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Regulation of the AKT/P53 Signaling Pathway by Translated Control Tumor Protein Inhibits Apoptosis and Promotes Hyperplasia of the Mammary Glands

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Abbreviations

TCTP:Translationally controlled tumor protein

HMG:hyperplasia of the mammary glands

H&E:Directory of open access journals

E2:Estradiol

LH:Luteinizing hormone

FSH:Follicle-stimulating hormone

PROG:Progesterone

IHC:Immunohistochemistry

mIHC:Multiplex Immunofluorescence

ABSTRACT

This study aims to explore the mechanism by which the Translationally controlled tumor protein (TCTP) promotes hyperplasia of the mammary glands (HMG). In this study, the TCTP ($Tpt1^{flox/flox}$) and TCTP ($Tpt1^{KI/KI}$) gene mice were respectively mated with MMTV-Cre to obtain mice with mammary glands-specific TCTP knockout (TCTP ^{cKO}) and overexpression (TCTP ^{KI}). Prepare the HMG animal model by using estrogen combined with progesterone. After the establishment of the TCTP ^{cKO} and TCTP ^{KI} mouse models, they are uniformly referred to as HMG ^{cKO} and HMG ^{KI}. Hematoxylin and Eosin (H&E) staining and hormone and receptor expression levels were detected. Detect the levels of TCTP, P53, p-AKT, as well as the indicators related to cell apoptosis and cell cycle in the breast tissues of each group. The H&E results showed that compared with the HMG group, the ductal cavity dilation and the number of milk glands in the breast tissue of the HMG ^{cKO} group were significantly reduced, while the HMG ^{KI} group exhibited obvious mammary gland hyperplasia. The results showed that compared with the HMG group, the expression levels of Ki67, E_2, FSH, LH, ERα, PR, TCTP, p-AKT, p-BAD, Bcl-2, Cyclin D1, CDK4 and CDK6 in the mammary tissues of the HMG ^{cKO} group were significantly decreased, while the expression levels of PROG, ERβ, P53, Bax and P27 were significantly increased. In the HMG ^{KI} group, the opposite results were observed. In our research, it was confirmed that TCTP inhibits cell apoptosis and promotes cell cycle progression by regulating the AKT/P53 signaling pathway,

leading to abnormal hyperplasia of the mammary glands. In HMG, by regulating the expression level of TCTP, the effect of treating HMG can be achieved.

Keywords: Hyperplasia of the mammary glands, Translationally controlled tumor protein , P53 protein

Introduction

Hyperplasia of the mammary glands (HMG) is a non-inflammatory and non-neoplastic disorder characterized by structural abnormalities of the breast resulting from varying degrees of hyperplasia and regressive changes in the mammary gland parenchyma and stroma[1]. HMG can affect the appearance and function of the breasts, cause pain, and significantly impact the quality of life of patients. What's more, the incidence of HMG has been on the rise in recent years, and it is more prominent among young women[2]. Unfortunately, HMG is often overlooked as a benign breast condition because the public's attention is mainly focused on the diagnosis and treatment of breast cancer[3,4]. Current clinical studies have shown that benign HMG can trigger carcinogenesis related to the onset of breast cancer, and is one of the important risk factors for the occurrence of breast cancer[5,6]. These findings underscore that HMG poses a substantial threat to the physical and psychological well-being of women. Current treatment strategies primarily include pharmacological approaches, such as estrogen-based therapy, and surgical excision. However, surgery does not address the underlying pathological mechanisms

of HMG, and drug-based treatments are often associated with significant side effects, complications, and high recurrence rates[1]. Therefore, exploring the pathogenesis of HMG and developing safe and effective drugs based on the mechanism have become the current research focus.

