BMC Cancer

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β2-microglobulin induced apoptosis of tumor cells via the ERK signaling pathway by directly interacting with HFE in HER2-overexpressing breast cancer

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Abstract

Background Our previous study demonstrated that β2-microglobulin (β2M) promoted $ER^+/HER2^-$ breast cancer survival via the SGK1/Bcl-2 signaling pathway. However, the role of β2M has not been investigated in $ER^-/HER2^+$ breast cancer. Here, we aimed to determine the role of β2M in $ER^-/HER2^+$ breast cancer.

Methods The interaction between β2M and HFE was confirmed by co-immunoprecipitation, mass spectrometry, yeast two-hybrid screening, and His pull-down. The knockdown and overexpression of β2M or HFE were performed in MDA-MB-453 cells, and ERK signaling pathway was subsequently analyzed via western blotting. Apoptotic cells were detected using flow cytometer. β2M, HFE, and p-ERK1/2 were examined in tumor and paired adjacent tissues via immunohistochemistry.

Results HFE was found to be an interacting protein of β2M in $ER^-/HER2^+$ breast cancer cells MDA-MB-453 by co-immunoprecipitation and mass spectrometry. A yeast two-hybrid system and His-pull down experiments verified that β2M directly interacted with HFE. β2M and HFE as a complex were mainly located in the cytoplasm, with some on the cytomembrane of MDA-MB-453 cells. In addition to breast cancer cells BT474, endogenous β2M directly interacted with HFE in breast cancer cells MDA-MB-453, MDA-MB-231, and MCF-7. β2M activated the ERK signaling pathway by interacting with HFE and induced apoptosis of MDA-MB-453 cells. The expression of HFE and p-ERK1/2 showed significantly high levels in HER2-overexpressing breast cancer tumor tissue compared with adjacent normal tissue, consistent with the results obtained from the cell experiments.

Conclusions β2M induced apoptosis of tumor cells via activation of the ERK signal pathway by directly interacting with HFE in HER2-overexpressing breast cancer.

Keywords β2M, Protein–protein interaction, Apoptosis, ERK signaling pathway, HER2-overexpressing breast cancer

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Background

β2-microglobulin (β2M) is a nonglycosylated protein with molecular weight of 12-kDa. It is part of the human major histocompatibility complex (MHC) class I molecules on the cell surface, acting as a light chain subunit of MHC class I molecules via non-covalent bonds. As an important structural protein, the key functions of β2M involve interaction with MHC class I molecules to stabilize the tertiary structure of the antigenic peptide presented to cytotoxic T lymphocytes to regulate $CD8^+$ T lymphocytes in host immune recognition of self- and non-self-antigens, and in immunoglobulin transport and iron metabolism [1–4]. Elevated levels of β2M were observed in cancer patients [5, 6]. Alterations of β2M genes and proteins lead to poor reaction to cancer immunotherapies by inhibiting antigen presentation [7]. In addition to the roles of β2M in immunity, other β2M functions with clinical relevance have been demonstrated, particularly the regulation of survival, proliferation, apoptosis, and metastasis of cancer cells [8, 9]. Nomura and others have a series of reports demonstrating that β2M is a growth stimulating factor and signaling molecule in several cancer cells [10–13]. Studies demonstrated that β2M played multiple roles in mediating tumorigenesis, angiogenesis, and osteomimicry, and that the hemochromatosis (HFE) protein interacted with β2M [10, 11, 14]. Existing data regarding the roles of β2M showed that β2M has two different functions, either tumor-promoting or suppressing, and the ability of β2M to act either as a positive or negative cell growth regulator is cancer cell context-dependent [15]. In summary, β2M acts as a growth-stimulating, angiogenesis-stimulating, epithelial-mesenchymal transition (EMT)-promoting, and bone metastasis-stimulating factor and a prognostic indicator, to promote cancer cell survival and metastasis in various solid tumor malignancies; however, β2M was also found to have a role as an apoptosis-inducible factor in hematological malignancies [15]. Kim et al. [16] reported that β2M promoted tumor growth via activation of the vascular endothelial growth factor receptor-2 (VEGFR-2)/Akt/mammalian target of rapamycin (mTOR) signaling pathway, and β2M was identified as a dickkopf-related protein 3 (DKK3) interacting protein. However, the mechanisms through which β2M promotes tumor cell survival and metastasis in cancer appear complicated, and its function is context dependent. Breast cancer is a remarkably heterogeneous malignant tumor, which is subtyped as four primary types based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) proteins: luminal A, luminal B, HER2-overexpressed, and basal-like [17]. Therefore, we speculated that the role of β2M in breast cancer may be associated with breast cancer heterogeneity, mediating diverse signaling pathways, and acting through disparate functions in different types of breast cancer. Our previous study demonstrated that β2M protein expression had significant differences in different types of breast cancer tissue, and negatively correlated with ER expression but did not correlate with HER2 expression. Furthermore, abnormal expression of β2M had different regulatory effects on $ER^+/HER2^-$ (MCF-7) and $ER^-/HER2^-$ (MDA-MB-231) breast cancer cells [18]. Another study of ours demonstrated that β2M promoted tumor survival via the serum and glucocorticoid-regulated kinase 1 (SGK1)/B-cell lymphoma 2 (Bcl-2) signaling pathway in $HER2^-/ER^+$ breast cancer and had no effect on $HER2^-/ER^-$ breast cancer [19]. A previous study demonstrated that β2M increased the sensitivity of the breast cancer cell line MCF-7 to doxorubicin, and that decreased β2M expression by antisense RNA made the cells resistant to doxorubicin [20]. However, it is unknown whether β2M has disparate roles affecting the progression and metastasis of tumors, or what its mechanism is in different types of breast cancer. In this study, we investigated the molecular mechanism of β2M affecting tumor progression in MDA-MB-453 breast cancer cells and the role of β2M in HER2-overexpressing breast cancer.

Methods

Cell lines, cell culture, antibodies, and tissue samples

The human breast cancer cell lines MCF-7 ($ER^+/HER2^-$) and MDA-MB-231 ($ER^-/HER2^-$) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-453 ($ER^-/HER2^+$) and BT474 ($ER^+/HER2^+$) cells were gifted by Prof. Xiao Wei from Capital Normal University. HEK-293T cells were gifted by Prof. Shengxiang Zhang from Lanzhou University. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Thermo Fisher Scientific, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Minhai Biotechnology, Beijing, China) at 37°C and 5% CO_2. The primary antibodies used in this study were anti-β2M specific monoclonal antibody (prepared and identified by our laboratory), anti-extracellular signal-regulated kinase 1 and 2 (ERK1/2), anti-phospho-ERK1/2 (Thr202/Tyr204; p-ERK1/2), anti-HFE, anti-calnexin (CANX), anti-calreticulin (CALR), anti-protein disulfide isomerase family A member 3 (PDIA3), anti-green fluorescent protein (GFP), anti-HA (Santa Cruz Biotechnology), and anti-β-actin (Beijing Bioss Biotechnology). Formalin-fixed, paraffin-embedded tumor tissues (30 cases) and adjacent tissues (30 cases), which were obtained from patients who were diagnosed with breast cancer based on clinical and histopathological evidence and underwent surgery at the Tumor Hospital

of Gansu Province, were provided by the Department of Pathology.

Western blotting (WB) analysis

Proteins were extracted using a Tissue or Cell Total Protein Extraction kit (Sangon Biotech, Shanghai, China). Approximately 40 μg of protein per lane were separated on 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked using tris-buffered saline with 0.1% Tween 20 (TBST) with 5% nonfat dry milk for 1 h, and the membranes were incubated with appropriate antibodies overnight at 4°C. After washing with TBST, the membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Shanghai, China). Reactive bands were visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Shanghai, China) and recorded with a ChemiDoc™ XRS+ imaging system. Signal intensities were quantified using Image Lab quantification software (Bio-Rad). β-actin was used as an internal control.

Co-immunoprecipitation (Co-IP) assay

To perform Co-IP**,** all cells were washed in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) and dissolved in cell lysis buffer (Shanghai Sangon Biotechnology, China). Lysates were incubated with anti-β2M and precipitated with protein A/G-agarose (Santa Cruz Biotechnology) [21]. The precipitated proteins were analyzed by WB.

