Mammary hydroxylated oestrogen activates the NLRP3 inflammasome in tumor-associated macrophages to promote breast cancer progression and metastasis

Han Zhao  
Xuzhou Medical University

Jiahao Xu  
Xuzhou Medical University

Ya'nan Zhong  
Xuzhou Medical University

Shiqing He  
the Affiliated Hospital of Xuzhou Medical University

Zhixiang Hao  
Xuzhou Medical University

Bei Zhang  
Xuzhou Central Hospital, Xuzhou Clinical School of Xuzhou Medical University

Zhao Liu  
the Affiliated Hospital of Xuzhou Medical University

Xueyan Zhou (✉️ zxy851107@xzhmu.edu.cn)  
Xuzhou Medical University

Research Article

Keywords: Oestrogen, metabolism, NLRP3 inflammasome, tumor microenvironment, breast cancer

Posted Date: September 29th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3381051/v1

License: ☕️ 📧 This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

**Background:** An imbalance of oestrogen homeostasis and an inflammatory tumor microenvironment (TME) are vital risk factors for the progression and metastasis of breast cancer.

**Methods:** The liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantitative analysis method for oestrogen active substances was used to detect the levels of oestrogen and its metabolites of 238 clinical serum samples, 6 clinical malignant breast cancer tissues and their corresponding non-tumor adjacent tissues and MMTV-PyMT mice mammary tissues. In vitro and in vivo experiments were conducted to investigate the correlation between oestrogen and tumor-associated macrophages (TAMs) and the role and mechanism of the NLRP3 inflammasome activated by oestrogen in promoting breast cancer progression and metastasis.

**Results:** Results of the present study demonstrated that tumor-associated macrophages (TAMs) were the main population of immune cells present in the breast TME. Thus, TAM-dependent tumor metastasis may be triggered by hydroxylated oestrogen via NOD-like receptor thermal protein domain associated protein 3 (NLRP3), inflammasome activation and IL-1β production. Mechanistically, TAM-derived inflammatory cytokines induced the expression of matrix metalloproteinases in breast tumor cells, leading to breast tumor invasion and metastasis. Collectively, these results revealed a previously unknown role of hydroxylated oestrogen in the reprogramming of the TME via NLRP3 inflammasome activation in TAMs. These mechanisms may facilitate breast cancer cell proliferation, migration and invasion.

**Conclusions:** The disorder of oestrogen metabolism in the breast, especially the abnormal elevation of hydroxylated oestrogen, may be a risk factor for the metastasis of breast tumors. Hydroxylated oestrogens act on TAMs to induce the generation of an inflammatory microenvironment in breast tumors, thereby regulating the progression of breast cancer.

**Background**

Breast cancer is the most common malignancy in females worldwide, and > 410,000 deaths are attributed to it every year [1]. Of the deaths caused by breast cancer, over 90% are attributed to metastasis-related complications [2]. Although significant advances have been made in diagnostics, surgery and the development of anti-cancer drugs, the therapeutic effects remain limited by the development of metastasis and therapeutic resistance.

Results of epidemiological and clinical studies demonstrated that factors associated with increased oestrogen levels in female patients throughout their lives may be associated with an increased risk of breast cancer development [3]. Oestrogen may promote the proliferation of breast cancer cells through the oestrogen receptor (ER), which acts as a transcription factor that regulates the expression of target genes encoding proteins with important biological functions [4]. Specific oestrogen metabolites, mainly catechol estrogen-3, 4-quinone (CE-3, 4-Q), may promote cancer progression through combining with DNA and forming urination adducts [5].
Results of our previous study demonstrated that oestrogen homeostasis is significantly dysregulated during the development of breast cancer [6]. The concentrations of hydroxylated oestrogens, such as 4-hydroxyestradiol/estrone (4-OHE2/1) and 2-hydroxyestrone (2-OHE2/1), are significantly higher in the breast tissue of experimental model rats with breast cancer [7]. However, the precise role of oestrogen in tumor progression, including the various forms of oestrogen metabolites, is not fully understood. In addition, the mechanisms underlying oestrogen homeostatic reprogramming in the progression and metastasis of breast cancer remains to be elucidated.

The inflammatory microenvironment generated by the infiltration of macrophages is an early event in the occurrence and development of breast cancer, and it is also a major indicator of a poor prognosis in patients with breast cancer [8–9]. Tumor-associated macrophages (TAMs) that infiltrate inside and around solid tumors account for > 50% of breast cancer-infiltrating immune cells [10–11]. TAMs secrete and produce growth factors, proinflammatory cytokines and chemokines in the breast tumor microenvironment (TME) [12]. Results of a previous study demonstrated that in the initial stage of breast cancer, TAMs in breast adipose tissue secrete and produce inflammatory cytokines, which create an inflammatory microenvironment that causes mutations and promotes tumor growth. In addition, TAMs increase the occurrence of ER+ breast cancer metastasis [13]. Results of a previous study demonstrated that TAMs produce a variety of cytokines that effectively stimulate the proliferation of tumor cells [14]. Therefore, the inflammatory microenvironment of breast tumors plays an important role in the development of breast cancer.

TAMs are the main cell type that express the functional inflammasome component, NLRP3 (NOD-like receptor thermal protein domain associated protein 3). As the malignancy of tumors increases, the number of macrophages that express NLRP3 increases significantly, and these macrophages are associated with the invasion and metastasis of breast cancer [15]. The NLRP3 inflammasome is composed of NLRP3 and the aptamer protein, apoptosis-associated speck-like protein containing a CARD (ASC), which activates the proinflammatory cytokine, IL-1β. On activation, IL-1β is matured and released via proteolytic enzyme caspase-1 activation [16]. Results of a previous study confirmed that IL-1β promoted the progression and metastasis of tumor cells through regulating the expression of matrix metalloproteinases (MMPs) [17]. The activation of the NLRP3 inflammasome is closely associated with the metastasis and exacerbation of breast cancer; however, the regulatory effects of oestrogen on the NLRP3 is yet to be fully elucidated.