In our earlier studies, we found that the translationally controlled tumor protein (TCTP) protein was highly expressed in HMG[7]. However, there is no report yet on whether the TCTP protein is the key factor in regulating the occurrence of HMG. TCTP is a multifunctional protein that is universally expressed in all eukaryotes. At the cellular and organ levels, it is involved in fundamental biological processes such as cell proliferation and cell apoptosis, and plays a crucial role in various pathological conditions including cancer and cardiovascular diseases[8,9]. It is worth noting that TCTP is highly expressed in breast cancer. At the cellular level, by knocking down the expression of TCTP, the structure of breast cancer cells can be reshaped, forming duct-like structures similar to normal breast epithelium, thus establishing TCTP as a major regulatory factor in the occurrence of breast cancer[10,11]. As a protein that is highly expressed in both HMG and breast cancer, exploring the therapeutic mechanism by which TCTP causes the occurrence of HMG and breast cancer, and developing therapeutic drugs based on TCTP in the future could be a two-pronged solution. Therefore, in our study, we established mouse models with specific knockout and overexpression of genes related to the mammary glands, and used the estrogen combined with progesterone to induce the HMG mouse model. We then utilized modern molecular biology techniques to explore the pathogenesis of TCTP in HMG.

Materials and Methods

Animal Experiments

By crossing TCTP knockout mice ($TPT1^{flox/flox}$) established on the C57BL/6J background with MMTV-Cre mice, mammary gland-specific knockout mice ($TPT1^{flox/flox,\ MMTV\text{-}Cre}$) were obtained, referred to as TCTP ^{cKO} in the article. Similarly, crossing TCTP overexpression mice ($TPT1^{KI/KI}$) with MMTV-Cre mice yielded mammary gland-specific overexpression mice ($TPT1^{KI/KI,\ MMTV\text{-}Cre}$), referred to as TCTP ^{KI} in the article. The aforementioned genetically modified mice were all purchased from Suzhou Cyagen Biotechnology Co., Ltd. The HMG animal model was established by consecutive intraperitoneal injections of estradiol benzoate (Shanghai General Pharmaceutical Co., Ltd.) at a dose of 0.5 mg/kg for 25 days, followed by intramuscular injections of progesterone (Shanghai General Pharmaceutical Co., Ltd.) at a dose of 4 mg/kg for 5 days. The control group received an equivalent volume of corn oil. We selected 16 female mice each of the wild-type (WT), TCTP ^{cKO}, and TCTP ^{KI} genotypes, with body weights ranging from 22 to 24 g, and divided them into a control group and an HMG model group using a random number table. These mice were used to study the expression of TCTP protein in the HMG model. Subsequently, wild-type (WT, n=16), TCTP ^{cKO} (n=13), and TCTP ^{KI} (n=13) mice were selected for mechanistic studies. Among these, the wild-type mice were further divided into a control group and an HMG group. The HMG group, TCTP cKO group, and TCTP ^{KI} group continued to undergo the establishment of a mammary hyperplasia model using estrogen combined with progesterone. After successful model preparation, for clearer and more consistent presentation in the subsequent article, TCTP ^{cKO} mice were uniformly labeled as HMG ^{cKO}, and TCTP ^{KI} mice were uniformly labeled as HMG ^{KI}. All experimental procedures strictly complied with the ARRIVE 2.0

guidelines.

Hematoxylin and Eosin (H&E) Staining

After fixation, embedding, and sectioning of mouse mammary gland tissues, pathological staining was performed using the H&E Staining Kit (Beyotime Biotechnology Co., Ltd., Cat# C0105S) according to the manufacturer ' s instructions. Morphological changes in the mammary gland tissues were observed under a light microscope at 400 × magnification. Pathological alterations in mammary tissues from each group were evaluated and scored based on the pathological scoring criteria for HMG tissues outlined in the Hyperplastic Mammary Gland Animal Model Preparation Guidelines (Draft). Statistical analysis was conducted on the recorded scores.

Serum Hormone Level Assay

Upon completion of the experimental protocol, blood specimens were obtained through retro-orbital plexus sampling under isoflurane anesthesia. Subsequent centrifugation (3000 × g, 15 min, 4°C) facilitated serum isolation for hormonal quantification. Serum concentrations of estradiol (E_2), progesterone (PROG), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (E2: MU30395; LH: MU30382; FSH: MU30265; PROG: MU30393; Wuhan Bioinlab Biotechnology Co., Ltd.), following standardized operational guidelines provided by the manufacturer. The ELISA kits used detect the total concentration of E_2,PROG, FSH and LH in serum (i.e., the sum of the free and protein-bound fractions)

Immunohistochemistry (IHC) Staining

Mammary gland tissue sections embedded in paraffin from each experimental cohort underwent standardized IHC processing.

