Estrogen Promotes Pituitary Prolactinoma by Upregulating TLR4 / NF- κ B/p38MAPK Pathway

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Primary research

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Abstract

Background: Prolactinomas have harmful effects on human health, and the pathogenesis is still unknown. Furthermore, the morbidity of women is much more than man, maybe related with estradiol level. Thus, it is important to reveal the pathogenesis and develop new therapeutic methods for prolactinomas.

Methods: Immunofluorescence analysis or Immunohistochemistry analysis were performed on the ER β , TLR4 and prolactin (PRL) expressions of pituitary gland in C57BL/6 mice and human prolactinoma specimen. In the present study, the role of TLR4 in prolactinoma was determined using estradiol-induced mice models in C57BL/6 wild-type (WT) and TLR4 $-/-$ mice. MMQ cells were treated with estradiol, fulvestrant, LPS or transfected with different TLR4 small interfering RNA, which to study ER β , TLR4 and PRL expression in MMQ cells. Co-immunoprecipitates analysis was used to investigate the interaction between ER β and TLR4.

Results: Immunofluorescence analysis or Immunohistochemistry analysis showed that PRL and TLR4 expression were co-located and increased in the pituitary gland of mice and human prolactinoma specimen compared with the control specimen. It was shown that PRL and TLR4 expression was co-located and increased significantly in the pituitary gland of estradiol-injected prolactinoma mice compared with the control mice. Knockout of TLR4 significantly inhibited tumor overgrowth, and PRL expression was decreased in estradiol-induced mice through regulating TLR4/NF- κ B/p38MAPK pathway. Estradiol promoted PRL expression through regulating TLR4/NF- κ B/p38MAPK pathway in vitro study, and pre-Inhibiting ER β or TLR4 reverse the effect, while simultaneously activating ER β and TLR4 enhanced PRL expression than activating single ER β or TLR4. Furthermore, ER β co-immunoprecipitates with endogenous TLR4 was assessed by co-immunoprecipitation analysis.

Conclusions: These results suggest that estradiol promoted prolactinoma development by activating the TLR4/NF- κ B/ p38MAPK pathway through Er β and TLR4 knockout inhibited the proliferation and secretion of prolactin in prolactinoma.

Background

Pituitary tumors are the most common tumors in the sella turcica region, accounting for about 15% of intracranial tumors[1, 2]. The rate of pituitary tumors detected at autopsy was about 10% in people without pituitary disease before birth, and up to 17% in healthy people by Magnetic Resonance Imaging(MRI)[3]. Prolactinomas are the most common subtype of pituitary tumors, accounting for about 40% of pituitary tumors[3, 4]. Bromocriptine is the only available drug for the treatment of prolactinoma in China, and drug resistance often occurs[5, 6]. Prolactinomas cause severe harm to the human body with various symptoms, such as amenorrhea, galactorrhea, sexual dysfunction, infertility and hyperprolactinemia[7–9]. At present, the pathogenesis of prolactinoma is not clear, and the drug

development and clinical therapeutic target exploration need to be studied urgently. Therefore, it is very necessary to study the pathogenesis of prolactinoma.

Estradiol-injected rats or mice is the most extensively acceptable prolactinoma animal models[10]. Estrogen plays an important role in the development of prolactinoma[11, 12], possible mechanism is binding to estrogen receptor (ERs), a nuclear steroid hormone receptor widely present in pituitary tissues[13], mainly including ER α and ER β . ER β and its isoforms have wider tissue distribution, including the gastrointestinal tract, lung, and brain, than the traditional estrogen receptor-ER α . The significance of ER β expression in tumors was first demonstrated in breast cancer. Several studies demonstrated that women with ER β -positive breast cancers treated with adjuvant tamoxifen have better survival. There is evidence that ER β may play an important role in ER α -/ER β +(proliferative) tumors [14, 15]. Furthermore, estradiol can promote IL-6 expression through ER β , and then stimulate the proliferation of pituitary cells and excessive secretion of prolactin, promoting the development of prolactinoma, which is also one of the pathogenesis of prolactinoma[16–18]. TLR4, an important member of the Toll-like protein family, locates in the cell membrane and cytoplasm as a pattern recognition receptor, which plays an important role in mediating natural immunity and inflammation. TLR4 is specifically activated by lipopolysaccharide (LPS) from gram-negative bacterial cell walls[19]. Activation of TLR4 on the respiratory epithelium activates myeloid differentiation primary response 88 (myd88) adaptor protein-dependent signaling, which phosphorylates and activates downstream signaling pathways, leading to host defense responses, including the production of inflammatory cytokines[20, 21]. TLR4 may also allow tumor cells to escape host immune surveillance through the MyD88 signaling pathway[22, 23]. Accumulative evidence showed that TLR4 overexpression might contribute to the development and progression of human malignancies, including liver cancer, lung cancer, breast cancer and ovarian cancer[24–27]. TLR4 is considered to have a very positive promoting effect in the development of many tumors.