The role of oestrogen signalling in auto-immunity is well-established, and the ER is expressed by multiple immune cell populations that affect regulation [18]. Typically, oestrogen causes the nuclear localization of the oestrogen/ER complex, which alters the transcription pattern of target genes, leading to tumor cell reprogramming and immune cell differentiation. However, oestrogen-induced non-canonical signalling pathway activation is also vital for the rapid function of oestrogen; however, the specific mechanism is yet to be fully elucidated [19]. Results of a previous study demonstrated that IL-1β, IL-18, NLRP3 and active caspase-1 levels are markedly increased in the hippocampus of ovariectomized female mice, suggesting that oestrogen deficiency leads to activation of the NLRP3, resulting in hippocampal
neuroinflammation, depression and anxiety [20]. Although the role of tumors, such as ovarian carcinoma, breast carcinoma and lung carcinoma, are regulated by oestrogen, the specific role of oestrogen in the regulation of tumor-associated immune cells remains unknown [21].

Results of numerous previous studies suggested that the TME plays a significant role during breast cancer development and progression, and in the regulation of responses to treatment [22–23]. Oestrogen homeostasis and elements of the immune system comprise the breast cancer TME. Thus, the present study aimed to determine the potential association between abnormal oestrogen homeostasis and cytokine-mediated TME reprogramming during breast cancer progression and metastasis. Results of the present study demonstrated that hydroxylated oestrogen accumulates in the mammary gland and activates the NLRP3 inflammasome in TAMs, leading to the secretion of IL-1β. Subsequently, IL-1β secretion enhances breast tumor progression and metastasis. Thus, further understanding the mechanisms that regulate oestrogen levels is vital for developing the current understanding of the TME, and may support the development of endocrine hormone-targeted cancer immunotherapy.

Methods

Clinical sample collection

The basic information of patients collected in this study is shown in Table 1. The specific inclusion and exclusion criteria of this study were consistent with those described in previous reports [24]. In brief, inclusion criteria: 1) Premenopausal patients with primary benign or malignant luminal A breast tumor. 2) Not taking tamoxifen within 2 years. 3) All the women enrolled in our study had never received receive radiotherapy, chemotherapy, oestrogen, progesterone and other systemic hormone therapy. Exclusion criteria: 1) Pregnancy, alcoholism and severe infection. 2) Patients with malignant tumors in other parts. 3) Liver and kidney dysfunction. 4) Accompanied by diabetes, hyperthyroidism and other diseases affecting endocrine metabolism. 5) Patients who have taken estrogen drugs in recent 3 months. 6) Patients who take drugs that affect blood lipid metabolism and liver and kidney function during medication. 7) Patients with whole blood sample volume less than 3 ml. 8) Patients with hemolysis and clotting of whole blood samples.

Animals and treatments

Four-week-old female MMTV-PyMT transgenic mice were obtained from Shanghai Southern Model Animal Center (Shanghai, China), and nontransgenic FVB/n female littermates were used as the negative controls. Mice in each group were randomly assigned. After the 4-week-old female mice were adaptively reared for one week, the mice in the treatment group were subcutaneously administered an oestradiol pellet (0.72 mg/60 days; SE-121, Innovative Research). The control mice were sham operated. In brief, FVB/n female mice were subjected to a sham operation (Control, FVB/n) or administered an oestrogen pellet (Oestradiol, FVB/n). MMTV-PyMT female mice were subjected to a sham operation (Control, MMTV-PyMT) or administered an oestrogen pellet (Oestradiol, MMTV-PyMT).
1.2 × 10^6 MCF-7 cells were implanted alone or 1.2 × 10^6 MCF-7 + 0.4 × 10^6 THP-1 cells in phosphate-buffered saline (PBS) (D8537, Sigma Aldrich) were administered to BALB/C-NU/NU (nude) mice via subcutaneous injection. The next day, the treatment group mice were intraperitoneally injected with 0.1 ml E2 (0.12mg/ml, E8875, Sigma Aldrich) or 0.1 ml 4-OHE2 (0.12mg/ml, MFCD00063335, Beijing Bailingwei Technology Co., Ltd.) once a day for four weeks. The control group was treated with oil. In brief, the mice were divided into the MCF-7+Oil (Control), MCF-7+E2, MCF-7+4-OHE2, MCF-7+THP-1+Oil (Control), MCF-7+THP-1+E2, and MCF-7+THP-1+4-OHE2 groups.

1.2 × 10^6 4T1 were implanted alone or 1.2 × 10^6 4T1 plus 0.4 × 10^6 THP-1 in phosphate-buffered saline (PBS) (D8537, Sigma Aldrich) were administered to the BALB/C mice via subcutaneous injection. The next day, the treatment group mice were intraperitoneally injected with 0.1 ml E2 (0.12mg/ml, E8875, Sigma Aldrich) or 0.1 ml 4-OHE2 (0.12mg/ml, MFCD00063335, Beijing Bailingwei Technology Co., Ltd.) once a day for four weeks. The control group mice were treated with sesame oil. In brief, the mice were divided into the 4T1+Oil (Control), 4T1+E2, 4T1+4-OHE2, 4T1+THP-1+Oil (Control), 4T1+THP-1+E2, and 4T1+THP-1+4-OHE2 groups. The long side (L) and the short side (W) were measured every 3 days, the tumor volume \( V = \frac{LW^2}{2} \).

The mice were anesthetized with 0.3% pentobarbital sodium (50mg/kg) by intraperitoneal injection and subsequently euthanized by cervical dislocation. Verification of death included cardiac and respiratory arrest, lack of reflexes and changes in mucosal color. Tumor diameter > 2.0 cm was considered a humane endpoint for euthanasia, but no mice reached this humane endpoint.