Mass spectrometry (MS)

The authenticated band of the Co-IP was cut from the SDS-PAGE gel and destained in a 50 mM NH_4HCO_3 and 50% acetonitrile solution. Following dehydration in 100 μL of 100% acetonitrile for 5 min, the sample was hydrated in 10 mM dithiothreitol and incubated at 55°C for 60 min, washed using 50 mM NH_4HCO_3, hydrated in 55 mM iodoacetamide, and incubated in the dark at room temperature for 45 min. The gel was resuspended in 10 ng/μL trypsin and hydrated in 50 mM NH_4HCO_3 for 60 min, and digested at 37°C overnight. The digested peptides were completely dried and resuspended in 20% acetonitrile and 0.1% formic acid. The 0.1% formic acid peptides solution was separated on a reversed-phase column, and analyzed by liquid phase mass spectrometry (PTM Biolabs, Hangzhou, China). Functional annotation and protein–protein interaction (PPI) analysis was performed using bioinformatics analysis.

Yeast two-hybrid (Y2H) screening and protein–protein interaction assay

The cDNA encoding full-length human β2M was cloned into *Bam*H I and *Nde* I restriction enzyme sites of the pGBKT7 yeast plasmid vector. The cDNA encoding full-length HFE was cloned into the same restriction enzyme sites of the pGADT7 yeast plasmid vector. The pGBKT7 and pGBKT7-β2M plasmid were transformed into yeast cells Y2HGold, and cultured in SD/-Try medium and SD/-Trp/-His/-Ade/-Leu medium to detect toxicity and self-activity of the plasmids in the yeast cells. The pGADT7-HFE plasmid was transformed into yeast cells that already contained the bait plasmid pGBKT7-β2M, and cultured on double (SD/-Leu/-Trp) and quadruple deficiency solid medium (SD/-Trp/-Ade/-His/-Leu). Blue colonies were the marker of binding activity between HFE and β2M. The plasmids pGADT7 + pGBKT7-β2M/pGADT7-HFE + pGBKT7 were negative controls, and pGADT7-T + pGBKT7-53 was a positive control.

Protein–protein interaction verification in HEK-293T cells

Eukaryotic expression vectors pEGFP-C1-β2M and pcDNA4/TO-HA-HFE were constructed and transfected into HEK-293T cells, and the interaction of β2M and HFE was measured by Co-IP using anti-GFP antibody.

His pull-down

The cDNA encoding full-length human *β2M* was cloned into the *EcoR* I and *XHo* I restriction site of the prokaryotic expression plasmid pET-28a, and plasmid pET-28a-β2M were transformed into *E. coli* cells BL21 (DE3). The BL21 (DE3) cells with pET-28a-β2M plasmid were cultured in LB medium and induced by 1 mM IPTG to express the fusion proteins His-β2M. To purify the His-β2M protein, the pellet was resuspended in 10 mL of 2 M urea and centrifugated at 10,000 rpm at 4°C for 20 min. The supernatant was discarded and the pellet was dissolved in 6 mL of 8 M Urea. To obtain active proteins, the denatured recombinant protein was refolded in 50 mM Tris (pH 8.5) buffers containing 150 mM NaCl and decreasing concentrations of urea, from 8 to 3 M. The refolded protein was purified by gel filtration chromatography using Sephacryl S-300 (Pharmacia, USA). To perform His pull-down, 100 μL of purified recombination His-β2M and 300 μL of total protein containing HA-HFE obtained from HEK-293T cells were added to Ni–NTA Agarose (QIAGEN, Germany) and incubated for 4 h at 4°C. The total protein from HEK-293T cells without HA-HFE expression was used as a control. The Ni–NTA Agarose were centrifuged at 2000 rpm for 5 min, and the precipitates were washed five times using washing buffer (10 mM PBS, pH 7.4) at 2000 rpm for 5 min. Then, the

precipitates were eluted five times using 10 mM PBS (pH 7.4) buffer containing 100 mM imidazole at 2000 rpm for 5 min. The buffer used for washing and the eluates were analyzed by WB using anti-β2M and anti-HA antibodies.

Overexpression of HFE or β2M in MDA-MB-453 cells

The cDNA encoding full-length human HFE or β2M was cloned into a mammalian expression plasmid to construct HFE or β2M expression vector. The HFE expression vector or β2M expression vector was transfected into MDA-MB-453 cells. Transfection was performed using a Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's protocols. Briefly, cells grown on six-well plates were transfected using 40 nM siRNA and 7.5 μL Lipofectamine RNAiMAX per well, and the medium was changed after 6 h. At approximately 48 h post-transfection, the cells were lysed and analyzed using WB analysis.

Silencing of the *β2M* gene or *HFE* gene by siRNA in MDA-MB-453 cells

Three siRNAs targeting different regions of *β2M* mRNA or *HFE* mRNA were designed and purchased from GenePharma (Suzhou, China). Scrambled siRNA (GenePharma) that did not target any gene was used as the control. All siRNAs are detailed in Table 1. siRNA transfection was performed using a Lipofectamine RNAiMAX reagent according to the manufacturer's protocols, and the cells were lysed and analyzed using WB analysis.

Apoptosis detection

Cells were plated in 6-well culture plates (1×10^6 cells/well), and 48 h after transfection were trypsinized and collected. Three replicate wells were set up for each set of experiments. The supernatant was discarded after centrifugation, and the cells were resuspended in 1 mL of PBS (0.01 M, pH 7.4) and then centrifuged and resuspended again. Next, 200 μL of Annexin V binding buffer and 5 μL FITC Annexin V were added to each tube. The reaction was mixed and then protected from light for 15 min. Propidium iodide was added to the cells (5 μL/tube) and incubated for 5 min. Apoptotic cells were detected using a BD FACSVerse flow cytometer within 1 h. A FITC Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, US) was used for the apoptosis detection.

Table 1 Sequences of siRNAs

Name	Sequence
siR-β2M	Sense, 5′-CCGACAUUGAAGUUGACUUTT-3′
	Antisense, 5′-AAGUCAACUUCAAUGUCGGTT-3′
siR-HFE	Sense, 5′-CCCUAGUCAUUGGAGUCAUTT-3′
	Antisense, 5′-AUGACUCCAAUGACUAGGGTT-3′
siR-NC	Sense, 5′- UUCUCCGAACGUGUCACGUTT-3′
	Antisense, 5′-ACGUGACACGUUCGGAGAATT -3′

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue specimens were obtained and handled by standard surgical oncology procedures. Serial 4-μm sections were prepared and stained using a biotin-streptavidin HRP detection systems (ZSGB-BIO, Beijing, China) with the primary antibodies. Immune complexes were visualized using a 3,3′-diaminobenzidine tetrahydrochloride substrate solution (ZSGB-BIO). The slides were then counterstained with hematoxylin and mounted. A negative control was prepared by replacing the primary antibody with PBS. The mean percentage of positive tumor cells was determined in at least five areas at × 400 magnification and assigned to one of the following five categories: (a) 0, < 5%; (b) 1, 5–25%; (c) 2, 25–50%; (d) 3, 50–75%; and (e) 4, > 75%. The intensity of immunostaining was scored as follows: (a) weak, 1 + ; (b) moderate, 2 + ; and (c) intense, 3 + . The percentage of positive tumor cells and the staining intensity were added to generate a weighted score for each case. Cases with weighted scores less than three were defined as negative, otherwise they were defined as positive.

Statistical analysis

All experiments were repeated at least three times, and statistical comparisons were performed using SPSS version 23.0. Data involving the silencing and overexpression experiments were presented as the mean ± SD and analyzed via two-tailed Student's t test. All data were analyzed for normality, and two-tailed Student's t test or nonparametric test were applied. All tests with $p < 0.05$ were statistically significant; $^{*}p < 0.05$, and $^{**}p < 0.01$.

