Tamoxifen induces fatty liver disease in breast cancer through the MAPK8/FoxO pathway

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Funding information
Innovation Capacity Support Plan of Shaanxi Province, Grant/Award Number: 2018TD-002

Abstract

Background: Prevention of metabolic complications of long-term adjuvant endocrine therapy in breast cancers remained a challenge. We aimed to investigate the molecular mechanism in the development of tamoxifen (TAM)-induced fatty liver in both estrogen receptor (ER)-positive and ER-negative breast cancer.

Methods and results: First, the direct protein targets (DPTs) of TAM were identified using DrugBank5.1.7. We found that mitogen-activated protein kinase 8 (MAPK8) was one DPT of TAM. We identified significant genes in breast cancer and fatty liver disease (FLD) using the MalaCards human disease database. Next, we analyzed the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of those significant genes in breast cancer and FLD using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). We found that overlapping KEGG pathways in these two diseases were MAPK signaling pathway, Forkhead box O (FoxO) signaling pathway, HIF-1 signaling pathway, AGE-RAGE signaling pathway in diabetic complications, and PI3K-Akt signaling pathway. Furthermore, the KEGG Mapper showed that the MAPK signaling pathway was related to the FoxO signaling pathway. Finally, the functional relevance of breast cancer and TAM-induced FLD was validated by Western blot analysis. We verified that TAM may induce fatty liver in breast cancer through the MAPK8/FoxO signaling pathway.

Conclusion: Bioinformatics analysis combined with conventional experiments may improve our understanding of the molecular mechanisms underlying side effects of cancer drugs, thereby making this method a new paradigm for guiding future studies on this issue.

KEYWORDS
bioinformatics analysis, breast cancer, fatty liver, FoxO signaling pathway, MAPK8, TAM
1 | BACKGROUND

Breast cancer is the most common cancer in women and the main cause of cancer-related death in women worldwide. Recently, obesity has been regarded as a risk factor for this disease, and fatty liver disease (FLD) and breast cancer have been found to share similar risk factors, including obesity and metabolic abnormalities. Hyperinsulinemia is also associated with both FLD and breast cancer, suggesting there is a mechanistic link between the two diseases.

Tamoxifen (TAM) is used for the treatment of breast cancer widely. It is noticeable, however, that hepatocyte steatosis has been described in studies of patients with breast cancer because of TAM, and TAM is known to induce this condition in half of the patients within the first 2 years of TAM treatment. Therapeutic intervention to prevent TAM-induced hepatocyte steatosis may improve the safety of TAM usage. Thus, there is an urgent need to find effective paradigms to clarify the functional mechanisms underlying breast cancer and TAM-induced FLD.

In recent years, tumor databases and drug databases have developed and are continuously improving, especially drug databases, which combine drug action information and drug target genes are rapidly developing. Integrative analysis of tumor databases and drug databases derives a good technique to discover the mechanism underlying drug-induced diseases.

In this study, we identified direct protein targets (DPTs) of TAM using DrugBank. We found that mitogen-activated protein kinase 8 (MAPK8) was one DPT of TAM. Meanwhile, we identified significant genes in breast cancer and FLD using the MalaCards human disease database, and the results of Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that the MAPK and Forkhead box O (FoxO) signaling pathways were related to both breast cancer and FLD. Further, the KEGG Mapper showed that the MAPK signaling pathway was upstream of FoxO signaling pathway. Finally, we explored the functional relevance of TAM-induced fatty liver in breast cancer with the MTT assay, colony formation assay, flow cytometry, and Western blotting. The result showed that TAM may induce fatty liver in patients with breast cancer by interfering with the MAPK8/FoxO signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Recognition of DPTs of TAM

The DrugBank is a rich database that combines drug interaction information and drug target genes. It has been widely used for drug research since 2006. Manual literature searches for data are guided by PolySearch2, a text-mining tool developed for DrugBank annotation projects. The DPTs of TAM were driven from DrugBank by inputting TAM in the search box and clicking Targets.

2.2 | Identification of differentially expressed DPTs of TAM

The Gene Expression Profiling Interactive Analysis (GEPIA) is a tool. It is based on The Cancer Genome Atlas and GTEx data and delivers fast and customizable functionalities. There are rich functions including differential expression analysis, similar gene detection, correlation analysis, and patient survival analysis in GEPIA. First, a DPT in the search box was inputted and GEPIA was clicked, then the cancer type with breast cancer (BRCA) was chosen, and finally, the differential expression of DPT of TAM was identified.

2.3 | Analysis of significant genes in breast cancer and FLD

MalaCards is a database of human diseases and their annotations, whose architecture and strategy is based on the GeneCards database. MalaCards generates a web card for more than 20,000 human diseases in six global categories. When searched for breast cancer and fatty liver in the MalaCards, a table containing significant genes of breast cancer and fatty liver can be downloaded directly. Cytoscape is one of the most successful network biology analysis and visualization tools. The significant genes of breast cancer and fatty liver were visualized using Cytoscape 3.7.1.