Primary antibody incubation was conducted overnight at 4°C using the following reagents: Ki67 (1:5,000; Cat# 27309-1-AP), TCTP (1:100; Cat# T56981), ERβ (1:200; Cat# 14007-1-AP), PR (1:200; Cat# 25871-1-AP), Bax (1:1,000; Cat# 50599-2-Ig), Bcl-2 (1:400; Cat# 26593-1-AP), p-BAD (1:100; Cat# AF7427) and ERα (1:200; Cat# ET7110-60). Following primary antibody treatment, sections were exposed to HRP-conjugated secondary antibodies (1:300 dilution) for 1 hour at room temperature.

Post-staining procedures included hematoxylin counterstaining (10 min), sequential rinsing under running tap water, dehydration through an ethanol gradient, xylene-based clearing, and mounting with neutral resin. Three representative microscopic fields per group were imaged for quantitative analysis. Protein expression levels were evaluated using mean density values (Integrated Optical Density [IOD]/Area SUM) derived from Image-Pro Plus software (Media Cybernetics, USA), with statistical comparisons performed across experimental groups.

Multiplex Immunofluorescence (mIHC) staining

mIHC staining was performed on mammary gland tissue sections using a seven-color automated system (OPAL™ Kit, PerkinElmer) integrated with the BOND RX automated staining platform (Leica Biosystems). Tissue sections were initially baked at 65°C for 2 hours, followed by automated deparaffinization and heat-induced epitope retrieval (HIER) according to the manufacturer’s standardized protocol. Sequential incubations included rabbit monoclonal anti-P53 (1:200, red fluorescence) , mouse monoclonal anti-TCTP (1:150, green fluorescence) and mouse monoclonal anti-p-AKT (1:200, purple fluorescence), with nuclear counterstaining using 4',6-diamidino-2-phenylindole (DAPI, blue fluorescence).

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from mammary gland tissues using TRIzol® reagent (Thermo Fisher Scientific, Inc., USA), according to the manufacturer's instructions. Quantitative analysis of RNA was performed using a Nanodrop® 2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). The mRNA expression levels of Cyclin D1, CDK4, CDK6 and P27 were evaluated using RT-qPCR. The quantitative analysis was carried out using LightCycler® 480 System (Roche Diagnostics). RT-qPCR data were analysed by the $2-\Delta\Delta Cq$ method using β-actin as an internal control. Cyclin D1: forward 5-'ATTTCCAACCCACCCTCCAT-3′, reverse 5-′AGGGGGTCCTTGTTTAGCCA-3′. CDK4 □ forward 5-'GTCTATGGTCTGGCCCGAAG-3′, reverse 5-′CGGGTTCATATCGAGTGGCA-3′. CDK6 □ forward 5-'CCTCTCCTTCGTGAAGACTGC-3′, reverse 5-′CATAGCTGGACTGGAGCAGG-3′. P27 □ forward 5′-TCAAACGTCAGAGTGTCTAACG-3′, reverse 5′-CCGGGCCGAAGAGATTTCTG-3. β-actin: forward 5′-GTCACCCACACTGTGCCCA-3′, reverse 5′-AGCCAAGTCCAGACGCAGG-3.

Western blot assay

Mammary gland tissues were homogenized in ice-cold RIPA buffer supplemented with a protease/phosphatase inhibitor cocktail (Servicebio) using a cryogenic grinder (JXFSTPRP-CL model, Shanghai Jing Xin). Total protein concentrations were quantified via bicinchoninic acid (BCA) assay kit (Omni-Easy) according to the manufacturer’s protocol. Equal protein aliquots (20 μg per lane) were resolved on 10% gradient SDS-PAGE gels and electrotransferred to PVDF membranes using a semi-dry transfer system (CAVOY) at 15 V for 45 min. Membranes underwent