Results

HFE interacts with β2M in the breast cancer cell line MDA-MB-453

To identify the interacting protein of β2M in MDA-MB-453 breast cancer cells, we used Co-IP and MS. The anti-β2M monoclonal antibody (prepared by our laboratory) suitable for WB was chosen from seven potential monoclonal antibodies using MDA-MB-453 whole cell lysates (WCL). Only the 4G3 antibody was suitable for WB analysis (Fig. 1A). To further determine whether 4G3 was suitable for Co-IP, we precipitated β2M using the antibody in MDA-MB-453 and MCF-7 cells (Fig. 1B), and the SDS-PAGE results of the precipitated proteins from Co-IP presented several different bands (Fig. 1C).

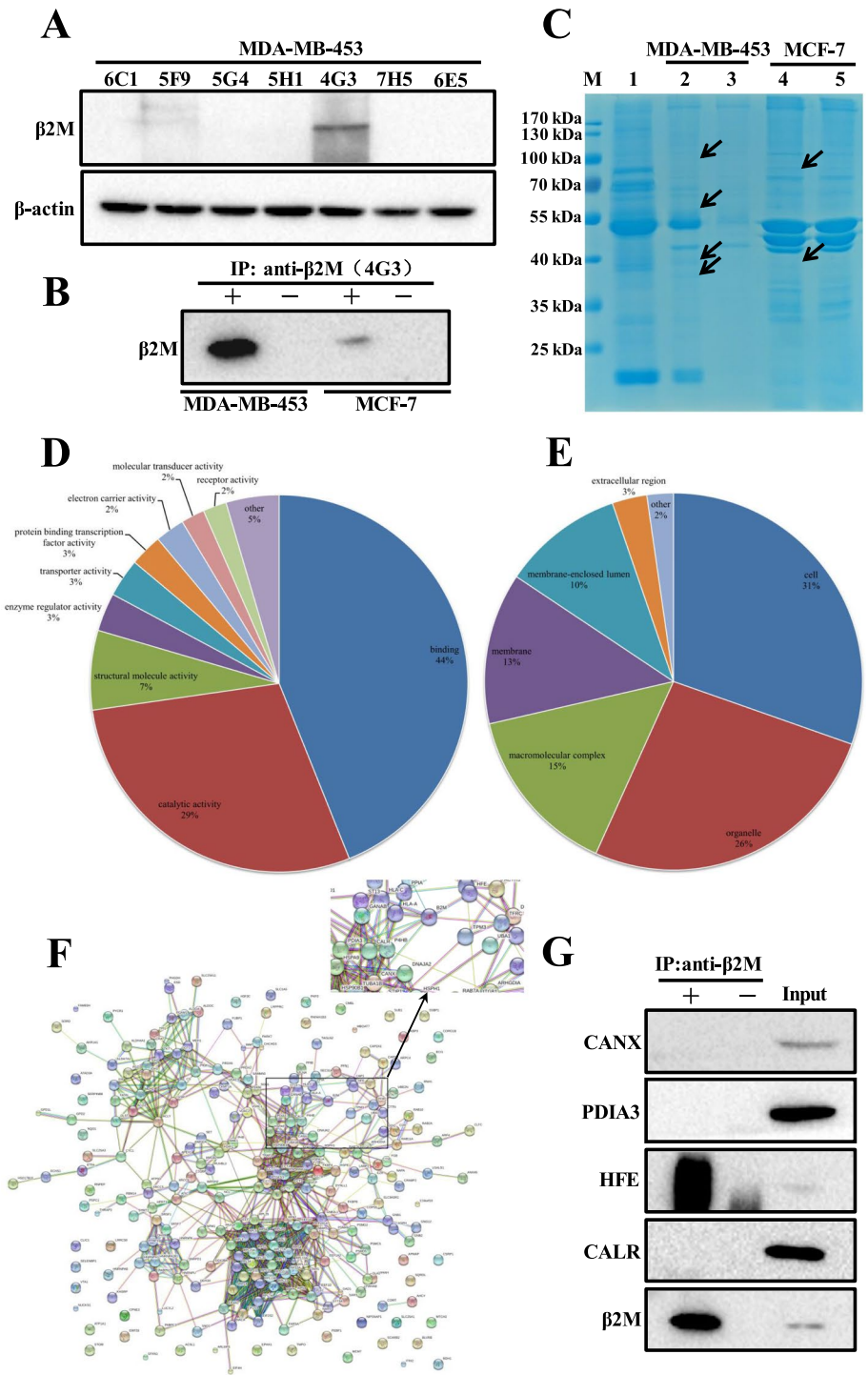

Fig. 1 Interaction of β2M with HFE protein. **A** The anti-β2M monoclonal antibody 4G3 was chosen from seven monoclonal antibodies by WB in MDA-MB-453 breast cancer cells. **B** β2M was precipitated by Co-IP using 4G3 antibody in MDA-MB-453 and MCF-7 breast cancer cells. **C** SDS-PAGE results show that the precipitated proteins from Co-IP presented several different bands. **D** Molecular function of the differentially expressed proteins. **E** The subcellular localization analysis of the differentially expressed proteins. **F** Protein–protein interaction analysis. **G** Co-IP verification of the interacting proteins

The mass spectrometry results of the precipitated proteins showed that there were 231 differentially expressed proteins, which were mainly involved in "binding", "catalytic", "structural molecular", "enzyme regulator", "transporter", "protein binding transcription factor", "electron carrier", "molecular transducer", and "receptor" functions (Fig. 1D). The subcellular location analysis revealed that differentially expressed proteins were mainly located in the cytoplasm, membrane, organelles, membrane-enclosed lumen, and so forth (Fig. 1E). PPI network analysis showed that β2M was well connected with multiple proteins, including HFE, CANX, PDIA3, and CALR (Fig. 1F). To verify the protein interactions, we performed a Co-IP and WB using β2M, HFE, CANX, PDIA3, and CALR antibodies with MDA-MB-453 breast cancer cell lysate. The WB results demonstrated that HFE, CANX, PDIA3, and CALR were expressed in MDA-MB-453 breast cancer cells, but only HFE was co-immunoprecipitated by β2M antibodies (Fig. 1G). In conclusion, these results strongly indicated that HFE is an interacting protein of β2M in MDA-MB-453 breast cancer cells.

β2M directly interacts with HFE in MDA-MB-453 breast cancer cells

To investigate whether β2M directly interacted with HFE in MDA-MB-453 cells, we used a Y2H system. The bait plasmid vector pGBKT7-β2M and prey vector pGADT7-HFE were successfully constructed, and their sequencing results confirmed the gene sequence of β2M and HFE (Supplementary Fig. S1A–D). To examine the toxicity and auto activation of pGBKT7-β2M, vectors were transformed into yeast cells Y2HGold and cultured in tryptophan-deficient solid medium (SD/-Trp), and the same volume of colonies appeared (Supplementary Fig. S2A), suggesting that pGBKT7-β2M did not cause toxicity to the yeast cells, and did not interfere with the growth of the yeast cells. Moreover, the colonies did not appear on tryptophan-, leucine-, histidine-, and adenine-deficient solid medium (SD/-Trp/-His/-Ade/-Leu), suggesting that pGBKT7-β2M vector had no autoactivation (Supplementary Fig. S2B). Vectors were transferred to yeast cells by co-transformation according to the following groups: empty control (pGADT7 + pGBKT7), experimental group (pGBKT7-β2M + pGADT7-HFE), two negative controls (pGADT7 + pGBKT7-β2M and pGADT7-HFE + pGBKT7), and positive control (pGADT7-T + pGBKT7-53). Cells were cultured on double (SD/-Leu/-Trp) or quadruple deficient solid mediums (SD/-Trp/-Ade/-His/-Leu) with or without the antibiotic aureobasidin A (AbA) and X-α-Gal (SD/-Trp/-Ade/-His/-Leu/A/X). Positive colonies (blue) grew only on SD/-Trp/-Ade/-His/-Leu/A/X agar plates of the experimental group and positive control, indicating that β2M directly interacted with HFE (Fig. 2A). To eliminate the false positive results, we performed Co-IP in HEK-293 T cells by co-transfecting pcDNA4/TO-HA-HFE and pEGFP-C1-β2M. The results of Co-IP using anti-GFP antibodies showed that GFP antibodies co-immunoprecipitated with exogenously expressed HFE (Fig. 2B), suggesting that exogenously expressed β2M interacted with HFE. To further verify the interaction of β2M with HFE, we performed His pull-down assay in vitro by expressing His-β2M in *E. coli* cells and expressing HA-HFE in HEK-293T cells, and the parental HEK-293T was used as the negative control. The results of WB verified that HA-HFE was pulled down by His-β2M (Fig. 2C, D), suggesting that β2M directly interacted with HFE in vitro. Collectively, these results strongly indicated that β2M directly interacted with HFE in the cellular conditions of MDA-MB-453 cells.