Activation of the downstream p38MAPK pathway by estrogen receptors such as ER β play an important role in the development of prolactinoma. Our previous research has confirmed that the tumor overgrowth, and PRL expression was decreased significantly in estradiol-induced MAPK14-/- mice[28]. It has been reported that ER β and TLR4 protein interact to conduct signaling pathway and activate downstream p38MAPK to promote tumor genesis in non-small cell lung cancer [24]. We confirm that the TLR4 expression is elevated in prolactinomas. In turn, blocking the TLR4 activation-mediated NF- κ B/p38MAPK pathway inhibited the pituitary tumor growth and PRL production. Therefore, our research provide evidence for the use of TLR4 or p38MAPK inhibitors for prolactinoma treatments, and elucidate the molecular mechanism of estradiol-induced prolactinoma.

Materials And Methods

Human pituitary gland specimens

Pituitary gland paraffin samples were collected from 20 prolactinoma patients and 10 patients with other pituitary diseases, all of whom underwent surgical excision at the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology. All samples of participants were anonymized. The present study was approved by the Institutional Research Ethics Committee of the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology.

Animals and cell lines

Female C57BL/6 mice (weighing 20 ± 2 g) were purchased from Hubei Experimental Animal Center. Toll-like receptor 4 deficient (TLR4^{-/-}) mice were purchased from GemPharmatech co., Ltd. (Nanjing, China). All animal experiments were approved by the Ethics Committee of Tongren Hospital Affiliated to Wuhan University, The Third Hospital of Wuhan. All the mice were housed and bred in specific pathogen-free (SPF) grade cages and provided with autoclaved food and water. All animal experiments have complied with the Animal Research: Reporting of In Vivo Experiments guidelines and have been conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The MMQ cell line was purchased from the Beijing Beina Chuanglian Biotechnology Research Institute, Beijing, China.

Prolactinoma animal models

The Estradiol-treated rat is a well-known model of pituitary lactotroph hyperplasia and hyperprolactinaemia [10, 29]. The prolactinoma model was established by intraperitoneal injection of estradiol oil in mice (concentration of 1 mg/1 mL, 0.1 mL for each mouse once), once every 5 days and lasted for 50 days. The mice were divided into four groups (n=10): control group, estradiol-induced prolactinomas group, TLR4^{-/-} group, estradiol-induced TLR4^{-/-} group. All mice were prepared into prolactinomas model by intraperitoneally injecting estradiol oil except control group and TLR4^{-/-} group mice. The control group and TLR4^{-/-} group mice were injected with 1 mL of Castor oil for each mouse.

Cell culture

MMQ cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (SeraPro, South America) at 37°C under 5% CO₂. MMQ cells (5×10^5 per well) seeded in six-well plates were treated with lipopolysaccharide (LPS, MCE), or 17 β -estradiol (E2, MCE), or fulvestrant (Ful, MCE) for 48 h.

RNA interference

In total, three different sets of small interfering (si)RNA sequences against TLR4 were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences of the three siRNA sequences used were: i) GCTATAGCTTCACCAATTT; ii) GCAGCAGGTCGAAT

TGTAT; and iii) CCTAGAACATGTGGATCTT. A total of 2×10^5 MMQ cells/well were seeded into a 6-well plate and transfected with the different TLR4 siRNA separately. The cells were transfected using

Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA or protein was extracted 48 h later.