blocking with 5% non-fat milk in TBST (2 h, RT) followed by overnight incubation (4°C) with primary antibodies: TCTP (1:2000; Cat# T56981), P53 (1:1000; Cat# CY5047), Bax (1:10,000; Cat# 50599-2-Ig), Bcl-2 (1:1000; Cat# 26593-1-AP), BAD (1:1000; Cat# AF6471), p-BAD (1:1000; Cat# AF7427), p-AKT (1:5000; Cat# 66444-1-Ig), AKT (1:6000; Cat# 10176-2-AP) and GAPDH (1:10,000; Cat# T0004). After TBST washes, membranes were probed with species-matched HRP- conjugated secondary antibodies (1:10,000; Abclonal) for 1 h at RT. Protein signals were visualized using ECL detection reagent (KeyGen BioTECH) and captured with a high-sensitivity chemiluminescence imager (Amersham 680RGB). Band intensities were normalized to GAPDH via densitometric analysis using ImageJ (v1.53k, Rawak Software).

Statistical Analysis

All quantitative data are expressed as mean ± SD. Intergroup differences were analyzed by one-way ANOVA, with post hoc comparisons (Tukey's test for homoscedastic data or Tamhane's T2 test for heteroscedastic data) based on variance homogeneity assessment. Statistical analyses were performed using SPSS 24.0 (IBM, USA), and a two-tailed significance threshold of $P < 0.05$ was applied.

Results

TCTP protein is highly expressed in HMG mice

In wild-type mice, the HMG model was induced by estrogen combined with progesterone. Through the detection of TCTP protein expression levels, it was found that compared with the control group, the expression of TCTP protein in the mammary glands tissues of the HMG group mice was significantly increased ($P<0.05$) (Figure 1A-B, 1E). Furthermore, in TCTP ^{cKO} and TCTP ^{KI} mice, we used the estrogen combined with progesterone-induced

HMG model to investigate the expression level of TCTP protein. The results showed that compared with the control group, the expression of TCTP protein in the mammary tissue of TCTP ^{cKO} mice slightly increased, while in the mammary tissue of TCTP ^{KI} mice, the expression of TCTP protein significantly increased (P<0.05) (Figure 1C-D, 1E). The hyperplasia of mammary tissues in each group of mice was analyzed using H&E staining technique. Compared with the control group, the mammary tissues of estrogen and progesterone-induced HMG mice showed obvious ductal cavity dilation and an increase in the number of mammary alveoli (P<0.05) (Figure 1F-G). However, the mammary glands of TCTP ^{cKO} mice was significantly less severe than that of the WT HMG group, while the mammary glands of TCTP ^{KI} mice was more severe than that of the WT HMG group (P<0.05) (Figure 1F-G). The Ki67 protein, as an important indicator for evaluating cell proliferation, we conducted a detection of the expression level of the Ki67 protein in mouse mammary tissues. The results showed that compared with the control group, the expression level of the ki67 protein in the HMG group was significantly increased (P<0.05) (Figure 1F,1H). Further comparison revealed that compared with the WT HMG group, the expression level of the ki67 protein in the TCTP ^{cKO} group was decreased, while the expression level of the ki67 protein in the TCTP ^{KI} group was significantly increased (P<0.05) (Figure 1F,1H).

TCTP protein affected hormone levels in HMG mice

Furthermore, we measured the expression levels of E_2 (Figure 2A), FSH (Figure 2B), PROG (Figure 2C), and LH (Figure 2D) in serum samples from each group. Our research indicates that in the mouse model of hyperplasia of the mammary glands induced by estrogen combined with progesterone, the expression levels of E_2 (Figure 2A), FSH (Figure 2B), and LH (Figure 2D) significantly