β2M/HFE complex is primarily located in the cytoplasm and secondarily located on the cytomembrane of breast cancer cells

To ascertain the cellular location of β2M/HFE in breast cancer cells, MDA-MB-453 breast cancer cells were stained by green fluorescent-labeled anti-β2M antibody and red fluorescent-labeled anti-HFE antibody, and observed using a confocal laser scanning microscope (CLSM). Most red and green regions appeared in the cytoplasm, with some on the cytomembrane. The yellow overlapped regions mainly appeared in the cytoplasm, with some appearing on the cytomembrane (Fig. 3A). These results suggest that β2M and HFE as a complex were mainly located in the cytoplasm, with some on the cytomembrane of the breast cancer cells.

Endogenous β2M directly interacts with HFE in MDA-MB-453, MDA-MB-231, and MCF-7 cells, but not in BT474 cells

To investigate whether β2M interacted with HFE in other types of breast cancer cells, we synchronously performed Co-IP in four types of breast cell lines, including MDA-MB-453 ($ER^-/PR^-/HER^+$), MDA-MB-231 ($ER^-/PR^-/HER^-$), BT474 ($ER^+/PR^+/HER^+$), and MCF-7 ($ER^-/PR^-/HER^+$) in vitro. As shown in Fig. 3B, β2M antibody co-immunoprecipitated HFE from MDA-MB-453, MDA-MB-231, and MCF-7 cells. However, the β2M antibody could not immunoprecipitate HFE from BT474 cells, which may be partly due to undetectable levels of HFE (Supplementary Fig. S3).

Abnormal expression of HFE can affect the activation of the ERK signaling pathway, but β2M can not, in MDA-MB-453 cells

Our previous study confirmed that β2M promoted tumor survival via the SGK1/Bcl-2 signaling pathway in MCF-7 cells and had no effect on MDA-MB-231 cells [19]; however, the effect of β2M on HER^+ breast cancer cells was unclear. Here, we investigated the effects and mechanism

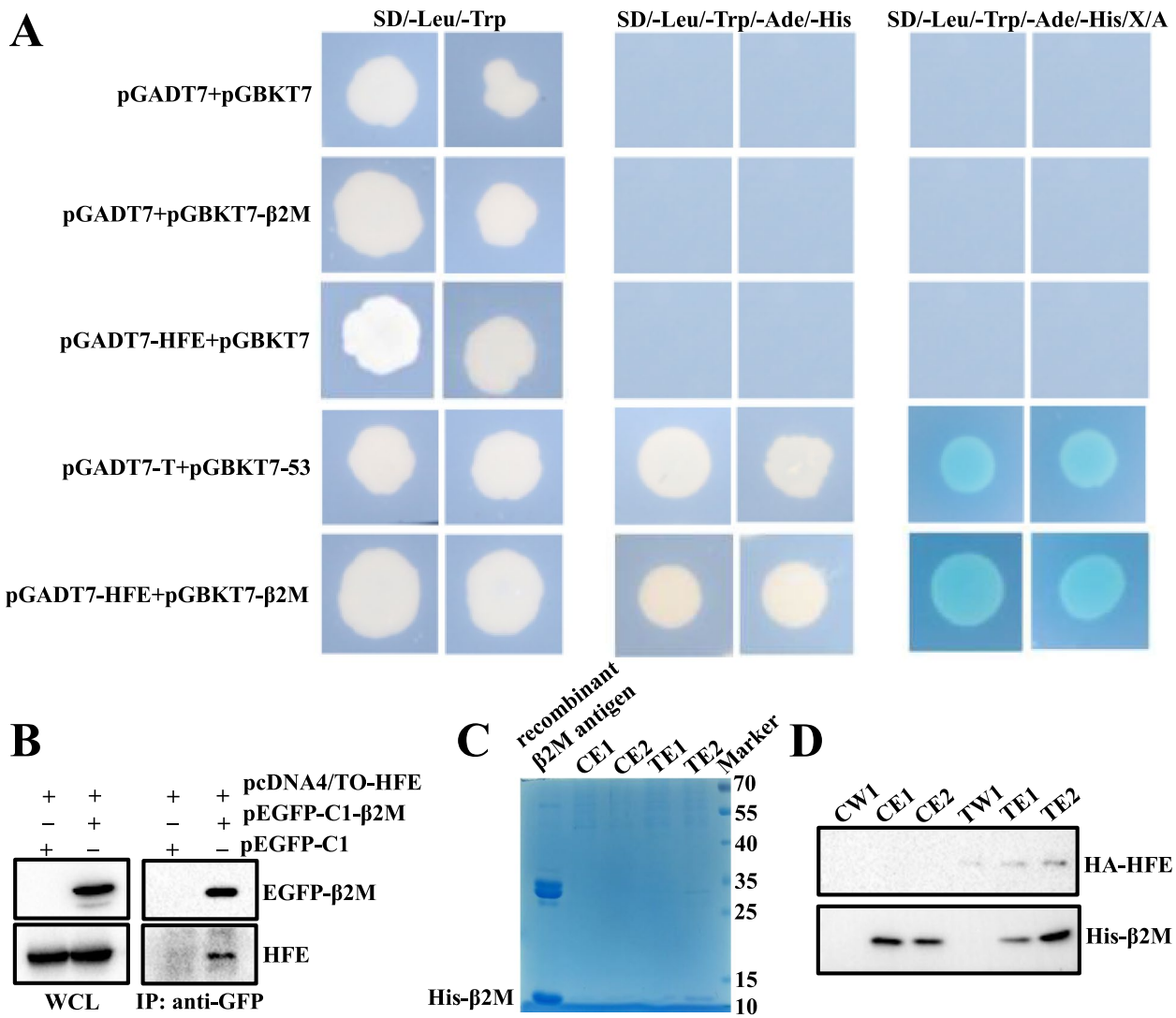

Fig. 2 Verification of β2M directly interacting with HFE. **A** Verification of the yeast two-hybrid system (Y2H) in vivo. Blue colonies grew only on SD/-Trp/-Ade/-His/-Leu/A/X agar plates of the experimental group and positive control. **B** GFP antibodies co-immunoprecipitated exogenously expressed HFE. **C**, **D** His pull-down assay of the β2M and HFE complex. His-β2M expressed in *E. coli* and HA-HFE expressed in HEK-293T were pulled down, and analyzed by Coomassie blue staining (**C**) and WB (**D**). Parental HEK-293T was used as a negative control. CE1 and CE2, the eluate samples of the negative control by different concentrations of imidazole. TE1 and TE2, the eluate samples of the experimental group by different concentrations of imidazole. CW, the washing sample of the negative control by washing buffer. TW, the washing sample of the experimental group by washing buffer

of β2M on HER^+ breast cancer cells. Since β2M was expressed at relatively high levels and HFE was expressed at low levels in MDA-MB-453 cells (Supplementary Fig. S3), we performed genetic manipulation of *β2M* or *HFE* in MDA-MB-453 cells. As shown in Fig. 4A–D, HFE overexpression significantly increased p-ERK/ERK levels, and silencing the *β2M* gene did not rescue p-ERK/ERK levels induced by HFE overexpression, and silencing the *HFE* gene significantly decreased p-ERK/ERK levels, suggesting that abnormal expression of HFE can affect the activation of the ERK signaling pathway in MDA-MB-453 cells. However, β2M overexpression and silencing the *β2M* gene did not significantly increase or decrease p-ERK/ERK levels (Fig. 4E, F). These results suggest that β2M can not activate the ERK signaling pathway independently in MDA-MB-453 cells.