Enzyme-linked immunosorbent assay (ELISA)

The blood samples of mice were collected from the orbit. The serum was then isolated by centrifugation immediately. For the PRL ELISA, 50 μ L of animal serum and 100 μ L HRP labeled detection antibody were used to coat a 96-well plate 1 h at 37°C. After being washed 5 times with 0.1% Tween (PBS solution). Samples were incubated with the 50 μ L substrate A and B at 37°C for 15 min and then add stop solution for 50 μ L, and 450 nm was set as the absorbance wavelength. The PRL ELISA kit was purchased from LunChangShuo Biotech Co. #SU-B20246 (Xiamen, China).

Western blotting method

The pituitary glands of mice were obtained and stored at -80°C. The samples were placed in protein lysis buffer with protease inhibitors (Roche Diagnostics, Basel, Switzerland) to lyse the tissue protein in the homogenizing tubes and were subsequently homogenized using the tissue homogenizer (Wuhan Servicebio Technology Co., Ltd.). The protein concentrations of animal tissues or cell lysates were determined based on the Bradford protein assay. Equal amounts of proteins were separated with 10% or 12% SDS-PAGE, electro-transferred on nitrocellulose membranes and then blocked with 5% milk for 1 h. The membranes were then incubated with primary antibodies at 4°C overnight. The used antibodies were as follows: prolactin (PRL) antibody from rabbit (Affinity Biosciences, 1:6000), PRL antibody from goat (R&D Systems, 1:6000), TLR4 antibody from rabbit (Affinity Biosciences, 1:2000), ER β antibody from rabbit (Affinity Biosciences, 1:2000), Myd88 antibody from rabbit (Affinity Biosciences, 1:2000), p38MAPK antibody from rabbit (Affinity Biosciences, 1:1000), NF- κ B p65 antibody from mouse (Proteintech, Wuhan, China, 1:2000) and β -actin antibody from mouse (Abclonal Biotech Co., Wuhan, China, 1:3000). After three washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and were visualized by ECL Western blotting detection reagents (Shanghai Jiapeng) with the Fluor-S-Multi Imager (Shanghai Jiapeng, JP-K600, Shanghai). All protein expressions of samples were quantified by Quantity One 4.2.1 software. The band intensity was normalized to the β -actin band.

Immunofluorescence assay

The tissue specimens of animal or human pituitary glands were routinely formalin-fixed, paraffin-embedded and sectioned in 5 μ m pieces for immunofluorescence assays. Paraffin sections were dewaxed with water and then filled with ethylenediaminetetraacetic acid antigen repair buffer (pH 8.0) at 70°C for 20 min. After the sections were slightly dried, they were rinsed with flowing water, and incubated with BSA for 30 min. Primary antibody against TLR4 (1:1000, Affinity Biosciences), ER β (1:2000, Affinity Biosciences) and PRL (1:1000, R&D Systems) were added and incubated with sections overnight at 4°C. After three PBS washes, the sections were incubated with secondary antibody for 50 min and then sealed with anti-fluorescence quenching tablet. The fluorescence images of sections were acquired under a Leica microscope and analyzed using Leica Application Suite X Software (Leica DFC450 C, Leica Microsystems Inc., Germany).

Immunohistochemistry

The human and mice pituitary gland or tumor specimens were paraformaldehyde-fixed and paraffin-embedded and were cut into 3 mm-thick sections. After fixation onto glass slides, they were rehydrated in an ethanol gradient. Furthermore, 3% of H₂O₂ solution was added to devitalize the endogenous catalase, and 0.01 M of sodium citrate solution was added to extract the antigen. The slides were then stained with anti-TLR4 (1: 1000, Affinity Biosciences), ERβ (1: 2000, Affinity Biosciences), p38MAPK antibody from rabbit (Affinity Biosciences, 1: 1000), and PRL (1: 1000, R&D Systems) antibody after blocked by 5 % of BSA. All tissues were visualized by incubation with goat anti-rabbit immunoglobulin G (IgG) (H+L)-HRP (1:100, Wuhan Servicebio, China). After being immunostained with a 3,3'-diaminobenzidine tetrahydrochloride immunohistochemistry color development kit (Wuhan Servicebio, China), the slides were fixed with Mayer's hematoxylin. The stained tissues were photographed and analyzed using a Leica microscope (Leica DFC450 C, Leica Microsystems Inc., Germany) at 100× and 200× and 400× magnifications. The quantification of the expressions was assessed by manual counting of positive and negative cells per highpower visual field.