2.4 | Analysis of KEGG pathways in breast cancer and fatty liver

Search tool for the Retrieval of Interacting Genes (STRING) is a public web-based tool that can evaluate the protein-protein interaction network, KEGG pathways, and gene ontology terms. We analyzed KEGG pathways in the significant genes of breast cancer and FLD using STRING. When those significant genes were searched (with organism being Homo sapiens), the analysis result showed the KEGG pathways in breast cancer and FLD. And the result was visualized using OriginPro 2015. KEGG Mapper is a suite of KEGG mapping tools available at the KEGG website; we mapped MAPK signaling pathway and FoxO signaling pathway using this tool.
| Searched drug (1/1) | Target symbol | Target (17) |
|---------------------|---------------|-------------|
| Tamoxifen           | ESR2          | Q92731      | Estrogen receptor beta |
|                     | ESR1          | P03372      | Estrogen receptor alpha |
|                     | MAPK8         | P45983      | Mitogen-activated protein kinase 8 |
|                     | SHBG          | P04278      | Sex hormone-binding globulin |
|                     | ESRGG         | P62508      | Estrogen-related receptor gamma |
|                     | NR1I2         | Q75469      | Nuclear receptor subfamily 1 group I member 2 |
|                     | KCNH2         | Q12809      | Potassium voltage-gated channel subfamily H member 2 |
|                     | AR            | P10275      | Androgen receptor |
|                     | EBP           | Q15125      | 3-beta-Hydroxysteroid-Delta(8),Delta(7)-isomerase |
|                     | Protein group | Q05513      | Protein kinase C zeta type |
|                     |               | Q04759      | Protein kinase C theta type |
|                     |               | P41743      | Protein kinase C iota type |
|                     |               | P05129      | Protein kinase C gamma type |
|                     |               | Q02156      | Protein kinase C epsilon type |
|                     |               | Q05655      | Protein kinase C delta type |
|                     |               | P05771      | Protein kinase C beta type |
|                     |               | P17252      | Protein kinase C alpha type |

### 2.5 Cell culture and reagents

Both the human breast cancer cell lines and the human liver cell lines were obtained from The American Type Culture Collection (Manassas, VA). MCF-7, MDA-MB-231, and LO2 cells were cultured under standard cell culture conditions in Dulbecco’s Modified Eagle’s Medium containing 10% serum at 37°C in a humidified atmosphere with 5% CO₂. T47D and ZR-75 cells were cultured under standard cell culture conditions in RPMI-1640 medium containing 10% serum at 37°C in a humidified atmosphere with 5% CO₂. TAM (C₂₆H₂₉NO; molecular weight: 371.51) purchased from MedChemExpress (MCE) was dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 27 mmol/L initially. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO). Triglyceride Assay Kit was purchased from Jiancheng (Nanjing, China). Antibodies were purchased from Cell Signaling Technology (Danvers, MA) and Proteintech Group Inc. (Rosemont, IL).

### 2.6 Cell viability assay

Cancer cell lines (MCF-7, T47D, ZR-75, and MDA-MB-231) were plated in 96-well plates at a density of 1 × 10³ cells per well and allowed to adhere overnight, and then treated at various concentrations (0, 5, 10, 20, 30, and 40 µmol/L) of TAM. At the indicated time points (0, 12, 24, and 36 hours), cell viability was assessed by the MTT assay and was measured using a multiwell microplate reader (BIO-TEC Inc., Richmond, VA) at an absorbance of 490 nm.

### 2.7 Colony formation assay

A total of 1000 cells in the control group and 20 000 cells in the drug group were seeded into six-well cell culture clusters and allowed to adhere overnight. Then TAM was added to the cells for 24 hours, after which media was replaced with drug-free media. Cells were cultured for an additional 10 days to allow the colonies to form. At the related time points, colonies were fixed in 4% paraformaldehyde and then stained with 0.1% crystal violet solution, rinsed, and imaged. The number of colonies >0.5 mm in diameter was counted using a microscope (Nikon Eclipse Ti-S, Tokyo, Japan) at a magnification of 20x and 40x.

### 2.8 Apoptosis assay

Cell apoptosis was assessed by flow cytometry with PE Annexin V Apoptosis Detection Kit I (Becton Dickinson Biosciences, Franklin Lakes, NJ) according to the manufacturer’s instructions. Briefly, cancer cells were seeded in 6-well plates at a density of 1 × 10⁵ cells per well. After being starved overnight, cells were treated with fresh medium containing various concentrations of TAM for 24 hours. Then cells were trypsinized, washed with phosphate-buffered saline (PBS), and stained with PE Annexin V. The percentage
of apoptotic cells was quantified by flow cytometry using a FACSCalibur instrument (BD Biosciences). The total apoptosis rate was calculated by summing the rate of early apoptotic cells (7-AAD−/PE Annexin V+) and late apoptotic cells (7-AAD+/PE Annexin V+).

2.9 | Oil Red O Staining

LO2 cells were grown in 6-well cell culture clusters and treated at various concentrations (0, 5, 10, 20, 30, and 40 µmol/L) of TAM after 24 hours. Then they were washed with PBS and fixed in paraformaldehyde solution for 10 minutes at room temperature. After fixation, cells were gently washed with ddH2O and stained with a working solution of 0.5 g Oil Red O for 30 minutes. The stained hepatocytes were washed three times with PBS to remove the unincorporated dye, and then examined by laser scanning confocal microscopy.