β2M and HFE interaction induced tumor cell apoptosis in MDA-MB-453 cells

To investigate the function of β2M and HFE interaction in breast cancer cell apoptosis, we detected the apoptosis rate of silencing the *β2M* gene, HFE overexpressed,

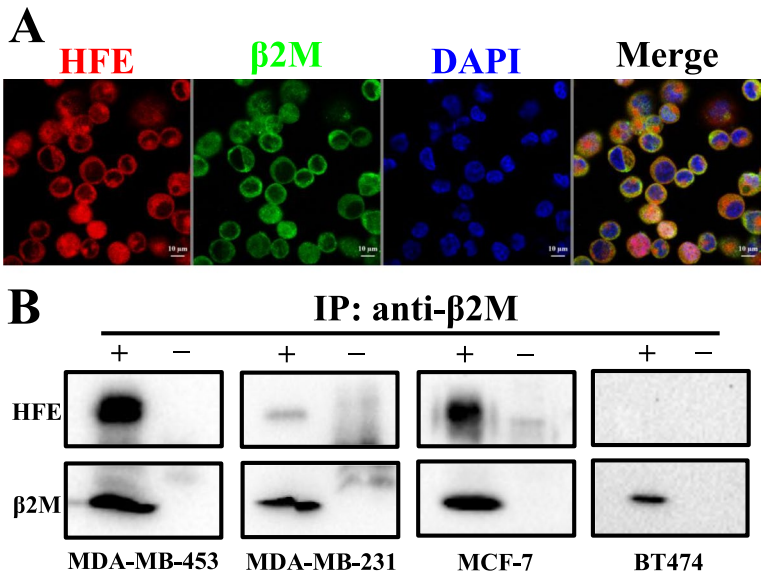

Fig. 3 The localization of the β2M/HFE complex and interaction of endogenous β2M with HFE. **A** MDA-MB-453 cells were stained by green-fluorescence labeled anti-β2M antibody and red-fluorescence labeled anti-HFE antibody. β2M and HFE fluorescence staining shows that most yellow regions (red and green overlap) appear in the cytoplasm, and some on the cytomembrane. **B** Co-IP was synchronously performed using β2M antibody in four types of breast cell lines. β2M antibody co-immunoprecipitated HFE from MDA-MB-453, MDA-MB-231, and MCF-7 cells, but not from BT474 cells

and *β2M* siRNA and pcDNA4/TO-HFE co-transfected MDA-MB-453 cells by flow cytometry. HFE overexpression significantly induced tumor cell apoptosis (Fig. 5A), and silencing the *β2M* gene reversed apoptosis induction of tumor cells mediated by HFE overexpression (Fig. 5B). However, silencing the *β2M* gene without HFE overexpression did not affect tumor cell apoptosis (Fig. 5C). This result suggests that β2M and HFE interaction induced apoptosis of MDA-MB-453 cells.

Expression levels of HFE and p-ERK1/2 proteins are significantly different between tumor tissues and adjacent normal tissues of patients with HER2-overexpressing breast cancer

Our previous study demonstrated that β2M protein expression had 84.61% positive rate in HER2-overexpressing breast cancer tissue [18]. Therefore, to verify the molecular mechanism of β2M in human $HER2^+$ breast cancer, we investigated the protein expression of β2M, HFE, and p-ERK1/2 in tumor tissue and adjacent normal tissue of patients with $HER2^+$ breast cancer (HER2-overexpressing) by IHC. Figure 6A–D shows the representative images of immunostaining of HER2-overexpressing breast cancer tissues. The expression levels of HFE and p-ERK1/2 were significantly higher in the tumor tissues compared with the adjacent tissues ($p < 0.05$) (Fig. 6E). These results were consistent with the results obtained from the experiments on cells, and strongly indicate that β2M and HFE are both involved in tumor progression of HER2-overexpressing breast cancer via activation of the ERK signaling pathway.

Discussion

Existing experimental studies have shown that β2M is a prognostic factor in tumor growth, angiogenesis, EMT, and bone metastasis in various solid malignancies [15]. Our previous studies have demonstrated that β2M expression varied significantly in the four different

(See figure on next page.)

Fig. 4 HFE activates ERK signaling pathways in MDA-MB-453 cells. **A** HFE overexpression significantly increased levels of p-ERK/ERK in MDA-MB-453 cells. **B** Silencing of the *β2M* gene did not reverse the increased p-ERK/ERK induced by HFE overexpression in MDA-MB-453 cells. **C** Both β2M and HFE overexpression significantly increased levels of p-ERK/ERK in MDA-MB-453 cells. **D** Silencing of the *HFE* gene significantly decrease p-ERK/ERK levels in MDA-MB-453 cells. **E** β2M overexpression did not significantly increase p-ERK/ERK levels in MDA-MB-453 cells. **F** Silencing of the *β2M* gene did not significantly decrease p-ERK/ERK levels in MDA-MB-453 cells. The upper panels in A–F show the representative immunoblots images, and the lower panels in A–F show the relative protein signal intensity which was quantitatively analyzed in the immunoblots (upper panels) using Image Lab software and shown as the bar graphs

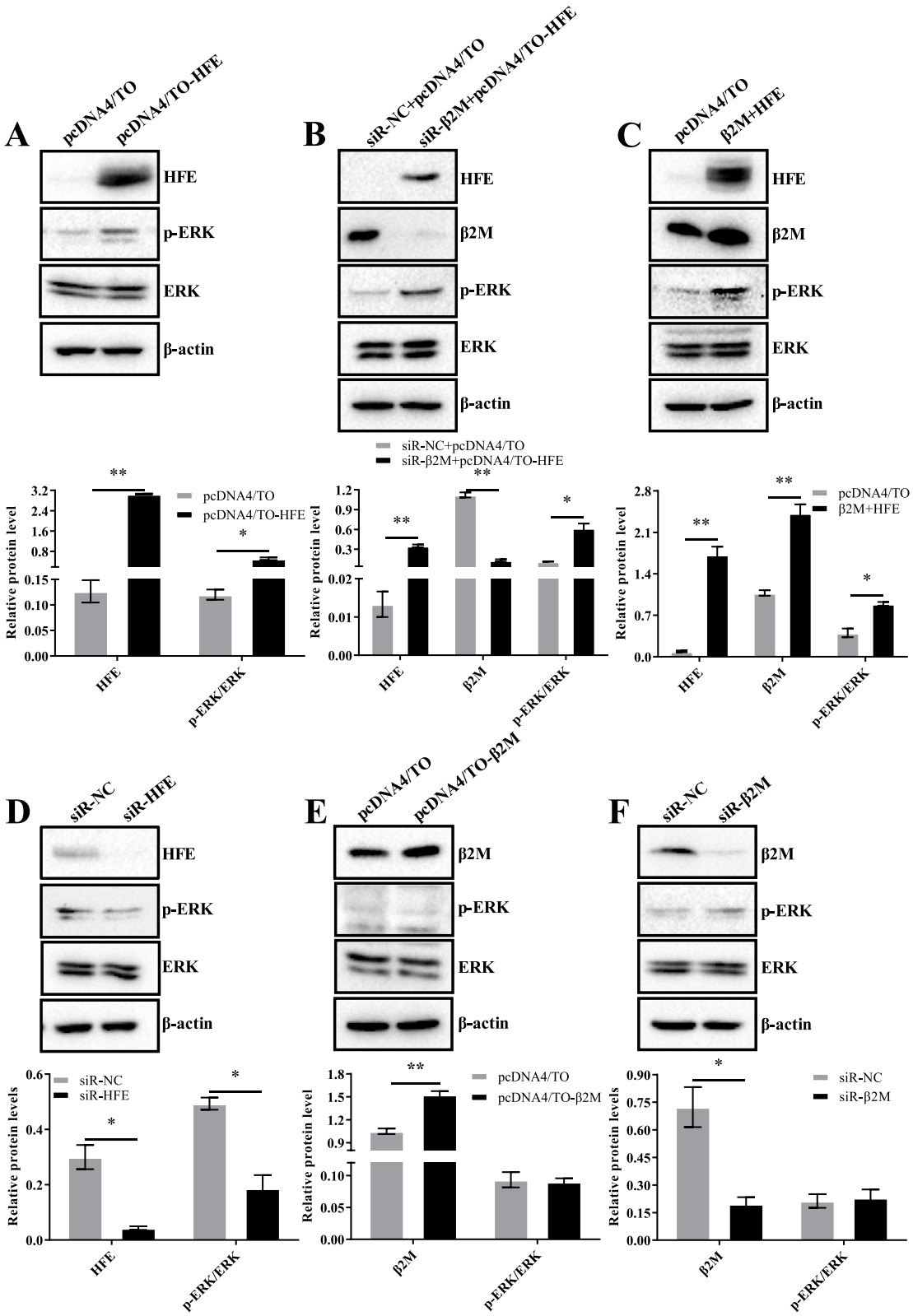

Fig. 4 (See legend on previous page.)