Co-immunoprecipitation assay

Cultured MMQ cells were placed into 10cm dishes and treated with E2. The cells were then washed in phosphate-buffered saline (PBS) and resuspended in 1 ml of IP lysis buffer supplemented with phosphatase inhibitor and protease inhibitor cocktails (Wuhan Servicebio, China), and the cell lysates were ultrasonicated. After rotating at 4°C for 30 min, the cell lysates were collected and precleared by centrifugation at 12000 rpm at 4°C for 10 min. Add 1.0μg IgG (the same species as the antibody used for IP experiment) and 20μL protein A/G beads to the supernatant of negative control (IgG) group (fully mixed before use). Add 20μL protein A/G beads directly to the experimental group, shake and incubate at 4°C for 1h and centrifuged for 5min(2000g) at 4°C, supernatant was taken. For each pull-down, 5 μg of anti-ERβ Ab (dilution 1:3000, 14007-1-AP; Proteintech) or anti-TLR4 Ab (dilution 1:500, SC-293072; Santa) was added to the normalized lysate, and the mixture was incubated overnight at 4°C. Immune complexes were then precipitated with protein A/G plus agarose (Millipore). Immunoprecipitates were collected by centrifugation (2000g, 4°C, 5min) and washed gently with lysis buffer. Immunoprecipitation samples were mixed with protein loading buffer for immunoblotting.

Statistical analysis

All quantitative data are presented as mean ± S.D. Statistical comparison were analyzed by the one-way ANOVA or Student's t-test using SPSS software (version 19.0). P value < 0.05 was considered statistically significant.

Results

ERβ correlates with TLR4 expression positively in prolactinoma tissue

To identify the potential role of ER β and TLR4 in the development and progression of prolactinoma, the pituitary glands of 20 prolactinoma patients and 10 patients with other pituitary diseases were collected and the expression of ER β and TLR4 were evaluated by immunohistochemistry. Our results showed that expression of ER β was negative in pituitary gland tissues while positive in tumor tissue, and that TLR4 was highly expressed in prolactinoma tissue than that of control. (Fig. 1A, C) Compared with pituitary gland tissues, the H-score of both ER β ($P<0.01$) and TLR4 ($P<0.01$) were increased significantly in prolactinoma tissues. (Fig. 1B, D) Furthermore, the H-score of ER β -positive tissues was highly correlated with the level of TLR4 overexpression in prolactinoma tissues (Spearman's correlation coefficient, $r=0.873$, $P<0.001$; Fig. 1F). TLR4 protein was found to be co-located with PRL protein in five samples of human prolactinomas by immunofluorescence. (Fig. 1E)

ER β activation promotes TLR4/NF- κ B/p38MAPK expression in MMQ cells

To study whether estradiol activation promotes TLR4/NF- κ B/p38MAPK expression, MMQ cells were treated with different concentrations of estradiol at different time points. Our results showed that 1 μ M of estradiol was most effective in inducing expression of PRL at 48h after administration of MMQ cells, meanwhile, PRL protein expression was increased significantly ($P<0.05$), along with elevated ER β ($P<0.01$), TLR4 ($P<0.01$), NF- κ Bp65 ($P<0.05$) and p38MAPK ($P<0.05$) proteins detected via Western blotting. Fulvestrant is a highly effective estrogen receptor antagonist, 1 μ M of Fulvestrant inhibited the expression of ER β and TLR4 at 48 h after administration of MMQ cells most significantly. Therefore, 1 μ M of estradiol and 1 μ M of fulvestrant were chosen as last induction concentration. (Fig. 2A, B, F, G and H) The up-regulation of the TLR4/NF- κ B/p38MAPK pathway proteins induced by estradiol was inhibited after preadministration of fulvestrant by blocking ER β . In comparison with estradiol-induced MMQ cells, PRL proteins of those cells treated with preadministration of fulvestrant were decreased significantly, and PRL proteins ($P>0.05$) did not change significantly in estradiol-induced MMQ cells in conjunction with change in ER β ($P>0.05$), TLR4 ($P>0.05$), Myd88 ($P>0.05$), NF- κ Bp65 ($P>0.05$) and p38MAPK ($P>0.05$) proteins quantified via Western blotting. (Fig. 2D and J)

TLR4 knockdown by siRNA inhibit PRL protein expression in MMQ cells

MMQ cells were transfected with different sets of TLR4 siRNA. Western blotting was used to determine the protein expression levels of TLR4. Compared with the NC group, transfection of TLR4 siRNA significantly decreased the expression of TLR4 in MMQ cells. ($P<0.01$, Fig. 2C and I) Activation of ER β and TLR4 expression synergistically promotes PRL protein expression in MMQ cells.

