indicated that EIF4EBP1 enhanced the resistance of T47D-R cells to TAM. In addition, our GSEA results may provide new insights into the molecular mechanism of TAM resistance. In brief, EIF4EBP1 could be a marker for the early diagnosis and a therapeutic target for the therapy of TAM resistance.

Research perspectives

Further studies should explore the potential function and molecular mechanism of EIF4EBP1 in TAM-resistant BC cells through *in vitro* and *in vivo* experiments.

FOOTNOTES

Author contributions: Li SN and Yang S contributed to conception, design and writing of the manuscript; Hui TL, Mi YZ, Zhang X and Wang HQ performed the research; Cheng M, Gao W, Geng CZ and Li SN contributed to analysis and interpretation of data; and all authors read and approved the final manuscript.

Institutional review board statement: The study protocol was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Approval No. 2022KY396).

Clinical trial registration statement: Human tissues were used for immunohistochemistry and real-time reverse transcription polymerase chain reaction to observe the expression of EIF4EBP1. All participants signed informed consent, but it did not interfere with the normal treatment activities of participants. Therefore, it does not belong to the scope of clinical trial registration, and the approval of clinical trial registration cannot be provided.

Informed consent statement: Informed consent was obtained from all patients at the time of sample collection.

Conflict-of-interest statement: All the authors report having no relevant conflicts of interest for this article.

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