**Cell culture and treatment**

MCF-7, 4T1 and MDA-MB-231 were received from Dr. Yanyan Yu. THP-1 was purchased from the Chinese Academy of Sciences. All the cell lines tested negative for mycoplasma. THP-1 were treated with 100ng/mL PMA (P8139, Sigma Aldrich) for 48 hours before the experiment to differentiate into macrophages. Then, the THP-1 were separately treated with E2 (E8875, Sigma Aldrich), 2-OHE2 (MFCD00010490, Beijing Bailingwei Technology Co., Ltd), or 4-OHE2 (MFCD00063335, Beijing Bailingwei Technology Co., Ltd.) for 24 h. Then, conditioned media (CM) was harvested and used to stimulate MCF-7 OR MDA-MB-231 cells.

**LC-MS/MS**

**LC-MS/MS sample preparation**

Serum sample: mix 400 ul serum with 10 ul 50 nM D5-E2, and extract with 1.2 ml ethyl acetate; Tissue sample: 20mg of tissue sample was added with 200ul PBS, and then prepared into tissue homogenate. 10 ul 50 nM D5-E2 was added, and 1.2 ml of ethyl acetate was used for extraction. 700 ul of the supernatant was taken, and the ethyl acetate extract was evaporated with Speed Vac (Thermo Savant, Waltham, MA). 0.1 M Na2CO3/NaHCO3 buffer solution and 1 g/L danoyl chloride solution were used to
derive the residue. Due to their chemical similarity, d5-E2 is used as an internal standard for measuring oestrogen.

**Quantification of oestrogens using LC-MS/MS method**

**Chromatographic conditions**

The chromatographic column is Agilent ZORBAX Extend-C18 (2.1 × 100 mm, 1.8 μm), mobile phase: A (water+0.1% formic acid), B (acetonitrile+0.1% formic acid), gradient elution: 0~2 min, 30% A; 2~3.5 min, 30%~20% A; 3.5~11 min, 20% A; 11~11.5 min, 20%~30% A; 11.5~17 min, 30% A.

**Mass spectrum conditions**

ESI ion source: curtain air 35 psi; Blowing air 8; Ionization voltage 5500 V; The temperature is 550 °C; Spray air 55 psi; Auxiliary heating air 55 psi; Scanning mode: MRM, positive ion detection; MRM parameter settings of each target analyte and internal standard are shown in Table 2. The method of LC-MS/MS was carried out according to the method previously reported [25].

**Flow cytometry analysis**

Breast tumors isolated from female MMTV PyMT mice were separated into single-cell suspensions and incubated with 7AAD Viability Staining Solution (B350877, Biolegend, diluted 1:100), FITC anti-mouse CD45 (B343304, Biolegend, diluted 1:100), PE anti-mouse/human CD11b Antibody (101207, Biolegend, diluted 1:100) and APC anti-mouse F4/80 Antibody (123116, Biolegend, diluted 1:100). All the analyses were conducted within FACS Diva Version 6.1.3.

**Reverse transcriptase-quantitative PCR (RT-qPCR)**

RNA was extracted by using TRIZOL® (Invitrogen). The analysis was performed using the LightCycler®480 II to measure mRNA expression. The primers sequences are listed in Table 3. The relative gene expression was analyzed by using the comparative Ct (2-ΔΔCq) method with genes normalized to GAPDH (mRNA) or β-Actin (mRNA) [26].

**Enzyme-linked immunosorbent assay (ELISA)**

The ELISA was performed using a human IL-1β ELISA Kit (LP-H01925, Lanpai Bio, Shanghai) following the manufacturer's instructions. The IL-1β concentrations in the samples were estimated by the standard curve.

**Western blotting**

After total protein extraction, SDS–PAGE separate the proteins, and their levels were measured by immunoblotting with specific antibodies. The primary antibodies included antibodies against NLRP3 (15101, Cell Signaling Technology), ASC (13833, Cell Signaling Technology), MMP2 (66366-Ig,
Proteintech), MMP9 (10375-2-AP, Proteintech), IL-18 (BS6823, Bioworld) and GAPDH (AP0063, Bioworld). Odyssey® Sa (LICOR, USA) quantifies the intensities of the bands. GAPDH expression was used as a control to quantify target protein signal intensities.

**Wound healing assay**

MCF-7 and MDA-MB-231 were cultivated into 6-well plates and cultured to 90% confluence. The monolayers of cells were scratched with a micropipette tip to form a wound on the plate. Cells were washed twice, dead cells were removed by PBS, and serum-free medium containing exosomes (50 µg) was added to the chambers. Three replicate wells were established in 6-well plates for each experimental condition.

**Trans-well migration assay**

MCF-7 or MDA-MB-231 co-incubated with THP-1 cells in conditioned medium (CM) which was a serum-free medium were seeded into the upper chamber. Add 600 µL complete medium into the lower chamber, and incubate it for 24 hours in an incubator at 37°C. Take out the chamber, clean it twice with PBS, and dry it with a cotton swab. Cells were fixed in 4% paraformaldehyde, and wiped dry with cotton swab. Dye with 0.1% crystal violet for 30min, and wash them twice with PBS. After the chamber was air-dried, the cell migration was observed with an inverted microscope under a random field of vision.

**Immunohistochemistry (IHC)**

Refer to previous reports for the operation process of IHC [27]. The main primary antibodies used in IHC are as follows: NLRP3 (19771-1-AP, Proteintech), CD163 (16646-1-AP, Proteintech), F4/80 (70076, Cell Signaling Technology) and CD68 (28058-1-AP, Proteintech).

**Statistical analysis**

Use Prism for statistical analysis. Student's test or one-way ANOVA was used to compare multiple groups. *P*<0.05 for all data sets was considered statistically significant. Kaplan Meier analysis was used to calculate survival differences between groups.