After confirming that exposure to E2 induced endogenous interaction of ER β and TLR4 and increased TLR4 protein levels, the PRL expression of the MMQ cell was assessed and mediated by exposure to E2, LPS or combined administration of E2+LPS. In addition, the role of TLR4 in prolactinoma by setting up

siTLR4 and E2+siTLR4 group was observed. Compared with the E2-treated group, PRL protein expression decreased significantly ($P<0.05$), along with decreased ER β ($P<0.05$), TLR4 ($P<0.01$), Myd88 ($P<0.05$), NF- κ B p65 ($P<0.05$) and p38MAPK ($P<0.01$) proteins in E2+siTLR4 cell groups detected via Western blotting. Our results suggest that TLR4 knockdown downregulated the TLR4/NF- κ B/p38MAPK pathway activation induced by estradiol, and inhibited PRL protein expression in MMQ cells ($P<0.05$). Furthermore, Western blotting revealed that the cells treated with E2+LPS significantly increased PRL ($P<0.05$), ER β ($P<0.05$), TLR4 ($P<0.05$), Myd88 ($P<0.05$), NF- κ Bp65 ($P<0.05$) and p38MAPK ($P<0.05$) protein expression, compared with the only E2 or LPS treated groups. (Fig. 2E and K) Taken together, our findings indicated that activation of ER β and TLR4 synergistically promoted the PRL expression and secretion in MMQ cells.

ER β co-interacts with TLR4 protein in MMQ cell lines

The result showed that TLR4 protein co-immunoprecipitates with endogenous ER β . After pre-precipitated TLR4 labeling, ER β proteins were also expressed. (Fig. 3)

ES mice model was established successfully with the volume elevation of pituitary gland

The resultsshowed that the volume of the pituitary gland and tumor/body weight (Fig. 4A, B, $P<0.01$) were elevated significantly in estradiol-induced prolactinoma mice compared with WT mice, The ELISA results showed that serum PRL levels of prolactinomas model mice were significantly elevated compared with those of WT mice (Fig. 4C, $P<0.01$), indicating that the prolactinoma model was established successfully.

The ER β and TLR4/NF- κ B/p38MAPK pathway activated in the pituitary gland of estradiol-induced prolactinomas mice.

Western blot results showed that compared with WT mice, the PRL protein of pituitary glands increased significantly in estradiol-induced prolactinomas mice ($P<0.01$). Furthermore, the expression of ER β ($P<0.05$), TLR4 ($P<0.01$), Myd88 ($P<0.05$), NF- κ Bp65 ($P<0.01$), p38MAPK ($P<0.05$), BMI1 ($P<0.01$) and Bcl2/Bax ($P<0.05$) proteins of the pituitary glands in prolactinomas mice were increased significantly compared with those in control mice (Fig. 4D and E). In summary, The ER β and TLR4/NF- κ B/p38MAPK pathway activated in the pituitary gland of estradiol-induced prolactinomas mice.

TLR4 knockout inhibited the pituitary tumor overgrowth and PRL expression and secretion in prolactinoma mice

We observed the effects of TLR4 knockout on the pituitary tumor growth, PRL expression and secretion in estradiol-induced prolactinoma mice. In comparison with WT mice, the pituitary gland weight/body weight ($P<0.001$) and serum PRL levels ($P<0.01$) were increased significantly in estradiol-induced prolactinoma mice, along with elevated ER β ($P<0.05$), TLR4 ($P<0.05$), Myd88 ($P<0.01$), NF- κ Bp65 ($P<0.05$), p38MAPK ($P<0.05$) and PRL ($P<0.05$), BMI1 ($P<0.01$) and Bcl2/Bax ($P<0.05$) proteins detected via Western