increased, while the expression level of PROG (Figure 2C) significantly decreased (P<0.05). It is worth noting that compared with the HMG group, the expression levels of E_2 (Figure 2A), FSH (Figure 2B), and LH (Figure 2D) in the HMG ^{cKO} group were significantly lower, while the expression level of PROG (Figure 2C) was significantly higher (P<0.05). However, in the HMG ^{KI} group, the expression levels of E_2 (Figure 2A), FSH (Figure 2B), and LH (Figure 2D) were significantly higher, while the expression level of PROG (Figure 2C) was significantly lower (P<0.05). Furthermore, the expression levels of ERα, ERβ and PR proteins in the mammary tissues of HMG mice were detected by IHC (Figure 2E-H). Compared with the control group, the expression levels of ERα and PR in the breast tissues of the HMG, HMG ^{cKO} and HMG ^{KI} groups were significantly increased, while the expression level of ERβ was significantly decreased (P<0.05). Compared with the HMG group, the expression of ERα and PR in the mammary tissues of the HMG ^{cKO} group was significantly decreased, while the expression of ERβ was significantly increased (P<0.05) (Figure 2E-H). However, in the HMG ^{KI} group, compared with the HMG group, the expression of ERα and PR in the mammary tissues of mice was significantly increased, while the expression of ERβ was significantly decreased (P<0.05) (Figure 2E-H).

The TCTP/AKT/P53 signaling pathway regulates HMG

In order to further explore the possible mechanism of action of the TCTP protein during the occurrence of HMG, we conducted an examination of the expression levels of TCTP, AKT and P53 proteins. The results showed that, compared with the control group, the expression of TCTP and p-AKT proteins in the mammary tissues of mice in the HMG group was significantly increased, while the expression of P53 protein was decreased (P < 0.05) (Figures 3A-D).

Furthermore, in the mammary tissues of HMG ^{cKO} mice, we observed that the expression of P53 protein significantly increased after TCTP knockout, while the expressions of p-AKT protein and TCTP protein significantly decreased. However, this phenomenon was reversed in the HMG ^{KI} group compared to the HMG ^{cKO} group (Figures 3A-D). Finally, mIHC assays were performed to assess TCTP, p-AKT and P53 expression patterns in mammary tissues of all groups (Figure 3E). The results indicated that in the HMG group, TCTP and p-AKT protein intensity was significantly elevated, primarily localized in the cytoplasm, whereas P53 expression intensity was markedly reduced, with predominant nuclear localization. In the HMG ^{cKO} group, the expression of TCTP and p-AKT protein was not significantly observed, while the expression of P53 protein was significantly increased (Figure 3E). In the HMG ^{KI} group, the expression of TCTP and p-AKT protein was significantly enhanced, while the expression of P53 protein was significantly reduced (Figure 3E).

TCTP promotes the occurrence of HMG by inhibiting cell apoptosis

We analyzed the expression of apoptosis-related proteins Bcl-2, Bax and p-BAD in mammary gland tissues across groups using Western blot (Figure 4A). The results demonstrated that compared with the control group, the HMG model group exhibited a significant increase in Bcl-2 and p-BAD protein expression and a marked decrease in Bax protein expression in mammary tissues (P<0.05) (Figure 4B-D). Compared with the HMG group, the expression of Bax protein in the mammary tissues of mice in the HMG ^{cKO} group increased, while the expression of Bcl-2 and p-BAD protein slightly decreased. However, in the HMG ^{KI} group, we found that compared with the HMG group, the expression of Bcl-2

and p-BAD protein in the mouse mammary tissue was significantly increased, while the expression of Bax protein was significantly decreased (P<0.05) (Figure 4B-D). Furthermore, IHC analysis of Bcl-2, Bax and p-BAD expression in mammary tissues revealed findings consistent with the Western blot results through quantitative statistical evaluation (Figure 4E-H).

TCTP promotes the occurrence of HMG by facilitating the cell cycle

The expression levels of key regulatory genes of the cell cycle in the mammary tissues of four groups of mice were detected using RT-PCR technology. The results showed that compared with the control group, the expression levels of Cyclin D1, CDK and CKD6 significantly increased during hyperplasia of the mammary glands, while the expression level of P27 decreased significantly in all groups except the HMG ^{cKO} group, which showed a significant increase (P<0.05) (Figure 4I-L). Furthermore, compared with the HMG group, the expression levels of Cyclin D1, CDK4, and CDK6 mRNA in the mammary tissues of mice in the HMG ^{cKO} group were significantly decreased, while the expression level of P27 mRNA was significantly increased (P<0.05) (Figure 4I-L). However, in the HMG ^{KI} group, it was found that compared with the HMG group, the mRNA expression levels of CDK4 and CDK6 in the mouse mammary tissue were significantly increased, while the mRNA expression level of P27 was significantly decreased (P<0.05) (Figure 4I-L).