2.10 | Triglyceride measurement

LO2 cells were preincubated in a 6 cm cell culture dish for 24 hours and then cultured in DMEM with TAM (0, 10, 15, 20, 30, and 40 µmol/L). After 24 hours of incubation, cells were transferred into an Eppendorf tube (1.5 mL) and centrifuged at 800 rpm for 5 minutes. Cell pellets were washed with PBS and centrifuged again at 800 rpm for 5 minutes. Total triglyceride (TG) was extracted by RIPA Lysis
| Term ID   | Term description                          | False discovery rate | Matching proteins in your network (labels)                                                                 |
|-----------|--------------------------------------------|----------------------|---------------------------------------------------------------------------------------------------------|
| hsa05200  | Pathways in cancer                         | 1.04E-38             | AKT1, ALK, APC, AR, BCL2, BRAF, BRCA2, CASP3, CASP8, CCND1, CCND2, CDH1, CDK2, CDK4, CDKN1A, CDKN1B, CTNNB1, EGFR, EGFR, EGF, EGF, ERBB2, ESR1, ESR2, FGFR3, FGFR2, FGF, FGF, GNAS, HRAS, IGF1, IGF2, ITGB1, KRAS, MAPK8, MDM2, MTOR, MYC, NCOA3, NOTCH1, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TGFB2, TP53, VEGFA, VEGFC, WNT10B |
| hsa05224  | Breast cancer                              | 1.50E-28             | AKT1, APC, BRAF, BRCA1, BRCA2, CCND1, CDK4, CDKN1A, CTNNB1, EGFR, EGFR, ERBB2, ESR1, ESR2, FGFR3, HRAS, IGF1R, KRAS, MTOR, MYC, NCOA3, NOTCH1, PGR, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, VEGFC, WNT10B |
| hsa04151  | PI3K-Akt signaling pathway                 | 4.37E-27             | AKT1, BCL2, BRAF, CCND1, CDK2, CDK4, CDKN1A, CDKN1B, EGF, EGFR, ERBB2, ESR1, ESR2, FGFR3, HRAS, IGF1R, KRAS, MTOR, MYC, NCOA3, NOTCH1, PGR, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, VEGFC, WNT10B |
| hsa05206  | MicroRNAs in cancer                        | 4.37E-27             | APC, ATM, BCL2, BRCA1, CASP3, CCND1, CCND2, CDC25A, CDKN1A, CDKN1B, EEFNA3, EGFR, EGF, EGF, ERBB2, ERBB3, ERBB4, FGFR3, FGFR2, FGF, HRAS, IGF1R, IGF2, ITGB1, KRAS, MDM2, MTOR, MYC, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, WNT10B |
| hsa05215  | Prostate cancer                            | 1.01E-26             | AKT1, AR, BCL2, BRAF, CCND1, CDK2, CDKN1A, CTNNB1, EGFR, ERBB2, ESR1, ESR2, GNAS, HRAS, IGF1R, KRAS, MDM2, MTOR, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, WNT10B |
| hsa05122  | Endocrine resistance                       | 2.14E-25             | AKT1, BCL2, BRAF, CCND1, CDK4, CDKN1A, CDKN1B, EGFR, ERBB2, ESR1, ESR2, GNAS, HRAS, IGF1R, KRAS, MAPK8, MDM2, MTOR, NCOA3, NOTCH1, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, WNT10B |
| hsa05226  | Gastric cancer                             | 1.23E-24             | AKT1, APC, BCL2, BRAF, CCND1, CDH1, CDK2, CDKN1A, CDKN1B, CTNNB1, EGFR, EGFR, ERBB2, FGFR2, HRAS, IGF1R, KRAS, MTOR, MYC, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, WNT10B |
| hsa05210  | Colorectal cancer                          | 2.96E-23             | AKT1, APC, BCL2, BRAF, CASP3, CCND1, CDKN1A, CTNNB1, EGFR, EGFR, HRAS, KRAS, MAPK8, MTOR, MYC, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, WNT10B |
| hsa05205  | Proteoglycans in cancer                    | 4.23E-22             | AKT1, BRAF, CASP3, CCND1, CDKN1A, CTNNB1, EGFR, ERBB2, ERBB3, ERBB4, ESR1, HRAS, IGF1R, IGF2, ITGB1, KRAS, MDM2, MTOR, MYC, PIK3CA, TGFA, TGFB1, TP53, VEGFA, WNT10B |
| hsa05212  | Pancreatic cancer                          | 3.41E-21             | AKT1, BRAF, BRCA2, CCND1, CDK4, CDKN1A, EGF, EGFR, ERBB2, KRAS, MAPK8, MTOR, PIK3CA, SMAD4, TGFA, TGFB1, TP53, VEGFA |
| hsa05165  | Human papillomavirus infection             | 3.57E-21             | AKT1, APC, ATM, CASP3, CASP8, CCND1, CCND2, CDK2, CDK4, CDKN1A, CDKN1B, CTNNB1, EGFR, EGFR, ERBB2, ERBB3, ERBB4, ESR1, HRAS, IGF1R, IGF2, ITGB1, KRAS, MDM2, MTOR, NOTCH1, PIK3CA, PTEN, SMAD4, TGFA, TGFB1, TP53, VEGFA, WNT10B |
| hsa01521  | EGFR tyrosine kinase inhibitor resistance   | 6.48E-21             | AKT1, AXL, BCL2, BRAF, EGFR, EGFR, ERBB2, ERBB3, FGFR2, HRAS, IGF1R, KRAS, MTOR, NRG1, PIK3CA, PTEN, TGFA, VEGFA |
| hsa04068  | FoxO signaling pathway                     | 3.36E-20             | AKT1, ATM, BRAF, CCND1, CCND2, CDK2, CDKN1A, CDKN1B, EGFR, EGFR, ERBB2, ERBB3, ErbB2, HRAS, IGF1R, KRAS, MAPK8, MDM2, PIK3CA, PTEN, SMAD4, TGFB1 |
| hsa04218  | Cellular senescence                        | 3.58E-20             | AKT1, ATM, CCND1, CCND2, CDC25A, CDK1, CDK2, CDK4, CDKN1A, CHEK1, CHEK2, HRAS, KRAS, MDM2, MTOR, MYC, NBN, PIK3CA, PTEN, TGFB1, TP53 |