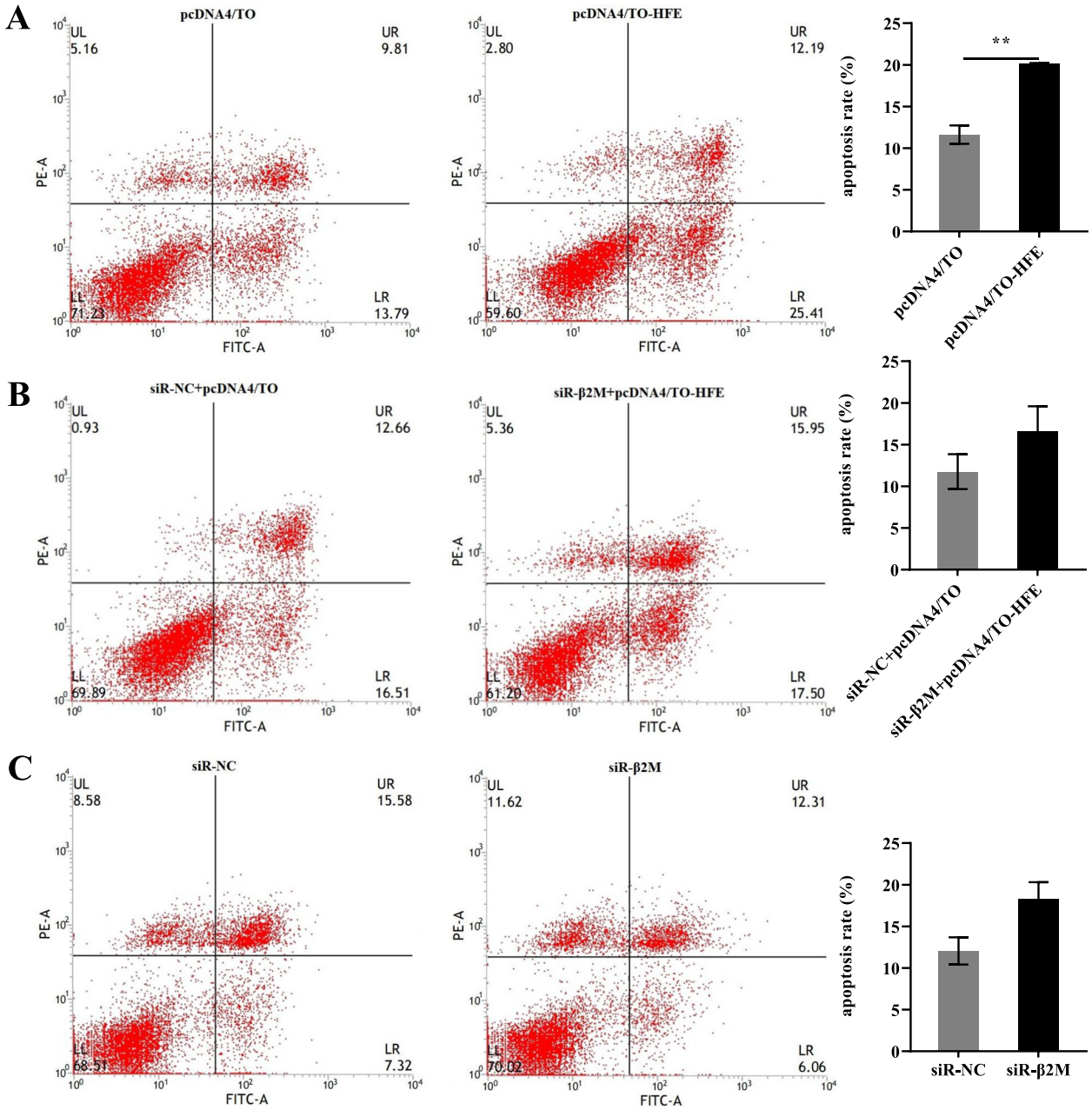

Fig. 5 β2M and HFE interactions induced tumor cell apoptosis in MDA-MB-453 cells. **A** HFE overexpression significantly induced tumor cell apoptosis. **B** Silencing of the *β2M* gene reversed the apoptosis of the tumor cells mediated by HFE overexpression. **C** Silencing the *β2M* gene did not affect tumor cell apoptosis

molecular types of breast cancer, with significant differences in ER^+ and ER^-, but not between $HER2^+$ and $HER2^-$ breast cancer cells, and that β2M protein expression had a negative association with ER expression [18]. Therefore, we speculated that β2M may be involved in the regulation of different types of breast cancer via different signaling pathways and mechanisms. Subsequently, our study demonstrated that β2M promoted tumor survival via the SGK1 signaling pathway in $ER^+/HER2^-$ breast cancer and had no effect on and $ER^-/HER2^-$ breast cancer [19]. However, the molecular mechanism of β2M in $HER2^+$ breast cancer required further investigation. The present study provides new evidence that β2M induced tumor cells apoptosis via the ERK signaling pathway by directly interacting with HFE in HER2-overexpressing breast cancer (Fig. 7). First, Co-IP, MS, and WB results

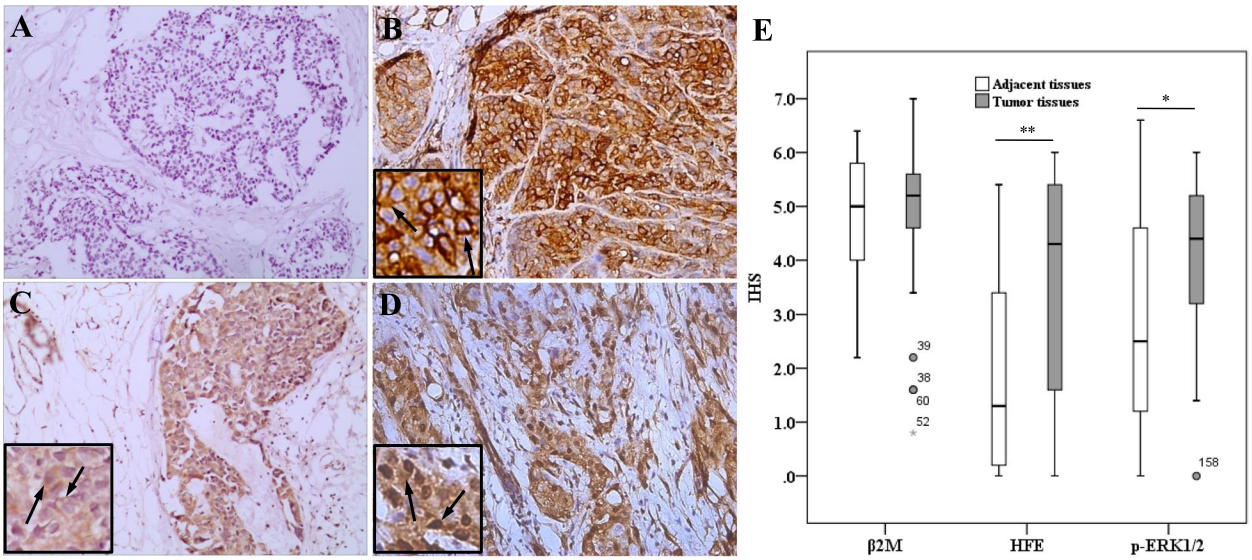

Fig. 6 The expression of β2M, HFE, and p-ERK1/2 in the tumor and adjacent tissues. **A-D** Representative images of immunostaining in HER2-overexpressing breast cancer tissues. **A** Cancer tissues stained without the primary antibody as a negative control. **B** β2M was expressed in cytoplasm and membrane. **C** HFE was expressed in most of the cytoplasm, with very little on the membrane. **D** p-ERK1/2 was expressed in the cytoplasm or nucleus. **E** Box plots of protein expression in tumor and adjacent tissues