**Results**

**Oestrogen homeostasis is disrupted in patients with breast cancer.**

The levels of E1, E2, E3 and 12 oestrogen metabolites were measured in serum samples obtained from 80 premenopausal patients with malignant metastatic breast cancer (Malignant group), 80 premenopausal patients with benign breast cancer (Benign group) and 78 matched controls (Normal group) using liquid chromatography-tandem mass spectrometry analysis (LC/MS-MS). Compared with those in the Normal group, the levels of E2, E3, 16 epiE3, 17-epiE3, 2-OHE2, 4-OHE2 and 4-MeOE2 were significantly increased in the Malignant group (Fig. 1a). Moreover, E2 and 4-OHE2 levels were significantly elevated in patients...
with malignant breast cancer compared with patients with benign breast cancer (Fig. 1a). Measuring 15 types of oestrogen in six pairs of cancer tissues and para-cancerous tissues from patients diagnosed with malignant breast cancer demonstrated that the concentrations of E1, E2, 16-epiE3 and 2-OHE2 or 4-OHE2 were significantly increased in malignant breast cancer tissues compared with para-cancerous tissues (Fig. 1b), indicating that oestrogen homeostasis is dysregulated in breast cancer, and that oestrogens and hydroxylated metabolites are abnormally accumulated in patients with breast cancer. In addition, results of the present study demonstrated that this metabolic disorder is intimately associated with breast cancer metastasis. Notably, results of the present study demonstrated that oestrogens and their hydroxylated metabolites are closely associated with the progression of breast cancer, and these results are consistent with those of a previous study [28].

Subsequently, multivariate analyses, including Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA), were performed to identify potential oestrogen homeostatic differences among the Normal, Benign and Malignant groups. Results of the analysis demonstrated that the metabolic profile varied widely among the three groups, and that hydroxylated oestrogen was the key metabolite in the development of breast cancer. These results suggested that differences in oestrogen metabolism are observed during the progression of breast cancer (Fig. 1c). Similarly, results of the analysis of para-cancerous and cancerous tissues also revealed the dysregulation of oestrogen homeostasis in patients with breast cancer (Fig. 1d).

To determine the association between overall oestrogen levels and the progression of breast cancer, oestrogen composition was observed in an MMTV-PyMT murine model of breast cancer, with FVB/n littermates that did not spontaneously develop disease used as controls. Results of the present study were showed in Additional file 1 and demonstrated that the levels of E2, E3, 16-epiE3 and 2-OHE2 or 4-OHE2 in MMTV-PyMT breast tissues increased significantly (Additional file 1: Fig. S1), indicating that proto-oestrogens and hydroxylated oestrogens accumulate in the mammary tissue of mice with malignant breast cancer.

**Mammary levels of hydroxylated oestrogen are positively correlated with the levels of inflammation in the TME.**

To elucidate the potential association between the imbalance of oestrogen metabolism and the breast TME in vivo, the levels of oestrogen and associated metabolites in breast tumor tissues were measured using LC-MS/MS, and alterations in macrophage abundance were assessed in breast tumor tissues using flow cytometry. Notably, the MMTV-PyMT murine model of breast cancer was used for these studies, and the mice were administered oestradiol sustained-release tablets (Fig. 2a). A single-cell suspension was prepared as previously described [29]. Results of the present study demonstrated that the levels of E2, 2-OHE2 and 4-OHE2 were significantly increased following the subcutaneous administration of oestrogen sustained-release tablets in mice (Fig. 2b). Subsequently, the percentage of CD11b + F4/80 + cells in the CD45 + 7AAD cell population in the single-cell suspension isolated from the tumor tissue of MMTV-PyMT mice were investigated using flow cytometry. As shown in Fig. 2c, tumor cells derived from MMTV-PyMT
mice treated with estradiol sustained-release tablets contained a significantly higher number of the CD11b + F4/80 + cells, indicating that oestrogen contributes to macrophage infiltration in the microenvironment of breast tumors; thus, promoting the generation of the tumor inflammatory microenvironment (Fig. 2c). Moreover, IHC staining of tumor tissues of the treated group also demonstrated that the expression of the macrophage surface marker, CD68, was higher than that in the sham operation group (Fig. 2d). Results of the present study suggested that tumors in oestrogen-treated mice were significantly larger than tumors in the mice subjected to the sham operation (Fig. 2e). In addition, nuclei in the tumor tissues of mice in the treatment group were enlarged and deeply stained, and the tumor cells were more closely aligned (Fig. 2f). Collectively, these results revealed that the occurrence and development of the breast cancer immune microenvironment may be induced by mammary hydroxylated oestrogens that promote the growth of breast cancer tumors.

Hydroxylated oestrogen activates TAMs to mediate breast tumor progression and metastasis.

Compared with the control group, E2 promoted the proliferation and migration of MCF-7 breast cancer cells (Fig. 3a), whereas 2-OHE2 and 4-OHE2 did not, and these results are consistent with those of previous studies [30–31] (Fig. 3b). TAMs are an important part of the TME that play a vital role in promoting the progression and metastasis of breast cancer. Thus, the present study aimed to determine whether hydroxylated oestrogen acted on TAMs to induce the generation of an inflammatory microenvironment in breast tumors, thereby regulating the progression of breast cancer.

In the present study, THP-1 cells were treated with 10 nM E2, 1 µM 2-OHE2, 1 µM 4-OHE2 or 50 ng/mL Lipopolysaccharide (LPS) for 24 h. Subsequently, the conditioned medium (CM) of THP-1 cells were collected and transferred into MCF-7 cells (ER positive) and MDA-MB-231 cells (ER negative) to establish a co-culture system (Fig. 3c). As displayed in Fig. 3d-f, the migration, proliferation and invasion of the hydroxylated oestrogen administration group was significantly enhanced compared with the control group, which indicated that hydroxylated oestrogens require the participation of macrophages to induce the progression and metastasis of MCF-7 cells. As shown in Additional file 2, consistent with the results observed in MCF-7 cells, the migration and invasion of MDA-MB-231 cells were markedly upregulated following the addition of the CM of THP-1 cells treated with hydroxylated oestrogen (Additional file 2: Fig. S2a and b). Previous literature reported that oestrogen promotes the progression of breast cancer through activating ER [32]. Collectively, these results indicated that hydroxylated oestrogen promotes the occurrence and development of breast cancer; however, regulating the inflammatory microenvironment of breast cancer is ER-independent.