(Continues)
| Term ID | Term description                        | False discovery rate | Matching proteins in your network (labels)                                                                                                                                 |
|---------|-----------------------------------------|----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| hsa05225| Hepatocellular carcinoma                | 7.66E-20             | AKT1,APC,BRAF,CCND1,CDK4,CDKN1A,CTNNB1,EGFR,HRAS,IGF1R,IGF2,KRAS,MTOR,MYC,PIK3CA,PTEN,SMAD4,TGFA,TGFB1,TP53,WNT10B                                                   |
| hsa05213| Endometrial cancer                      | 9.33E-20             | AKT1,APC,BRAF,CCND1,CDH1,CDKN1A,CTNNB1,EGF,EGFR,ERBB2,HRAS,KRAS,MYC,PIK3CA,PTEN,TP53                                                                                   |
| hsa05161| Hepatitis B                             | 1.25E-19             | AKT1,BCL2,CASp3,CASp8,CCND1,CDK2,CDK4,CDKN1A,CDKN1B,CDK2,CDK4,CCND2,CDK1,CDK2,CDK4,EGFR,HRAS,KRAS,MAPK8,MYC,PCNA,PIK3CA,PTEN,SMAD4,TGFB1,TP53 |
| hsa04012| ErbB signaling pathway                  | 3.87E-19             | AKT1,BRAF,CDKN1A,CDKN1B,EGF,EGFR,ERBB2,ERBB3,ERBB4,HRAS,KRAS,MAPK8,MTOR,MYC,NRG1,PIK3CA,TGFA                                                                         |
| hsa04115| p53 Signaling pathway                   | 7.09E-19             | ATM,CASP3,CASP8,CCND1,CCND2,CDK1,CDK2,CDK4,CDKN1A,CDK1,CDK2,CDK4,CCND1,CDK2,CDK4,EGF,EGFR,ERBB2,HRAS,KRAS,MAPK8,MTOR,MYC,TP53,VEGFA |
| hsa05214| Glioma                                  | 7.09E-19             | AKT1,BRAF,CCND1,CDK4,CDKN1A,EGF,EGFR,HRAS,IGF1R,HRAS,MDM2,MTOR,PIK3CA,PTEN,TGFA,TP53                                                                               |
| hsa05218| Melanoma                                | 1.43E-18             | AKT1,BRAF,CCND1,CDH1,CDK4,CDKN1A,EGF,EGFR,FGF3,HRAS,IGF1R,HRAS,MDM2,PIK3CA,PTEN,TP53                                                                             |
| hsa05219| Bladder cancer                          | 1.63E-18             | BRAF,CCND1,CDH1,CDK4,CDKN1A,EGF,EGFR,ERBB2,HRAS,KRAS,MDM2,MYC,TP53,VEGFA                                                                                         |
| hsa04110| Cell cycle                              | 4.82E-18             | ATM,CCND1,CCND2,CD25A,CD1,CDK2,CDK4,CDKN1A,CDKN1B,CHEK1,CHEK2,EP300,MDM2,MYC,PCNA,PIK3CA,TGFB1,TP53,VEGFA,VEGCF                                                   |
| hsa05166| HTLV-I infection                        | 9.96E-18             | AKT1,APC,ATM,CCND1,CCND2,CDK4,CDKN1A,CHEK1,CHEK2,CTNNB1,EP300,HRAS,KRAS,MAPK8,MYC,PCNA,PIK3CA,TGFB1,TP53,WNT10B,XBP1                                                   |
| hsa04010| MAPK signaling pathway                  | 1.55E-17             | AKT1,BRAF,CASP3,EFNA3,EGF,EGFR,ERBB2,ERBB3,ERBB4,FGF3,FGF2,FGF,HRAS,IGF1R,IGF2,KRAS,MAPK8,MYC,TP53,VEGFA,VEGFC,VEGFC,VEGFC,VEGFA |
| hsa05223| Nonsmall cell lung cancer               | 4.20E-16             | AKT1,ALK,BRAF,CCND1,CDK4,CDKN1A,EGF,EGFR,ERBB2,HRAS,KRAS,PIK3CA,TGFA,TP53                                                                                           |
| hsa04510| Focal adhesion                          | 5.04E-16             | AKT1,BCAR1,BCL2,BRAF,CCND1,CCND2,CTNNB1,EGF,EGFR,ERBB2,FGF,HRAS,IGF1R,ITGB1,MAPK8,PIK3CA,PTEN,VEGFA,VEGFC                                                              |
| hsa04015| Rap1 signaling pathway                  | 8.13E-16             | AKT1,BCAR1,BRAF,CDH1,CTNNB1,EFNA3,EGF,EGFR,FGF3,FGFR2,FGF,HRAS,IGF1R,ITGB1,MAPK8,PIK3CA,PTEN,VEGFA,VEGFC                                                              |
| hsa04933| AGE-RAGE signaling pathway in diabetic complications | 2.09E-15             | AKT1,BCL2,CASP3,CCND1,CDK4,CDKN1B,FGF,HRAS,KRAS,MAPK8,PIK3CA,SMAD4,TGFB1,VEGFA,VEGFC                                                                               |
| hsa05220| Chronic myeloid leukemia                | 2.09E-15             | AKT1,BRAF,CCND1,CDK4,CDKN1A,CDK1B,HRAS,KRAS,MDM2,MYC,PIK3CA,SMAD4,TGFB1,TP53                                                                                     |
| hsa04915| Estrogen signaling pathway              | 6.00E-15             | AKT1,BCL2,CTSD,EGFR,ESR1,ESR2,GNAS,HRAS,KRAS,KRT14,KRT19,NCOA3,PGK,PIK3CA,TPF1,TGFA                                                                               |
| hsa03440| Homologous recombination                | 7.23E-14             | ATM,BARD1,BRCA1,BRCA2,BRIP1,FAM175A,NBN,PALB2,RAD54L,XRCC2,XRCC3                                                                                                |
| hsa05230| Central carbon metabolism in cancer     | 2.66E-13             | AKT1,EGFR,ERBB2,FGFR2,HRAS,IDH1,HRAS,MTOR,MYC,PIK3CA,TPEN,TP53                                                                                                      |
| hsa04919| Thyroid hormone signaling pathway       | 3.24E-13             | AKT1,CCND1,CTNNB1,EP300,ESR1,HRAS,KRAS,MDM2,MTOR,MYC,NCOA3,NOTCH1,PIK3CA,TP53                                                                                       |
| hsa05222| Small cell lung cancer                  | 4.60E-13             | AKT1,BCL2,CASP3,CCND1,CDK2,CDK4,CDKN1A,CDKN1B,ITGB1,MYC,PIK3CA,PTEN,TP53                                                                                           |