Discussion

An imbalance in the levels of estrogen and progesterone in the body can lead to excessive breast hyperplasia and incomplete recovery, resulting in continuous hyperplasia of glandular tissue[13]. Therefore, in the preparation of HMG animal models, estrogen

combined with progesterone is also commonly used for induction[14]. Furthermore, in the HMG model, rats are usually used as experimental animals. However, in our study, since we needed to use mice with specific knockout and overexpression of TCTP genes in mammary tissue, we chose mice as the experimental animals. H&E staining revealed significant ductal dilation and increased acinar density in the mammary tissues of HMG group mice, consistent with our earlier observations in rats, confirming the validity of estrogen-progesterone-induced HMG modeling in mice[7,12]. At the same time, we observed that compared with the wild-type HMG mice, the degree of breast tissue hyperplasia in the HMG ^{cKO} group was significantly reduced, while the degree of breast tissue hyperplasia in the TCTP ^{KI} group was significantly increased. The expression of Ki67 protein was consistent with the results of H&E staining, indicating that TCTP plays a promoting role in the occurrence of HMG.

Current understanding posits that HMG pathogenesis is associated with an imbalance in E_2 and PROG secretion, characterized by relative E_2 excess and PROG deficiency[15]. E_2 stimulates mammary epithelial cell proliferation, whereas PROG promotes lobuloalveolar development and counteracts E_2-induced hyperplastic stimuli[16]. Elevated LH levels enhance ovarian androgen secretion, potentially contributing to hyperandrogenemia and HMG progression[17]. FSH drives follicular maturation and ovarian E_2 production [18]. In our study, the expression levels of E_2, LH, FSH and PROG in the mouse serum were detected. It was found that compared with the control group, the expression levels of E_2, LH and FSH in the HMG group were increased, while the expression level of PROG decreased significantly. This trend was more obvious in the HMG ^{KI} group. However, in the HMG ^{cKO} group,

we observed that compared with the HMG group, the expressions of E_2, LH, and FSH in the mouse serum were decreased, while the expression level of PROG was significantly increased. ERα and ERβ are ligand-regulated transcription factors that play crucial roles in mammary cell proliferation and differentiation [19,20]. Studies have shown that in breast cancer, the expression of ERα is significantly increased and can serve as both a biological marker and therapeutic target, while ERβ expression is markedly reduced [21]. PR, another key regulatory protein in the female reproductive system, also plays important roles in mammary gland development, proliferation and differentiation [22]. Notably, ER and PR are functionally interconnected, and both can reflect tissue proliferation and differentiation status to some extent [22,23]. In our study, the expression levels of ERα, ERβ and PR proteins in the mammary tissues of each group of mice were detected using IHC technology. The results showed that compared with the control group, the expression levels of ERα and PR in the mammary tissues of mice in the HMG group significantly increased, while the expression level of ERβ significantly decreased. This result was consistent with the findings of the rat study. Additionally, in the HMG ^{cKO} group, we found that compared with the HMG group, the expression levels of ERα and PR in the mammary tissues of mice significantly decreased, while the expression level of ERβ significantly increased. In the TCTP ^{KI} group, this phenomenon reversed. These results indicate that by regulating the expression of the TCTP protein in breast tissue, it is possible to effectively alleviate the occurrence of HMG caused by abnormal sex hormones.

TCTP/Tpt1, also known as histamine release factor, p23 or fortilin, is a multifunctional protein that exists in almost all