verified that HFE is an interacting protein of β2M in MDA-MB-453 breast cancer cells ($ER^-/HER2^+$). Second, yeast two-hybrid, Co-IP of HEK-293T cells expressing β2M and HFE, and His pull-down experiments demonstrated that β2M directly interacted with HFE. Third, Co-IP of endogenous β2M and HFE in different types of breast cancer cells showed that endogenous β2M and HFE interacted in MCF-7 ($ER^+/HER2^-$), MDA-MB-453 ($ER^-/HER2^+$), and MDA-MB-231 ($ER^-/HER2^-$) cells, but no interaction occurred in BT474 ($ER^+/HER2^+$) cells. These results further suggest that β2M was involved in tumor progression through different signaling pathways in different types of breast cancer. The consequence of this in MCF-7 cells was consistent with a previous report [14], and the conclusions for other types of breast cancer cells were shown for the first time. Fourth, the CLSM

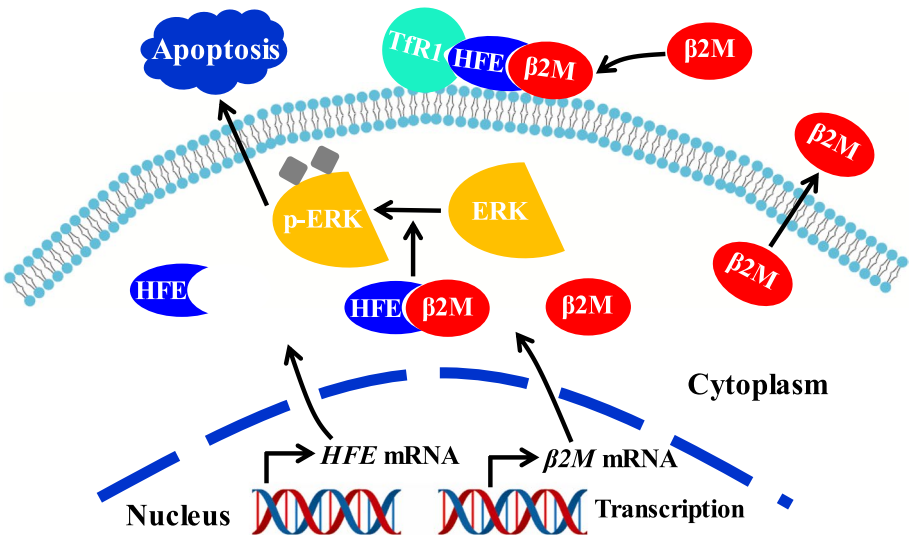

Fig. 7 A diagram showing the proposed mechanism of β2M role in breast cancer. The interaction between β2M and HFE phosphorylates ERK, which induces apoptosis of breast cancer cells. The conclusion that β2M/HFE complex binds with TfR1 on cell membrane comes from our unpublished research data

observation showed that most of β2M and HFE were localized in the cytoplasm in the form of a complex, with some β2M/HFE complexes localized on the cytomembrane of breast cancer cells. It was reported that HFE protein normally formed a stable complex with the TfR1, which is the molecule responsible for receptor-mediated endocytosis of iron-bound transferrin [22]. It was found that HFE physically interacted with β2M in prostate cancer cells, and that β2M/HFE played a key role in regulating iron homeostasis in cancer cells, mediated by interacting with the transferrin receptor complex [14]. In the present study, it was further confirmed that the β2M/HFE complex was also located on the cell membrane, and further experiments verified that the β2M/HFE complex bound to transferrin receptor 1 (TfR1) (the data has not been published). Fifth, HFE overexpression significantly increased p-ERK/ERK levels, silencing the *HFE* gene significantly decreased p-ERK/ERK levels, and simultaneous silencing of the *β2M* gene did not rescue p-ERK/ERK levels induced by HFE overexpression; however, β2M overexpression and silencing the *β2M* gene did not affect the p-ERK/ERK levels in MDA-MB-453 ($ER^-/HER2^+$) breast cancer cells in vitro. These results demonstrated that HFE overexpression activated the ERK signaling pathway in $ER^-/HER2^+$ breast cancer cells in vitro, whereas it needs further research that HFE dependently activate the ERK signaling pathway or by directly interacting with β2M in MDA-MB-453 cells. Sixth, HFE overexpression significantly induced tumor cell apoptosis, and simultaneously silencing the *β2M* gene rescued tumor cell apoptosis induced by HFE overexpression. And the silencing of *β2M* gene did not affect tumor cell apoptosis in MDA-MB-453 cells. This result demonstrated that β2M and HFE interacted to induce tumor cell apoptosis of HER2-overexpressing breast cancer. Seventh, IHC demonstrated that expression levels of HFE and p-ERK1/2 were significantly higher in tumor tissue compared with the adjacent normal tissues in HER2-overexpressing breast cancer. This is consistent with cell experiments in vitro. We demonstrated that HFE activated ERK signaling pathways in $ER^-/HER2^+$ MDA-MB-453 breast cancer cells, and this molecular mechanism needs further study. ERK is an important member of the mitogen-activated protein kinase (MAPK) family and controls cell proliferation, differentiation, and apoptosis via signaling feedback. ERK1/2 activation is associated with anti-apoptotic functions by regulating cell proliferation and differentiation [23]. Blocking ERK activation inhibited apoptosis of melanoma cells [24]. Activating ERK and p38 signaling protected osteocytes from apoptosis [25]. Previous studies demonstrated that β2M promoted tumor cell survival via activation of the ERK signaling pathway in renal cancer [11, 13]. However, there are several studies demonstrated that ERK1/2 signaling can be pro-apoptotic [26]. The ERK inhibitor significantly reduced apoptosis of PC12 cells [27]. An experiment proved that ERK1/2 activation also have anti-proliferation and apoptotic effects of a quinone-based small molecule compound in colon cancer cells [28]. The studies related to pro-apoptotic mechanism of ERK demonstrated that ERK can facilitate cells apoptosis through phosphorylation of MFN1 (profusion protein mitofusin 1), as phosphorylated MFN1 has a high affinity to the pro-apoptotic protein Bak [29]. The present study demonstrated that β2M induced apoptosis of tumor cells via activation of the ERK signal pathway by directly interacting with HFE in HER2-overexpressing breast cancer cells. However, the molecular mechanism of β2M inducing apoptosis in $HER2^+$ breast cancer required further investigation.

Our previous study has demonstrated that β2M had different regulating mechanisms between ER^- and ER^+ breast cancer that were $HER2^-$ [19]. In the present study, the interaction between β2M and HFE could not be detected in BT474 cells, which may be related to ER expression, and the specific mechanism needs to be further studied.

Conclusions

In summary, we demonstrated that β2M induced apoptosis of tumor cells via activation of the ERK signal pathway by directly interacting with HFE in HER2-overexpressing breast cancer.