blotting or IHC. Furthermore, compared with those in estradiol-induced prolactinoma mice, the pituitary gland weight/body weight ($P<0.001$) and serum PRL levels ($P<0.01$) were decreased significantly in estradiol-induced TLR4^{-/-} mice in conjunction with reductions in ER β ($P<0.05$), TLR4 ($P<0.05$), Myd88 ($P<0.05$), NF- κ Bp65 ($P<0.01$), p38MAPK ($P<0.01$), PRL ($P<0.01$), BMI1 ($P<0.01$) and Bcl2/Bax ($P<0.05$) proteins quantified via Western blotting or IHC. Compared with those in TLR4^{-/-} mice, the pituitary gland weight/body weight ($P>0.05$) and serum PRL levels ($P>0.05$) did not change significantly in estradiol-induced TLR4^{-/-} mice in conjunction with change in ER β ($P>0.05$), TLR4 ($P>0.05$), Myd88 ($P>0.05$), NF- κ Bp65 ($P>0.05$), p38MAPK ($P>0.05$), PRL ($P>0.05$), BMI1 ($P<0.01$) and Bcl2/Bax ($P<0.05$) proteins quantified via Western blotting or IHC (Fig. 5A, B, C, D, E and F). Furthermore, immunofluorescence analysis showed that comparison with WT mice, the expression of ER β , TLR4 and PRL proteins increased significantly in the pituitary gland of estradiol-induced prolactinoma mice, but this phenomenon disappeared in TLR4 knockout mice. (Fig. 5G) This is consistent with our previous western blot results.

Discussion

The current theory on the pathogenesis of prolactinomas includes the effects of endocrine hormone, abnormal expression of genes, and microRNA irregular expression[30, 31]. However, the exact pathogenesis of prolactinomas was still not clear. The gender difference in the occurrence of prolactinoma has long existed in the clinical, especially in the period of 20-30 years old, the incidence ratio of male and female even reached 1:10. The reason for this phenomenon may be the high estrogen level of women at this stage.[32] The role of estrogen is well established in promoting prolactinoma, DRD2 Knockout mice and estradiol-injected rats or mice are the most extensively acceptable prolactinoma animal models[10, 28, 29], Estradiol-injected mice was selected in the present study to clarify the role of estradiol in prolactinoma. ERs can regulate Toll-like receptor (TLR) signaling pathways in the immune system[33], Additionally, a previous study reported that estrogen promotes upregulation of TLR4/NF- κ B/p38MAPK activation via ER β [24]. Based on these previously results, we suppose that estrogen-activated ER β /TLR4/NF- κ B/p38MAPK pathway participated in the pathogenesis of prolactinomas.

TLR4 plays a fundamental role in pathogen recognition and activation of innate immunity, and is often overexpressed in malignant and tumour-infiltrating immune cells. TLR4 plays a crucial role in mesenchymal stem cell (MSC) induced inhibition of natural killer (NK) cell function. TLR4 ultimately activates the transcription factor NF- κ B by linking to MyD88, which is required for the expression of cytokines, chemokines, and other stimulating molecules such as TNF- α , IL-1 β , IL-6, IL-8, IL-12, and MIP 1 α . [34] Various stimulators induce activation of the p38MAPK, an important kinase involved in cell proliferation and apoptosis.[35] The proliferation-related proteins B-cell-specific Moloney murine leukemia virus insertion site1 (BMI1), Bcl2, and Bax were determined in the pituitary gland via western blot. The BMI1 and Bcl2/Bax protein expression of estradiol-treated mice are increased significantly compared with WT mice. However, this effect reversed in estradiol-treated TLR4^{-/-} mice. The results proved that estrogen-induced prolactinoma may be related to pituitary proliferation.

TLR4 is highly expressed in various kinds of tumor cells, and activation of TLR4 mediated p38MAPK promotes tumor growth and proliferation[36, 37]. Therefore, TLR4 can be a therapeutic target. In this study, immunohistochemistry analysis showed that ER β and TLR4 were overexpressed, and TLR4 was co-located with PRL protein in human prolactinoma tissues by immunofluorescence analysis. The high expression and positive correlation of TLR4 and ER β are first found in clinical samples of prolactinoma. Furthermore, TLR4 co-located with PRL, a marker of prolactinoma, laying a foundation for subsequent in vitro and in vivo studies.