(Continues)
Table 2 (Continued)

| Term ID | Term description | False discovery rate | Matching proteins in your network (labels) |
|---------|------------------|----------------------|-------------------------------------------|
| has05167 | Kaposi’s sarcoma-associated herpesvirus infection | 5.10E-13 | AKT1,CASP3,CASP8,CCND1,CDK4,CDKN1A,CTNNB1,EP300,HRAS,KRAS,MAPK8,MTOR,MYC,PIK3CA,TP53,VEGFA |
| has05203 | Viral carcinogenesis | 5.10E-13 | CASP3,CASP8,CCND1,CCND2,CDK1,CDK2,CDK4,CDKN1A,CDKN1B,CHEK1,EP300,HRAS,KRAS,MDM2,PIK3CA,TP53 |
| has04014 | Ras signaling pathway | 1.13E-11 | AKT1,EFNA3,EGF,EGFR,FGF3,FGFR2,FGF6,HRAS,IGF1R,IGF2,KRAS,MAPK8,PIK3CA,TGFA,VEGFA,VEGFC |
| has04917 | Prolactin signaling pathway | 1.13E-11 | AKT1,CCND1,CCND2,CYP17A1,ESR1,ESR2,HRAS,KRAS,MAPK8,PIK3CA |
| has01524 | Platinum drug resistance | 1.26E-11 | AKT1,ATM,BCL2,BRCA1,CASP3,CASP8,CDKN1A,ERBB2,MDM2,PIK3CA,TGFA |
| has04066 | HIF-1 signaling pathway | 1.73E-11 | AKT1,BCL2,CDKN1A,CDKN1B,EGF,EGFR,EP300,ERBB2,IGF1R,MTOR,PIK3CA,TGFA |
| has05216 | Thyroid cancer | 4.03E-11 | BRAF,CCND1,CDH1,CDKN1A,CTNNB1,HRAS,KRAS,MYC,TP53 |
| has04934 | Cushing’s syndrome | 1.42E-10 | AHR,APC,BRAF,CCND1,CDK2,CDK4,CDKN1A,CDKN1B,CTNNB1,CYP17A1,ESR2,ESR1 |
| has04914 | Progesterone-mediated oocyte maturation | 2.08E-10 | AKT1,AURKA,BRAF,CDCA5A,CDK1,CDK2,EGF1R,HRAS,MAPK8,PIK3CA |
| has05211 | Renal cell carcinoma | 2.08E-10 | AKT1,BRAF,CCDN1A,EP300,HRAS,KRAS,PIK3CA,TGFA,TFGB1,VEGFA |
| has04630 | Jak-STAT signaling pathway | 2.23E-10 | AKT1,BCL2,CCND1,CCND2,CDK1,CDK4,CDKN1A,EGF,EGFR,EP300,HRAS,MTOR,MYC,PIK3CA,PRLR |
| has04140 | Autophagy - animal | 3.25E-09 | AKT1,BCL2,CTSD,HRAS,IGF1R,KRAS,MAPK8,MTOR,PIK3CA,PTEN,RB1CC1 |
| has04926 | Relaxin signaling pathway | 4.68E-09 | AKT1,EGFR,FGF,GNAS,HRAS,MAPK8,MTOR,PIK3CA,PTEN,RB1CC1 |
| has04210 | Apoptosis | 6.65E-09 | AKT1,ATM,BCL2,CASP3,CASP8,CTSD,HRAS,KRAS,MAPK8,PIK3CA,TP53 |
| has04550 | Signaling pathways regulating pluripotency of stem cells | 8.10E-09 | AKT1,APC,CTNNB1,FGFR2,HRAS,IGF1R,KRAS,MYC,PIK3CA,SMAD4,WNT10B |

Buffer (Fisher, Pittsburgh, PA). The concentration of TG was determined using the TG Assay Kit (Jiancheng, Nanjing, China) and normalized by protein concentration according to the manufacturer’s instructions.