eukaryotic organisms[9]. TCTP is involved in a large number of important cellular processes related to cell growth, cell cycle progression, malignant transformation and inhibition of cell apoptosis[8,24]. Currently, many studies have shown that TCTP can play a role in the occurrence and development of tumors by inhibiting cell apoptosis, especially the endogenous apoptotic pathway[25-27]. In the study of lung cancer found that overexpression of TCTP protein would reduce the expression of E-cadherin and p53, thereby increasing the migration and invasion ability of cancer cells [28]. Current research indicates that the dysregulation or mutation of TCTP expression level can lead to cell cycle arrest, microtubule stability and changes in cell morphology, thereby inducing pathological processes such as tumors [29]. In tumor research, it has been discovered that TCTP can inhibit the expression of P53 by regulating the AKT signaling pathway, thereby preventing apoptosis caused by external stress [29,30]. In the animal model of hyperplasia of the mammary glands, it was found that the expression of phosphorylated AKT was abnormally increased, and by inhibiting cell apoptosis, it promoted the occurrence of hyperplasia of the mammary glands [31]. Current research indicates that activated AKT phosphorylates BAD. The phosphorylation of BAD leads to the separation of BAD from mitochondrial Bcl-2, as well as the binding of p-BAD to the 14-3-3 scaffold protein, thereby inactivating the pro-apoptotic function of BAD [32]. In our study, it was found that the expressions of TCTP and p-AKT proteins increased in the breast tissues of mice with breast hyperplasia, while the expression of P53 protein decreased. At the same time, the expressions of p-BAD and Bcl-2 proteins increased, while the expression of Bax protein significantly decreased. Furthermore, in the HMG ^{CKO} group, we observed a significant

decrease in the expression of TCTP and p-AKT, alongside a marked increase in P53 protein levels within the mammary gland tissues. This was accompanied by reduced expression of Bcl-2 and p-BAD proteins and a notable upregulation of Bax. It is worth noting that completely opposite results were observed in the HMG ^{KI} group. In addition, studies have shown that blocking TCTP at the P53 binding site can upregulate the expression of P53, thereby inducing a dose-dependent reduction in the levels of CDK2, CDK4, CDK6, cyclin D1 and cyclin D3, leading to G0/G1 cell cycle arrest [33]. The essence of HMG is that breast cells undergo excessive proliferation, and cell proliferation is controlled by the cell cycle. Estrogen can induce the overexpression of the Cyclin D1 protein. When the expression of Cyclin D1 protein increases, it can make cell proliferation more active[34]. In our study, it was found that by knocking out the TCTP gene during the HMG process, the expressions of Cyclin D1, CDK4, and CDK6 could be inhibited, and the expression of P27 could be increased, which had a blocking effect on the cell cycle of breast cells. However, when TCTP was overexpressed, the opposite result occurred. This also indicates that TCTP, during the occurrence of HMG, alters the cell cycle and thereby influences the course of the disease.

Conclusion

In summary, in our study, by establishing mice with specific knockout and overexpression of TCTP genes for breast TCTP, we prepared the HMG model. We found that after TCTP knockdown during HMG occurrence, it could significantly inhibit the proliferation of mouse mammary glands, and pathological damage could be significantly alleviated, and the level of hormonal disorder could also be effectively alleviated. This phenomenon was clearly reversed in the HMG model with TCTP overexpression.

Furthermore, in the HMG model, we demonstrated that TCTP can inhibit apoptosis and promote the cell cycle through the AKT/P53 signaling pathway (Figure 5), which is also a possible mechanism for the formation of HMG. Additionally, our research has some limitations. It must be acknowledged that the MMTV-Cre driver system used in this study, although widely employed for mammary epithelial-specific genetic manipulation, may exhibit low-level extra-mammary activity under certain conditions. The systemic hormonal profile changes we observed in HMG ^{cKO} and HMG ^{KI} mammary hyperplasia mice could result from systemic endocrine feedback triggered by mammary-specific genetic manipulation, but the potential direct contribution of Cre activity in tissues such as the ovaries or pituitary gland cannot be entirely ruled out. Future studies employing rigorous tissue-specific Cre reporter assays and multi-organ TCTP expression profiling are warranted to definitively clarify the precise origin of these changes. Finally, in the subsequent research, through in vitro and in vivo experiments, the mechanism of action of TCTP in HMG will be fully elucidated, laying the foundation for the subsequent drug development.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author (Jun-Fei Zhang E-mail:zhangjunfei007@126.com) on reasonable request.

Author Contributions

ZJF, ML, MMY□LJ, WS and JXM designed the study. SY,CYM,YB and DHH performed multiple experiments and wrote the manuscript.