Western blot results also showed that the expression of ER β , TLR4 and PRL were increased in the pituitary tissues of estradiol-treated mice. Furthermore, TLR4 knockout significantly inhibited tumor overgrowth and PRL secretion, and ER β , TLR4 and PRL protein expression in estradiol-induced mice. It was further verified that TLR4 is a potential target of prolactinomas. It is reported that estradiol and ERs regulate inflammatory pathways of innate immune cells, including dendritic cells and macrophages[33]. As a classic protein regulating innate immunity and inflammation, TLR4 has many interactions with estrogen[38]. Therefore, excessive estrogen perhaps was a key cause that influenced immune and inflammatory processes in the body. The TLR4 activation effect of estradiol have been proved in vitro and in vivo experiments in our study, thus we have sufficient evidence to verify the role of TLR4 in estradiol-mediated prolactinoma. In order to verify the role of estradiol in the ER β /TLR4/NF- κ B/p38MAPK pathway in prolactinoma, the effects of inhibition and activation of ER β on TLR4/NF- κ B/p38MAPK pathway and PRL expression were investigated in MMQ cells in vitro. MMQ cells were treated with estradiol and fulvestrant, which can activate or inhibit the protein expression of ER β /TLR4/NF- κ B/p38MAPK pathway and PRL expression. Meanwhile, the expression of TLR4/NF- κ B/p38MAPK pathway was inhibited after the transfection knockdown of TLR4, and the expression of ER β was also decreased, which indicate that there may be an interaction between TLR4 and ER β . Then TLR4 protein directly binding to ER β protein were found by immunoprecipitation, these results indicate that both TLR4 and ER β play important roles in mediating the occurrence of prolactinoma. In addition, the expression of p38MAPK and PRL were increased significantly after treatment with LPS and E2 than with the single LPS or E2, which proves that the interaction of TLR4 and ER β can synergistically regulate prolactin expression and tumor growth.

Our study first reported the role of the TLR4/NF- κ B/p38MAPK pathway in prolactinoma and TLR4 knockout inhibited the proliferation and secretion of prolactin in prolactinoma mice effectively. The limitation of this study is that only paraffin specimens of human prolactinoma tissues were obtained, and more detailed clinical data were not obtained, such as the relationship between tumor size and TLR4 positive expression in tissues. Moreover, drugs targeting inhibition of TLR4/p38MAPK in pituitary tissue for prolactinoma have not been developed, further research is needed. In conclusion, previous studies of our group demonstrated that MAPK14 and NLRP3 knockout can inhibit the proliferation and secretion of prolactin in prolactinoma. Therefore, this study focused on exploring the role of their upstream protein TLR4 in prolactinoma and whether estradiol-mediated prolactinoma genesis is mediated by TLR4/NF- κ B/p38MAPK pathway. We reveal the correlation between the interacting ER β and TLR4 signaling pathways. In addition, we demonstrated that E2 or LPS promoted PRL expression and prolactin tumor

proliferation through the p38MAPK pathway. Further research on drugs targeting estrogen receptor and TLR4/NF- κ B/p38MAPK pathway is the focus of our research, and new drug development based on these targets may provide new treatment strategies for prolactinoma.

Conclusion

This study firstly demonstrated that estrogen activated TLR4/NF- κ B/p38MAPK pathway and promoted the development of prolactinomas, which provided new therapeutic targets for drug study in future.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Tongren Hospital Affiliated to Wuhan University, The Third Hospital of Wuhan. All samples and data from participants were anonymized and their written informed consent was obtained. The present study was approved by the Institutional Research Ethics Committee of the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication NO.85–23, revised 1996). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study and supporting our findings are included and can be found in the manuscript. The raw data can be provided by corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

YZ performed the majority of experiments and drafted the manuscript. QD, SW, QW, HZ and PN helped with experiments and analyzed the data. XW, YC and JW conceived the study, supervised the experiments and revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

ERβ and TLR4 were highly expressed in human prolactinoma specimens. (A)–(C) Images of ERβ and TLR4 expression in normal pituitary tumor–pituitary gland tissue pairs obtained in conjunction with immunohistochemistry (×100, scale bar represents 100 μm; ×200, scale bar represents 50 μm; ×400, scale bar represents 20 μm). Arrow indication is ERβ or TLR4 protein expression. (B)–(D) Histochemistry scores (H scores: [% weakly stained cells × 1] + [% moderately stained cells × 2] + [% strongly stained cells × 3]) of ERβ and TLR4 expression in 20 normal tissue pairs of the pituitary tumor and 10 pituitary gland. **P < 0.01 vs pituitary gland group (n = 10). (E) Fluorescence images of TLR4 and PRL protein expressions in normal human pituitary tumor–pituitary gland tissue pairs by immunofluorescence (×400). Scale bar represents 20 μm. Arrow indication is TLR4 or PRL protein expression. (F) The correlation between ERβ and TLR4 expression in human prolactinoma specimens. ERβ, estrogen receptor β; TLR4, Toll-like receptor 4.