2.11 Western blot analysis

Total proteins were extracted by RIPA Lysis Buffer and their concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Then Western blotting was performed. The 4-12% Bis-Tris precast gels (Bio-Rad, Hercules, CA) were used for electrophoresis. Equal volumes of cell total protein were loaded and subsequently electrotransferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat milk (Lab Scientific, Livingston, NJ), followed by incubation with primary and horseradish peroxidase–conjugated secondary antibodies overnight and 2 hours, respectively.20-26 Protein expression was visualized by enhanced chemiluminescence (GE, Buckinghamshire, UK). Images were captured using the ChemiDoc XRS imaging system (Bio-Rad), and Quantity One image software was used for densitometry analysis of each band. GAPDH was used as the internal loading control.

2.12 Statistics

The results are expressed as the mean ± SD. The lipid accumulation in LO2 cells with different TAM concentrations was
analyzed by analysis of variance using GraphPad Prism 6.0. Other data were analyzed by the Student’s t-test using GraphPad Prism 6.0. P values <0.05 were considered to be statistically significant. Each experiment was performed at least three times.

3 | RESULTS

3.1 | Bioinformatics analysis of TAM, breast cancer, and FLD

TAM was output as DB00675 (APRD00123) from DrugBank 5.1.4 with 17 primary DPTs (Table 1). It is noteworthy that MAPK8 was overexpressed in breast cancer samples compared to normal samples (Figure 1A). Significant genes and 41 hub genes in breast cancer were identified (Figure 1B). Significant genes in FLD are shown in Figure 1C.

The results of KEGG analysis of breast cancer are shown in Table 2, and the top 20 KEGG pathways in breast cancer are shown in Figure 2A. The results of KEGG analysis of FLD are shown in Table 3, and the top 20 KEGG pathways are shown in Figure 2B. The five overlapping KEGG pathways in both breast cancer and FLD were the phosphoinositide 3-kinase-Akt, FoxO, MAPK, hypoxia inducible factor-1, and advanced glycation end product receptor for advanced glycation end product (in diabetic complications) signaling pathways. Meanwhile, KEGG mapper (Figure 2C) showed that the MAPK signaling pathway was upstream of the FoxO signaling pathway.

3.2 | TAM inhibits the proliferation of breast cancer cells

The effects of TAM on the viability of breast cancer cells were evaluated. We found that TAM decreased the growth of breast cancer cell lines (MCF-7, T47D, ZR-75, and MDA-MB-231) in dose- and time-dependent manners (Figure 3A). Limited inhibitory effects on MCF-7, T47D, ZR-75, and MDA-MB-231 were observed even when the TAM concentrations were 25.56, 35.28, 31.14, and 39.68 µmol/L (IC50), respectively. These results indicate that TAM inhibits the growth of breast cancer cells at concentrations more than 25.56 µmol/L.
### TABLE 3  KEGG pathway in fatty liver