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Ethical Statement

All animal procedures were carried out in accordance with the regulations of the Animal Protection Committee of Ningxia Medical University, and all experimental procedures were approved by the Ethics Committee of the General Hospital of Ningxia Medical University(KYLL-2022-0094).

Conflict-of-interest statement

The author declared no conflicts of interest in this work.

Patient consent for publication

Not applicable.

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Figure 1 TCTP protein is highly expressed in HMG mice

A-B: Representative images (A) and statistical quantification

images (B) of TCTP protein expression in mammary tissue of the control group and the HMG group in WT mice analyzed by Western blot technique. **C-D:** Representative images (C) and statistical quantification images (D) of TCTP protein expression in mammary tissue of the control group and the HMG group in TCTP ^{cKO} and TCTP ^{KI} mice analyzed by Western blot technique. **E:** Representative images of TCTP protein expression in mammary tissues of the control group and the HMG group in WT, TCTP ^{cKO} and TCTP ^{KI} mice. **F:** Representative H&E images and representative images of Ki67 protein expression in mammary tissues of the control group and the HMG group in WT, TCTP ^{cKO} and TCTP ^{KI} mice. **G**: Statistical images of the pathological scores of mammary tissue in mammary tissues of the control group and the HMG group in WT, TCTP ^{cKO} and TCTP ^{KI} mice.. **H**: Statistical images of Ki67 protein expression in mammary tissues of the control group and the HMG group in WT, TCTP ^{cKO} and TCTP ^{KI} mice. Data are expressed as mean ± SD, compared with the control group, #P<0.05; compared with the WT HMG group, *P<0.05.

Figure 2 TCTP protein affected hormone levels in HMG mice

A-D: Expression levels of E_2 (A), FSH (B), PROG (C), and LH (D) in serum of control group, HMG group, HMG ^{cKO} group and HMG ^{KI} group. **E:** Representative images of ERα, ERβ, and PR protein expression in mammary tissues of the four groups of mice. **F-H**: Statistical images of ERα (F), ERβ (G) and PR (H) protein expression in mammary tissues of the four groups of mice. Data are expressed as mean ± SD, compared with the control group, #P<0.05; compared with the HMG group, *P<0.05.

Figure 3 The TCTP/AKT/P53 signaling pathway regulates HMG

A: Representative images of TCTP, P53 and p-AKT protein

expression in mammary tissue of control group, HMG group, HMG ^{cKO} group and HMG ^{KI} group mice analyzed by Western blot technique. **B-D**：Statistical quantification images of P53 (B), TCTP (C) and p-AKT (D) protein expression in mammary tissue of control group, HMG group, HMG ^{cKO} group and HMG ^{KI} group mice. **E:** Representative images of TCTP (green), P53 (red) and p-AKT (purple) protein expression in mammary tissue of control group, HMG group, HMG ^{cKO} group and HMG ^{KI} group mice analyzed by mIHC. Data are expressed as mean ± SD, compared with the control group, #P<0.05; compared with the HMG group, *P<0.05.

Figure 4 TCTP inhibits apoptosis and promotes the cell cycle during the occurrence of HMG. A: Representative images of Bax, p-BAD and Bcl-2 protein expression in mammary tissue of control group, HMG group, HMG ^{cKO} group and HMG ^{KI} group mice analyzed by Western blot technique. **B-D:** Statistical quantification images of Bax (B), Bcl-2 (C) and p-BAD (D) protein expression in mammary tissue of control group, HMG group, HMG ^{cKO} group and HMG ^{KI} group mice. **E:** Representative images of Bax, p-BAD and Bcl-2 protein expression in mammary tissues of the four groups of mice. **F-H**: Statistical images of Bcl-2 (F), Bax (G) and p-BAD (H) protein expression in mammary tissues of the four groups of mice. **I-L:** Statistical quantification images of CDK4 (I), CKD6 (J), P27 (K) and Cyclin D1 (L) mRNA expression in mammary tissue of control group, HMG group, HMG ^{cKO} group and HMG ^{KI} group mice.

Figure 5 The mechanism diagram shows that TCTP regulates the AKT/P53 signaling pathway, inhibiting cell apoptosis and promoting the cell cycle progression, which leads to the occurrence of HMG.