Figure 2

Protein expression of ER β , TLR4, and the downstream myd88/NF- κ B/p38MAPK axis in MMQ cell treated with estrogen and estrogen inhibitors. (A)–(F) The synchronized cells were exposed to E2 at different concentrations (0, 0.01, 0.1, 1 μ M) and Ful at different concentrations (0.1, 1 μ M) for 48 h. Protein expression of ER β , TLR4 axis was analyzed using western blot analysis. The data represent mean $\pm$ SD from three different experiments. E2 stimulated ER β , TLR4 axis response in the MMQ cell line in a dose-dependent manner. (B)–(G)–(H) The synchronized cells were treated with E2 at different time points (0, 6, 12, 24 and 48 h) at concentrations of 1 μ M. The data represent means $\pm$ SD from three different experiments. E2 stimulated ER β –TLR4 response in a time-dependent manner in the MMQ cell. (C)–(I) The knockdown efficiency of si-TLR4 was determined by Western blotting assay in MMQ cell. (D)–(J) Western blot analysis of ER β , TLR4 axis protein levels at 48 h in MMQ cells. Estrogen exposure significantly upregulated the expression of TLR4 and activated the myd88/NF- κ B/ p38MAPK pathway, while anti-estrogen drugs showed the opposite effect. (E)–(K) MMQ cells treated with negative control–E2 (1 μ M), si-TLR4-2, E2+ si-TLR4-2, LPS (1 μ g/ml) and E2+LPS. Data are presented as means $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001; #P < 0.05, ##P < 0.01, ###P < 0.001

Figure 3

ERβ co-interacts with TLR4 in MMQ cell, TLR4 co immunoprecipitates with endogenous ERβ as assessed by co-immunoprecipitation analysis.

Figure 4

Protein expression of ERβ, TLR4, myd88/NF-κB/p38MAPK axis in the pituitary gland of estradiol-induced prolactinomas (ES) mice. (A) Images of pituitary glands and (B) the pituitary gland weight/body weight in WT/ES(n=3). (C) Serum prolactin (PRL) levels of WT, estradiol-induced mice(n=3). (D) (E) ERβ, TLR4, myd88/NF-κB/p38MAPK axis protein expressions of pituitary gland in WT, estradiol-induced mice detected via Western blotting (n = 3).

Figure 5

TLR4 knockout inhibited the pituitary tumor overgrowth and PRL expression and secretion in estradiol-induced prolactinoma (ES) mice. (A) Images of pituitary glands and (B) the pituitary gland weight/body weight in WT, ES, TLR4(-/-) and ES + TLR4(-/-) mice (n = 10). (C) Serum prolactin (PRL) levels of WT, estradiol-induced, TLR4(-/-) and estradiol-induced TLR4(-/-) mice (n = 6). (D) ERβ, TLR4/myd88/NF-κB/p38MAPK axis protein expressions of pituitary gland in WT, estradiol-induced, TLR4(-/-) and estradiol-induced TLR4(-/-) mice detected via Western blotting (n = 3). (F) ERβ, TLR4, p38MAPK, PRL expression images of pituitary gland tissue in WT, estradiol-induced, TLR4(-/-) and estradiol-induced TLR4(-/-) mice obtained with IHC (×200). Scale bar represents 50 μM. Arrow indication is protein expression. (G) Fluorescence images of ERβ, TLR4 and PRL protein expressions in normal human pituitary tumor-pituitary gland tissue pairs by immunofluorescence (×400). Scale bar represents 20 μM. Arrow indication is ERβ, TLR4 or PRL protein expression. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs WT mice; #P < 0.05, ##P < 0.01, ###P < 0.001 vs ES mice; NS: P > 0.05.

Figure 6

Diagram of molecular mechanism of estradiol activate TLR4/NF-κB/p38MAPK pathway promoting prolactinoma development.