| Term ID   | Term description                                               | False discovery rate | Genes                                                                 |
|-----------|---------------------------------------------------------------|----------------------|----------------------------------------------------------------------|
| hsa04932  | Nonalcoholic fatty liver disease (NAFLD)                      | 3.44E-09             | ADIPOQ, CYP2E1, IL6, INS, LEP, PPARA, SREBF1                           |
| hsa04152  | AMPK signaling pathway                                         | 1.03E-06             | ACACA, ADIPOQ, FASN, INS, LEP, SREBF1                                |
| hsa04931  | Insulin resistance                                            | 1.68E-05             | IL6, INS, PPARA, SREBF1, TNF                                       |
| hsa04920  | Adipocytokine signaling pathway                                | 9.04E-05             | ADIPOQ, LEP, PPARA, TNF                                             |
| hsa04910  | Insulin signaling pathway                                      | 0.0009               | ACACA, FASN, INS, SREBF1                                           |
| hsa04930  | Type II diabetes mellitus                                      | 0.0009               | ADIPOQ, INS, TNF                                                    |
| hsa00061  | Fatty acid biosynthesis                                        | 0.0029               | ACACA, FASN                                                         |
| hsa04010  | MAPK signaling pathway                                         | 0.0107               | FGF21, INS, NLK, TNF                                               |
| hsa04068  | FoxO signaling pathway                                         | 0.0109               | IL6, INS, NLK                                                      |
| hsa01523  | Antifolate resistance                                         | 0.0117               | IL6, TNF                                                           |
| hsa05143  | African trypanosomiasis                                       | 0.0126               | IL6, TNF                                                           |
| hsa05332  | Graft-versus-host disease                                     | 0.0129               | IL6, TNF                                                           |
| hsa04940  | Type I diabetes mellitus                                      | 0.0138               | INS, TNF                                                           |
| hsa04975  | Fat digestion and absorption                                  | 0.0138               | APOB, MTTP                                                         |
| hsa01212  | Fatty acid metabolism                                         | 0.0171               | ACACA, FASN                                                        |
| hsa05144  | Malaria                                                       | 0.0171               | IL6, TNF                                                           |
| hsa05134  | Legionellosis                                                 | 0.0196               | IL6, TNF                                                           |
| hsa00590  | Arachidonic acid metabolism                                   | 0.0234               | CYP2E1, GGT1                                                       |
| hsa05321  | Inflammatory bowel disease (IBD)                              | 0.0234               | IL6, TNF                                                           |
| hsa03320  | PPAR signaling pathway                                        | 0.0288               | ADIPOQ, PPARA                                                      |
| hsa05133  | Pertussis                                                     | 0.0289               | IL6, TNF                                                           |
| hsa04060  | Cytokine-cytokine receptor interaction                        | 0.0321               | IL6, LEP, TNF                                                      |
| hsa05410  | Hypertrophic cardiomyopathy (HCM)                             | 0.0321               | IL6, TNF                                                           |
| hsa01100  | Metabolic pathways                                            | 0.0322               | ACACA, CYP2E1, FASN, GGT1, GPT, PNPLA3                              |
| hsa05323  | Rheumatoid arthritis                                          | 0.0322               | IL6, TNF                                                           |
| hsa04211  | Longevity regulating pathway                                  | 0.0324               | ADIPOQ, INS                                                        |
| hsa04657  | IL-17 signaling pathway                                       | 0.034                | IL6, TNF                                                           |
| hsa04640  | Hematopoietic cell lineage                                    | 0.0341               | IL6, TNF                                                           |
| hsa05146  | Amoebiasis                                                    | 0.0341               | IL6, TNF                                                           |
| hsa04066  | HIF-1 signaling pathway                                       | 0.0344               | IL6, INS                                                           |
| hsa04620  | Toll-like receptor signaling pathway                          | 0.0344               | IL6, TNF                                                           |
| hsa04922  | Glucagon signaling pathway                                    | 0.0344               | ACACA, PPARA                                                      |
| hsa04933  | AGE-RAGE signaling pathway in diabetic complications          | 0.0344               | IL6, TNF                                                           |
| hsa05142  | Chagas disease (American trypanosomiasis)                    | 0.0344               | IL6, TNF                                                           |
| hsa04668  | TNF signaling pathway                                         | 0.0354               | IL6, TNF                                                           |
| hsa04151  | PI3K-Akt signaling pathway                                    | 0.0418               | FGF21, IL6, INS                                                    |
| hsa05160  | Hepatitis C                                                   | 0.0481               | PPARA, TNF                                                         |

### 3.3 TAM inhibits clone formation and induces apoptosis of breast cancer cells

We determined the effects of TAM on the clone formation capability of breast cancer cells (MCF-7, T47D, ZR-75, and MDA-MB-231). Treatment with TAM markedly decreased the number of colonies compared to untreated cells (Figure 3B). Treatment of breast cancer cells with TAM caused an increase in apoptotic cells compared to untreated breast cancer cells (Figure 3C). These results demonstrate that TAM
**FIGURE 3**  A, TAM decreased the growth of breast cancer cell lines (MCF-7, T47D, ZR-75, and MDA-MB-231) in a dose- and time-dependent manner. B, The effect of TAM on clone formation capability of breast cancer cells. C, TAM-induced apoptosis of breast cancer cells. **P < .01, ***P < .001**

has potent effects against clone formation and induces the apoptosis of breast cancer cells.

### 3.4 | TAM induces lipid accumulation in LO2 Cells

We treated LO2 cells with various concentrations of TAM for 24 hours. Lipid accumulation was examined after Oil Red O staining. As shown in Figure 4A, TAM induced hepatocyte steatosis in LO2 cells, and cells treated with TAM accumulated significant amount of lipid droplets in a dose-dependent manner. Consistently, measurements of TG concentration in cell lysates showed that significant increases in TG were observed in LO2 cells treated with ≥10 µmol/L TAM (Figure 4B).

### 3.5 | TAM induces FLD by disrupting the MAPK8/FoxO signaling pathway

As shown in Figure 4C, as an upstream mediator of FoxO signaling, MAPK8 was suppressed in breast cancer cells (MCF-7, T47D, ZR-75, and MDA-MB-231) and liver cells...
FIGURE 4  A, TAM-induced hepatocyte steatosis in LO2 cells. B, Significant increases in TG were observed in LO2 cells treated with ≥10 µmol/L of TAM. C, Different transmission of MAPK8/FoxO signaling pathway in breast cancer cells (MCF-7, T47D, ZR-75, and MDA-MB-231) and liver cells (LO2) exposed to TAM. *P < .05, **P < .01, ***P < .001

(LO2) treated with TAM. Because of the variable differences between cell lines, the expression levels of FoxO proteins changed differently in breast cancer cell lines. When treated with TAM, p-FOXO3 and FOXO3 were up-expressed in MCF-7 cells; FOXO1 and p-FOXO3 were down-expressed in T47D cells; FOXO4 was up-expressed in T47D cells; FOXO1, p-FOXO3, and FOXO3 were up-expressed in ZR-75 cells; p-FOXO3 and FOXO4 were up-expressed in MDA-MB-231 cells; and FOXO3 and p-FOXO4 were down-expressed in MDA-MB-231 cells. And all proteins in liver cells (LO2) were down-expressed when exposed to TAM. These results indicate that TAM induces FLD by disrupting the MAPK8/FoxO signaling pathway in patients with breast cancer.