To further investigate whether hydroxylated oestrogen mediates breast cancer progression and metastasis through the TME, a suspension of MCF-7 cells and undifferentiated THP-1 monocytes were injected into BALB/C-NU/NU (nude) mice, and mice injected with MCF-7 cells alone were used as controls. Subsequently, mice in each group were intraperitoneally administered clear oil (Control), E2 and 4-OHE2 (Fig. 4a). Results of the present study demonstrated that following treatment with oestrogen, there was no significant difference in tumor volume in the group injected with MCF-7 cells alone (Fig. 4b and c). By
contrast, following treatment with oestrogen, the tumor volume in the group co-injected with MCF-7 + THP-1 cells was significantly larger than that in the control group, particularly following treatment with 4-OHE2 (Fig. 4b and c). As shown in Additional file 3, in highly metastatic ER negative 4T1 breast cancer cells treated with hydroxylated oestrogen, there was no significant difference in tumor volume among groups injected with 4T1 alone (Additional file 3: Fig. S3a and b). Following treatment with hydroxylated oestrogen, the tumor volume of mice co-injected with 4T1 and THP-1 cells was significantly larger compared with the control group (Additional file 3: Fig. S3a and b), suggesting that hydroxylated oestrogens act on TAMs to promote breast tumor development. H&E staining demonstrated that the tumor tissues of each MCF-7 cell-treated group exhibited invasive growth. When MCF-7 + THP-1 cells were co-injected and mice were treated with E2 or 4-OHE2, tumor tissues exhibited atypical hyperplasia accompanied by a large number of inflammatory cells (Fig. 4d). Moreover, F4/80, a mouse macrophage surface specific antibody, was used for immunohistochemical staining of tumor tissues. Results of the present study highlighted that there was no significant difference in the expression of F4/80 in the tumor tissues of mice injected with MCF-7 cells alone compared with the control group, following treatment with E2 and 4-OHE2. However, the expression of F4/80 in the tumor tissues of mice co-injected with MCF-7 and THP-1 cells was upregulated compared with the control group, following treatment with E2 and 4-OHE2. These results indicated that hydroxylated oestrogen promoted the recruitment of macrophages in vivo (Fig. 4e). Collectively, these results highlighted an ER-independent role of hydroxylated oestrogen in the reprogramming of the TME, which ultimately facilitates breast cancer progression and metastasis via the participation of macrophages.

The NLRP3 inflammasome is activated in mammary TAMs and functions as an oestrogen sensor.

To uncover the potential association between hydroxylated oestrogen and mammary TAMs in facilitating metastatic dissemination, mechanistic studies were carried out at a cellular level. Based on a literature review and previous research, the NLRP3 inflammasome remained the focus. As shown in Additional file 4, when treated with 10 nM E2, 1 µM 2-OHE2 or 1 µM 4-OHE2, NLRP3 was not upregulated in MCF-7 cells (Additional file 4: Fig. S4a and b). Studies were also carried out in THP-1 cells, and a LPS-treated group was used as a positive control. Results of the present study demonstrated that the protein levels of NLRP3 and ASC were markedly upregulated in THP-1 cells when treated with E2, 2-OHE2 or 4-OHE2 (Fig. 5a-c). In addition, the mRNA expression of IL-1β in the supernatants and the intracellular level of IL-1β were also increased (Fig. 5d and e). Thus, these results suggested that E2 and its hydroxylated metabolites may activate the NLRP3 inflammasome pathway in THP-1 cells.

The role of NLRP3 in oestrogens-mediated activation of inflammatory pathways in THP-1 cells was evaluated by transducing with lentivirus carrying a plasmid to silence NLRP3 expression (si-NLRP3 group) in THP-1 cells. Results of the present study demonstrated that NLRP3 and ASC protein expression levels were not upregulated when THP-1 cells were treated with E2 and its hydroxylated metabolites in the si-NLRP3 group (Fig. 5f-h; Additional file 5: Fig. S5a-c). However, IL-1β mRNA expression levels were not increased by 4-OHE2 or 2-OHE2 in THP-1 cells following NLRP3 knockdown (Fig. 5i; Additional file 5: Fig. S5d). As shown in Additional file 6, a specific NLRP3 inflammasome inhibitor MCC950, was used to
validate the off-target effects of NLRP3 siRNA and the results confirmed that the siRNA of NLRP3 exhibits good specificity (Additional file 6: Fig. S6a-c). Other inflammatory factors and chemokines were also investigated and shown in Additional file 7: Fig. S7.

In the co-culture system, the migration of MCF-7 cells was increased following treatment with 4-OHE2 or 2-OHE2 in the CON group, while the migration of the si-NLRP3 group was not significantly increased (Fig. 6a; Additional file 8: Fig. S8a). Notably, 4-OHE2 or 2-OHE2 treatment did not induce the proliferation of MCF-7 cells following NLRP3 knockdown (Fig. 6b; Additional file 8: Fig. S8b). These results further verified that E2 and its hydroxylated metabolites induce breast cancer metastasis in a manner that is dependent on the NLRP3 inflammasome of TAMs.

MMPs, such as MMP2 and MMP9, have been widely recognized as important indicators of malignant tumor invasion and metastasis. In the co-culture system, the protein expression levels of MMP2 and MMP9 in MCF-7 and MDA-MB-231 cells were significantly higher following treatment with E2, 2-OHE2 or 4-OHE2, compared with control treatment (Fig. 6c-e; Additional file 9: Fig. S9a-c). Following NLRP3 gene knockdown in the THP-1 co-culture system, MMP2 and MMP9 protein expression levels were not increased following 4-OHE2 or 2-OHE2 treatment (Fig. 6f-i; Additional file 10: Fig. S10a-c), suggesting that the inflammatory factors activated during oestrogen disorders promote tumor invasion and metastasis. Moreover, as shown in Additional file 11, following NLRP3 inhibited by MCC950 in the THP-1 co-culture system, MMP2 and MMP9 levels were also not increased (Additional file 11: Fig. S11a-c). Notably, in the co-culture system, treatment with E2, 2-OHE2 or 4-OHE2 also significantly upregulated the levels of mRNAs encoding MMP2 and MMP9 (Fig. 6j and k). In addition, following NLRP3 gene knockdown, MMP2 and MMP9 mRNA expression levels were not increased following 4-OHE2 or 2-OHE2 treatment (Fig. 6l and m; Additional file 12: Fig. S12a and b), which were consistent with protein levels detection results.