Abbreviations

β2M	β2-microglobulin
MHC	Major histocompatibility complex
HFE	Hemochromatosis
EMT	Epithelial–mesenchymal transition
VEGFR-2	Vascular endothelial growth factor receptor-2
mTOR	Mammalian target of rapamycin
DKK3	Dickkopf-related protein 3
ER	Estrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor receptor 2
SGK1	Serum and glucocorticoid-regulated kinase 1
Bcl-2	B-cell lymphoma 2
siRNA	Small interfering RNA
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
ERK1/2	Extracellular signal-regulated kinase 1 and 2
p-ERK1/2	Phospho-ERK1/2 (Thr202/Tyr204)
CANX	Calnexin
CALR	Calreticulin
PDIA3	Protein disulfide isomerase family A member 3
GFP	Green fluorescent protein
WB	Western blotting
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TBST	Tris-buffered saline with 0.1% Tween 20
Co-IP	Co-immunoprecipitation
PBS	Phosphate-buffered saline
MS	Mass spectrometry
PPI	Protein–protein interaction
Y2H	Yeast two-hybrid system
IHC	Immunohistochemistry

TfR1	Transferrin receptor 1
WCL	Whole cell lysates
AbA	Antibiotic aureobasidin A
CLSM	Confocal laser scanning microscope
MAPK	Mitogen-activated protein kinase
MFN1	Mitofusin 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-12757-x.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Acknowledgements
We thank. Wengui Shi, PhD, Yating Zhang, PhD, and Lexin Yang, PhD, from the Second Hospital of Lanzhou University for their help in this trial, and also thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Authors' contributions
KSL and HFD prepared the study design and analyzed the data, and wrote and revised the manuscript. DDC carried out the experiments, analyzed the data, and edited the figures. SYR, XWL, XLS, LEB and YX participated in the experiments. SSY, YRL, and XQL provided the cancer tissues and the adjacent tissues samples. All authors read and approved the manuscript.

Funding
This work was supported by the Natural Science Foundation of Gansu Province under Grant number 17JR5RA043, and the Health Scientific Research Plan of Gansu Province under Grant number GSWSKY-2019-14.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
This study was approved by the Medical Ethics Committee of Medicine and Science Research Institute of Gansu Province (approval number: A201603160006). Written informed consent was obtained from all patients prior to participation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 11 September 2023 Accepted: 2 August 2024
Published online: 12 August 2024

References

1. Yewdell JW, Reits E, Neefjes J. Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat Rev Immunol. 2003;3(12):952–61.
2. Liu F, Zhong F, Wu H, Che K, Shi J, Wu N, et al. Prevalence and associations of beta2-microglobulin mutations in MSI-H/dMMR cancers. Oncologist. 2023;28(3):e136–44.
3. Christianson GJ, Brooks W, Vekasi S, Manolfi EA, Niles J, Roopenian SL, et al. Beta2-microglobulin-deficient mice are protected from hypergammaglobulinemia and have defective antibody responses because of increased IgG catabolism. J Immunol. 1997;159(10):4781–92.
4. Josson S, Matsuoka Y, Gururajan M, Nomura T, Huang WC, Yang X, et al. Inhibition of β2-microglobulin/hemochromatosis enhances radiation sensitivity by induction of iron overload in prostate cancer cells. PLoS ONE. 2013;8(7):e68366.
5. Sequeira J, Sengupta S, Mhatre B. Serum beta-2 microglobulin analysis in patients with oral squamous cell carcinoma. Natl J Maxillofac Surg. 2021;12(2):227–32.
6. Kim HD, Cho H, Sohn BS, Park CS, Huh J, Ryu JS, et al. Prognostic significance of serum β2-microglobulin levels in patients with peripheral T-cell lymphoma not otherwise specified. Leuk Lymphoma. 2022;63(1):124–30.
7. Wang H, Liu B, Wei J. Beta2-microglobulin(β2M) in cancer immunotherapies: biological function, and remedy. Cancer Lett. 2021;517:96–104.
8. Shi C, Zhu Y, Su Y, Chung LW, Cheng T. β2-microglobulin: emerging as a promising cancer therapeutic target. Drug Discov Today. 2009;14(1–2):25–30.
9. Yang J, Yi Q. Killing tumor cells through their surface β_2-microglobulin or major histocompatibility complex class I molecules. Cancer. 2010;116(7):1638–45.
10. Huang WC, Wu D, Xie Z, Zhau HE, Nomura T, Zayzafoon M, et al. β2-microglobulin is a signaling and growth-promoting factor for human prostate cancer bone metastasis. Cancer Res. 2006;66(18):9108–16.
11. Nomura T, Huang W, Zhau HE, Wu D, Xie Z, Mimata H, et al. β_2-microglobulin promotes the growth of human renal cell carcinoma through the activation of the protein kinase A, cyclic AMP-responsive element-binding protein, and vascular endothelial growth factor axis. Clin Cancer Res. 2006;12(24):7294–305.
12. Yang J, Qian J, Wezeman M, Wang S, Lin P, Wang M, et al. Targeting β_2-microglobulin for induction of tumor apoptosis in human hematological malignancies. Cancer Cell. 2006;10(4):295–307.
13. Nomura T, Huang WC, Seo S, Zhau HE, Mimata H, Chung LW. Targeting β2-microglobulin mediated signaling as a novel therapeutic approach for human renal cell carcinoma. J Urol. 2007;178(1):292–300.
14. Josson S, Nomura T, Lin JT, Huang WC, Wu D, Zhau HE, et al. β2-microglobulin induces epithelial to mesenchymal transition and confers cancer lethality and bone metastasis in human cancer cells. Cancer Res. 2011;71(7):2600–10.
15. Nomura T, Huang WC, Zhau HE, Josson S, Mimata H, Chung LW. β2-Microglobulin-mediated signaling as a target for cancer therapy. Anticancer Agents Med Chem. 2014;14(3):343–52.
16. Kim BR, Lee EJ, Seo SH, Lee SH, Rho SB. Dickkopf-3 (DKK-3) obstructs VEGFR-2/Akt/mTOR signaling cascade by interacting of β_2-microglobulin (β2M) in ovarian tumorigenesis. Cell Signal. 2015;27(11):2150–9.
17. Jiang G, Zhang S, Yazdanparast A, Li M, Pawar AV, Liu Y, et al. Comprehensive comparison of molecular portraits between cell lines and tumors in breast cancer. BMC Genomics. 2016;17(Suppl 7):525.
18. Li KS, Du HF, Lian XW, Yang SS, Chai DD, Wang CY, et al. Characterization of β2-microglobulin expression in different types of breast cancer. BMC Cancer. 2014;14(1):750.
19. Chai DD, Li KS, Du HF, Yang SS, Yang R, Xu Y, et al. β2-microglobulin has a different regulatory molecular mechanism between ER^+ and ER^- breast cancer with $HER2^-$. BMC Cancer. 2019;19(1):223.
20. Ogretmen B, McCauley MD, Safa AR. Molecular mechanisms of loss of β_2-microglobulin expression in drug-resistant breast cancer sublines and its involvement in drug resistance. Biochemistry. 1998;37(33):11679–91.
21. Takahashi Y. Co-immunoprecipitation from Transfected Cells. Methods Mol Biol. 2015;1278:381–9.
22. Feder JN, Penny DM, Irrinki A, Lee VK, Lebron JA, Watson N, et al. The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. Proc Natl Acad Sci USA. 1998;95(4):1472–7.
23. Balmanno K, Cook SJ. Tumour cell survival signalling by the ERK1/2 pathway. Cell Death Differ. 2009;16(3):368–77.
24. Sugiura R. Fission yeast as a model system for studying cancer signaling and drug discovery: discovery of ACA-28 as a novel inducer of ERK-dependent apoptosis reveals a new cancer therapy. Yakugaku Zasshi. 2019;139(5):753–8.

25. He Z, Li H, Han X, Zhou F, Du J, Yang Y, et al. Irisin inhibits osteocyte apoptosis by activating the Erk signaling pathway in vitro and attenuates ALCT-induced osteoarthritis in mice. Bone. 2020;141:115573.
26. Cagnol S, Chambard JC. ERK and cell death: Mechanisms of ERK-induced cell death-apoptosis, autophagy and senescence. FEBS J. 2010;277(1):2–21.
27. Lee YJ, Choi SY, Yang JH. NMDA receptor-mediated ERK 1/2 pathway is involved in PFHxS-induced apoptosis of PC12 cells. Sci Total Environ. 2014;491–492:227–34.
28. Dubey NK, Peng BY, Lin CM, Wang PD, Wang JR, Chan CH, et al. NSC 95397 suppresses proliferation and induces apoptosis in colon cancer cells through MKP-1 and the ERK1/2 pathway. Int J Mol Sci. 2018;19(6):1625.
29. Pyakurel A, Savoia C, Hess D, Scorrano L. Extracellular regulated kinase phosphorylates mitofusin 1 to control mitochondrial morphology and apoptosis. Mol Cell. 2015;58(2):244–54.

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