4 | DISCUSSION

Breast cancer is the most common and aggressive cancer among women worldwide. TAM has been the gold standard treatment for all stages of estrogen receptor (ER)-positive breast cancer, and it is also effective against ER-negative breast cancer. However, TAM is associated with an increased risk of the development of FLD,27 and studies have reported that about 43% of breast cancer patients using TAM may develop FLD within the first 2 years,27-29 indicating the need to manage fatty liver with a positive strategy through early prevention. It is very urgent to find an effective paradigm for clarifying the functional mechanism underlying breast cancer and TAM-induced fatty liver.
In this study, we used a combination of bioinformatics analysis and conventional experiments to clarify the functional mechanisms underlying breast cancer and TAM-induced FLD. Bioinformatics analysis was done as follows: (a) DPTs of TAM were identified by DrugBank\textsuperscript{5.1.7}; (b) significant genes in breast cancer and fatty liver were identified by MalaCards; (c) KEGG pathways of those significant genes were analyzed using STRING; and (d) KEGG Mapper analysis was performed. We found that MAPK8 was one DPT of TAM, and significant genes of breast cancer and fatty liver were correlated with the MAPK and FoxO signaling pathways; the MAPK signaling pathway was found to be upstream of the FoxO signaling pathway. The functional relevance of breast cancer and TAM-induced fatty liver was validated by the experimental data. We verified that TAM may induce fatty liver in breast cancer through the MAPK8/FoxO signaling pathway.

MAPK8, also known as c-Jun NH2-terminal kinase-1 (JNK1), is a member of the MAPK family.\textsuperscript{30} Studies over-expressing a DN JNK1 mutant have demonstrated that TAM can stimulate JNK1 activity and interfere with the JNK pathway.\textsuperscript{31,32} Furthermore, it has been reported that TAM induces apoptosis of breast cancer cells through the JNK1 pathway.\textsuperscript{33} Sabio et al\textsuperscript{34} reported that JNK1 serves to prevent hepatic steatosis. Consistently, our study found that MAPK8 was a DPT of TAM (Table 1), which induces the apoptosis of breast cancer cells (Figure 3C) and steatosis in liver cells (Figure 4).

The FoxO family, which consists of FoxO1, FoxO3, FoxO4, and FoxO6, is known as a tumor suppressor that limits cell proliferation and induces apoptosis.\textsuperscript{35} However, paradoxical roles of FoxO proteins in cancer progression were recently described\textsuperscript{36}; for example, in acute and chronic myeloid leukemia, FoxO proteins maintain leukemia-initiating cells. These factors may also promote the invasion of breast cancer,\textsuperscript{37} and FoxO proteins contribute to treatment resistance in multiple cases, including targeted therapies.\textsuperscript{38} Hornsveld et al\textsuperscript{39} reported that FoxO proteins both suppress and support breast cancer progression. Dong\textsuperscript{40} claimed that FoxO proteins play critical roles in maintaining metabolic and cellular homeostasis in the liver, and their suppression may be involved in NAFLD development. In our study, we found that TAM can both upregulate and downregulate FoxOs and P-FoxOs in different breast cancer cell lines (MCF-7, T47D, ZR-75, and MDA-MB-231), which may predict different prognosis to types of breast cancer. Meanwhile, TAM down-regulated FoxOs in the LO2 liver cell line, which may induce FLD.

As determined using integrated bioinformatics analysis, the MAPK8/FoxO signaling pathway is important for the development of cancer and fatty liver. We confirmed that TAM can function through the MAPK8/FoxO signaling pathway in breast cancer cells (MCF-7, T47D, ZR-75, and MDA-MB-231) and liver cells (LO2). Thus, we predict that TAM induces fatty liver by interfering with the MAPK8/FoxO signaling pathway. However, further studies such as siRNA or shRNA directed against DPT (MAPK8) are urgently warranted to validate the prediction, and further mechanisms would be uncovered.

5 | CONCLUSIONS
In summary, combined bioinformatics analysis and experimental verification provided an effective and convenient approach for clarifying the molecular mechanism underlying TAM-induced FLD in breast cancer patients. Using existing drug and disease databases as the BioGPS, leading researchers combine web-based resources and experimental results with clinical application. This novel comprehensive research approach can be used to determine the molecular mechanism underlying the complicating effects of drugs in cancer treatment.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

FUNDING INFORMATION
Innovation Capacity Support Plan of Shaanxi Province 2018TD-002.

DATA AVAILABILITY STATEMENT
The data and materials used in the current study are available from the corresponding author on reasonable request.

ACKNOWLEDGMENTS
The authors acknowledge the Innovation Capacity Support Plan of Shaanxi Province for financial support (under Grant # 2018TD-002). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

AUTHOR CONTRIBUTIONS
Suxia Han and Jinlu Ma designed the research, Liuyun Gong wrote the manuscript and finished the experiment. Hanmin Tang and Zhenzhen Luo collected the data. Xinyue Tan and Lina Xie wrote the manuscript. Xiao Sun and Mengjiao Cai prepared reagents and materials. Yutiantian Lei and Chenchen He analyzed the data. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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**How to cite this article:** Gong L, Tang H, Luo Z, et al. Tamoxifen induces fatty liver disease in breast cancer through the MAPK8/FoxO pathway. *Clin Transl Med*. 2020;10:137–150.  
[https://doi.org/10.1002/ctm2.5](https://doi.org/10.1002/ctm2.5)