Abnormal hydroxylated oestrogen levels mediate activation of the inflammasome signalling pathway in mammary TAMs, and promote breast tumor progression and metastasis.

IHC staining analysis of a xenograft BALB/C-NU/NU (nude) mouse model confirmed that hydroxylated oestrogen did not upregulate NLRP3 in tumor tissues, when MCF-7 cells were injected alone. However, NLRP3 expression levels in mice co-injected with MCF-7 + THP-1 cells and treated with hydroxylated oestrogens were significantly higher than that in control mice (Fig. 7a). Moreover, the expression levels of NLRP3, ASC, MMP2 and MMP9 in mice co-injected with MCF-7 + THP-1 cells were significantly upregulated, compared with those in the mice injected with MCF-7 cells alone. These results suggested that activation of the inflammasome signalling pathway mediated by abnormal oestrogen levels promoted breast tumor progression and metastasis in TAMs (Fig. 7b; Additional file 13: Fig. S13a-d). Moreover, the NLRP3, IL-1β, MMP2 and MMP9 mRNA expression levels were also significantly higher in mice co-injected with MCF-7 + THP-1 cells, compared with those in mice injected with MCF-7 cells alone (Fig. 7c-f).

Similarly, IHC staining of gene-positive MMTV-PyMT mice confirmed that NLRP3 expression levels in the tumor tissues of the pellet-embedded group were significantly increased, compared with the tumor
tissues from the control group (Fig. 7g). In gene-positive MMTV-PyMT mice, NLRP3, ASC, MMP2 and MMP9 protein and mRNA expression levels were significantly increased following treatment with oestradiol sustained-release tablets, compared with those in the control group (Fig. 7h-l; Additional file 13: Fig. S13e-h). However, there was no significant difference between the mammary glands of FVB/n mice in the control group, or those treated with oestrogen sustained-release tablets (Fig. 7h-l; Additional file 13: Fig. S13e-h). Collectively, these findings demonstrated that mammary hydroxylated oestrogen activates the NLRP3 inflammatory pathway and increases the expression of MMPs to promote breast tumor metastasis with the participation of TAMs.

Validation of the association between hydroxylated oestrogen and the breast cancer immune microenvironment based on clinical data.

To further determine the potential association between inflammatory factors and the progression of breast cancer, IHC staining was performed using human breast tissue chips, to evaluate potential changes in the expression levels of NLRP3 and CD163. Notably, there were five cases in the normal control group (Normal group), 10 cases in the hyperplasia group (Hyperplasia group), 12 cases in the carcinoma in situ group (Carcinoma in situ group) and 21 cases in the invasive carcinoma group (Infiltrating carcinoma group). Compared with the Normal group, the levels of CD163, NLRP3 and MMP9 in the Carcinoma in situ group and Infiltrating carcinoma group were significantly increased (Fig. 8a-c), suggesting that with the rising risk of breast cancer metastasis, inflammatory cell infiltration increased. Therefore, we hypothesized that E2 and its hydroxylated metabolites may act on the inflammatory microenvironment of breast tumors to induce breast cancer metastasis. According to the database analysis, patients with ER+ breast cancer and high CD163 and NLRP3 expression levels exhibited a poor overall survival rate (Fig. 8d-e). Moreover, patients with breast cancer and high MMP expression levels also exhibited a poor prognosis (Fig. 8f), demonstrating a significant association between high TAM infiltration of the breast TME and a poor clinical prognosis.

Discussion

Notably, high levels of oestrogen accumulation and T lymphocyte infiltration are observed in breast cancer [33]. However, studies into the specific role of oestrogen in cancer remain focused on cancer cell proliferation [34], angiogenesis [35] and epithelial-mesenchymal transition (EMT) [36], while changes in oestrogen-induced cancer immune response remain unclear. Results of the present study demonstrated that oestrogens, particularly oestradiol and its hydroxylated metabolites, are markedly accumulated in mammary tissues. Moreover, TAM-dependent tumor metastasis may be triggered by oestrogens via NLRP3 inflammasome activation and IL-1β production. Mechanistically, IL-1β induces the expression of MMPs in breast tumor cells, leading to breast tumor progression and metastasis (Fig. 8g).

Results of previous epidemiological and clinical studies demonstrated that increases in endogenous oestrogens are associated with increases in breast cancer risk [33]. In addition, results of a previous study revealed that oestrogen promoted the progression of breast cancer through activating ER [32], and
specific oestrogen metabolites, such as CE-3, 4-Q, may promote cancer progression through mediating DNA damage [5]. However, prospective study demonstrated that the average oestradiol concentration in premenopausal patients with breast cancer was not significantly increased, compared with the control group [37]. Thus, the specific mechanisms underlying oestrogen homeostatic reprogramming in the progression and metastasis of breast cancer remains unclear.

In the present study, LC-MS/MS was used to quantify the abundance of oestrogens across multiple tissues, as previously described [25]. To investigate the potential correlation between oestrogen and breast cancer progression, 78 premenopausal healthy females, 80 premenopausal females with benign breast tumors and 80 premenopausal patients diagnosed with luminal A breast cancer, which is considered to be highly associated with oestrogen in clinical practice, were recruited in the present study. The specific inclusion and exclusion criteria of the present study were consistent with those described in previous reports [24]. Further information into the number of patients, patient age and BMI is displayed in Table 1. To fully delineate the role of the imbalance of oestrogen homeostasis in the progression of breast cancer, further investigations using larger sample sizes and molecular subtype stratification are required. Results of the present study demonstrated that the concentrations of numerous oestrogens were significantly increased in patients with breast cancer, particularly in patients with malignant metastatic breast cancer. Moreover, results of the present study demonstrated that oestrogen and its hydroxylated metabolites were significantly accumulated in the breast TME, compared with oestrogen levels in para-cancerous tissues. To the best of our knowledge, this is the first study to reveal the disruption of oestrogen homeostasis in the TME. These findings also suggested that altering oestrogen metabolism through lifestyle modifications or chemo-preventive strategies may exhibit potential in breast cancer prevention and treatment.

To further understand the interactions between hormonal metabolism and the progression of breast cancer, oestrogen profile analysis was carried out in a MMTV-PyMT murine model of breast cancer. The use of a MMTV-PyMT model in preclinical research helps to determine the role of macrophages in the progression of breast cancer, and macrophage-specific CD68 expression exhibits potential as a prognostic and predictive biomarker [38]. MMTV-PyMT transgenic mice are suitable models for investigating the potential interaction between oestrogen and macrophages in a number of disease stages, from in situ lesions to invasive carcinoma, and this is comparable with human disease progression [39]. Following the subcutaneous implanting of oestradiol sustained-release tablets in MMTV-PyMT mice, results of the present study revealed that the levels of prototypical oestrogen and its hydroxylated metabolites were positively correlated with the abundance of macrophages in the mouse mammary TME. Collectively, these results demonstrated that hydroxylated oestrogens in the mammary microenvironment, in addition to E2, are closely associated with the occurrence of immune inflammation.

At present, targeting tumor cells remains the main focus of research into anti-cancer treatment. However, recent advances in immunotherapy demonstrated that targeting the TME is effective in controlling tumor progression. TAMs are major cells that comprise the TME of breast cancer, accounting for > 50% in some cases [40]. Results of previous study confirmed that macrophages promote an inflammatory
microenvironment and metastasis through angiogenesis, tissue remodelling and adaptive immunosuppression [41]. In >80% of patients with breast cancer, increased macrophage density is associated with a poor prognosis [42]. Increased angiogenesis in patients with invasive breast cancer was associated with increased infiltration of TAMs, and negatively associated with clinical prognosis [43].

Results of the present study demonstrated that hydroxylated oestrogen accumulation in the mammary gland activates TAMs, and the subsequent effects on promoting breast cancer depends on TAMs in the immune microenvironment of the breast. These results suggested that the regulation of TAMs by oestrogen metabolites, hydroxylated oestrogens and parent oestrogens are important in the prevention of breast cancer progression and metastasis. Notably, following co-culturing of MCF-7 cells with THP-1 cells and subsequent treatment with oestrogen, the migration and proliferation of MCF-7 cells increased significantly, compared with MCF-7 cells treated with oestrogen alone. Moreover, the volume of transplanted tumors in nude mice co-injected with MCF-7 and THP-1 cells were increased significantly following oestrogen treatment, compared with mice injected with MCF-7 cells alone.

As the effects of oestrogen on the ER are key mechanisms in promoting the progression of breast cancer, ER-negative breast cancer cells, MDA-MB-231, and highly metastatic ER-negative 4T1 breast cancer cells were used in the present study, and these results were consistent with those observed using ER-positive MCF-7 cells. These results indicated that the oestrogen-driven formation of an inflammatory microenvironment requires the participation of TAMs. Notably, oestrogen promotes the proliferation, migration and invasion of both ER-positive and ER-negative breast cancer cells with the participation of TAMs, which suggests that the effects of oestrogen on promoting breast cancer are ER-independent. Notably, oestrogen binds to ER to promote the production of growth factors, thereby promoting the proliferation and invasion of breast cells [32]. However, results of previous studies demonstrated that breast cancer developed in ER knockout mice expressing the Wnt-1 oncogene, which indicated that oestrogen may promote the progression and metastasis of breast cancer through ER-independent mechanisms [44–45]. Results of the present study and those of previous studies demonstrated that the carcinogenic mechanisms of oestrogen in breast cancer may be either ER-mediated or non-ER-mediated.

Results of a previous study demonstrated that premenopausal females exhibited a higher risk of breast cancer than age-matched postmenopausal females [46]. However, results of a further study revealed that exogenous oestrogen potently inhibits tumorigenesis through multiple pathways, which may exhibit potential in the prevention of breast cancer [47]. Notably, oestrogen requires a specific environment to promote inflammation and breast cancer progression. The metabolic and immune microenvironments are important components of the TME, with close mutual regulatory associations. These associations are key for maintaining homeostasis under physiological conditions. Results of previous study demonstrated that 17β-oestradiol (E2) suppressed hepatocellular carcinoma growth via regulating the polarization of macrophages [48]. In a co-culture system of endometrial cancer cells and macrophages, oestrogen promoted the proliferation of tumor cells through inducing macrophages to secrete the inflammatory factor, IL-17 [49]. These findings revealed a potential association between oestrogen and TAM activation
in certain cancers. Immune cells, including CD4 T lymphocytes and fibroblasts, express ER, indicating that oestrogen may regulate immune function through binding to the ER on immune cells [50]. Notably, results of a previous study including 188 breast cancer clinical samples demonstrated that ERα was not expressed in immune cells infiltrating the TME [51]. Moreover, the regulatory effects of oestrogen on the immune response in the TME remain unclear, and the specific regulatory mechanisms require further validation.

Inflammasomes are cytoplasmic protein complexes that sense endogenous or exogenous pathogen-related molecular patterns or DAMPs, and subsequently regulate pro-inflammatory cytokines, including pro-IL-1β [52]. As the malignancy of tumors increases, the number of macrophages that express NLRP3 increases significantly, and these macrophages are associated with the invasion and metastasis of breast cancer [15]. Overactivation of NLRP3 is considered an important factor in the pathological development of the tumor inflammatory microenvironment [53]. Results of a previous study demonstrated that the disruption of NLRP3 or IL-18 diminishes tumor growth [54]. However, macrophages are considered mediators of tumor-promoting inflammation in cancer, and have not been previously implicated in sensing oestrogens through the NLRP3 inflammasome. Results of the present study demonstrated that numerous oestrogens activated NLRP3 in BALB/C-NU/NU (nude) mice with xenograft tumors, MMTV-PyMT mice implanted with oestrogen sustained-release tablets and MCF-7 or MDA-MB-231 cells co-cultured with THP-1 cells. Through targeting NLRP3 and IL-1β in mammary macrophages in multiple mouse models, results of the present study demonstrated that inflammasomes in TAMs play an important role in promoting tumor growth. In addition, results of the present study revealed that the NLRP3 inflammasome pathway, which regulates the secretion and maturation of IL-1β, is activated in TAMs in response to oestrogen treatment, suggesting a mechanism by which TAMs mediate tumor-promoting inflammation. Notably, results of the present study highlighted that NLRP3 is not expressed in breast cancer cells. NLRP3 was also not activated in breast cancer cells following treatment with oestrogen, whereas NLRP3 was highly expressed in macrophages and was strongly activated following treatment with oestrogen. Collectively, results of the present study revealed that the cancer-promoting effect of oestrogen requires the participation of macrophages. To further investigate potential changes in inflammatory factors and chemokines in THP-1 cells following treatment with oestrogens, the mRNA expression levels of NLRP3, IL-18, Caspase-1, TNF-α, IL-6, CCL-2 and CCL-5 were investigated. Results demonstrate that the mRNA expression levels of NLRP3, IL-18 and Caspase-1 were upregulated after treatment with E2, 2-OHE2 or 4-OHE2. However, there was no significant changes in the mRNA expression levels of proinflammatory cytokines such as TNF-α or IL-6, and chemokines such as CCL-2 or CCL-5. These results further confirmed that E2 and its hydroxylated metabolites could induce the expression of pro-inflammatory cytokines by activating the NLRP3 inflammasome pathway in THP-1 cells.

IL-1β released by TAMs promotes the invasiveness of breast cancer cells via upregulating the expression of MMPs in tumor cells, leading to tumor invasion and metastasis [17, 55]. Results of the present study demonstrated that following NLRP3 gene knockdown with siNLRP3 in the co-culture system, MMP2 and MMP9 protein expression levels of were not increased following hydroxylated oestrogens treatment.
Results of the present study demonstrated that the expression levels of Nlrp3, IL-1β, ASC, MMP2 and MMP9 in mice co-injected with MCF-7 + THP-1 cells were downregulated, compared with those in mice injected with MCF-7 cells alone. After in-depth literature research, we believe that the possible reason for this phenomenon may be due to the diverse functional phenotypes of THP-1 cells in vivo. THP-1 is a human leukemia monocytic cell line which are widely used for the study of the mechanisms underlying inflammatory disease and monocyte/macrophage [56]. Previous studies showed that THP-1 cells can differentiate into anti-inflammatory phenotype macrophages [57–58]. One study demonstrated that when THP-1 cells differentiate into anti-inflammatory phenotype macrophages, NF-κB/NLRP3 pathway inflammatory pathway is inhibited and ASC expression levels are downregulated [59]. The other study cocultured colon cancer cell lines (HCT8 or HCT116) with THP-1 and found IL-1β levels were reduced which downregulate the expression of MMP2 and MMP9 in tumor cells [60].

The present study focused on the role of oestrogen in the TME and the potential immunotherapeutic implications of targeting oestrogen in patients with breast cancer. Results of the present study confirmed that oestrogens, particularly hydroxylated oestrogens, accumulate in the mammary gland microenvironment and activate the NLRP3 inflammasome in TAMs, leading to the release of inflammatory factors, and the proliferation and invasiveness of breast cancer cells. The present study examined how the disruption of oestrogen metabolism potentiates breast cancer progression, and determined the association between abnormal oestrogen homeostasis with cytokine-mediated TME reprogramming during breast cancer progression and metastasis. These results highlighted the potential for oestrogen homeostasis regulation as a therapeutic target in cancer immunotherapy.

It is necessary to note several limitations of this study. First, clinical sample size included in present study is insufficient, especially in terms of tissue samples from breast cancer patients. Second, for better representation of the physiological function and phenotype of TAMs, primary macrophages and additional macrophage cell lines such as RAW 264.7 are required. Third, THP-1 cells can differentiate into diverse functional phenotypes in vivo, thus, the specific macrophages phenotype of THP-1 cells should be explored in the further research.

Conclusions

In conclusion, results of the present study revealed the mechanism by which the disruption of oestrogen metabolism potentiates breast cancer progression. In addition, results of the present study highlighted an ER-independent role of hydroxylated oestrogen in the reprogramming of the TME via NLRP3 inflammasome activation in TAMs, which facilitated breast cancer progression and metastasis. Results of the present study also demonstrated the association between abnormal oestrogen homeostasis and cytokine-mediated tumor microenvironment reprogramming in breast cancer progression and metastasis. Thus, the present study provided a novel theoretical basis for the regulation of oestrogen homeostasis as a therapeutic target in cancer immunotherapy.

Abbreviations
TME Tumor microenvironment, LC-MS/MS The liquid chromatography-tandem mass spectrometry, TAMs Tumor-associated macrophage, NLRP3 NOD-like receptor thermal protein domain associated protein 3, MMPs Matrix metalloproteinases, ER Oestrogen receptor, E1 Estrone, E2 Estradiol, E3 Estriol, 16-epiE3 16-epiestriol, 17-epiE3 17-epiestriol, 2-MeOE1 2-methoxyestrone, 3-MeOE1 13-methoxyestrone, 4-MeOE1 4-methoxyestrone, 2-MeOE2 2-methoxyestradiol, 4-MeOE2 4-methoxyestradiol, MRM Multiple reaction monitoring, 2-OHE1 2-hydroxyestrone, 4-OHE1 4-hydroxyestrone, 2-OHE2 2-hydroxyestradiol, 4-OHE2 4-hydroxyestradiol, 16α-OHE1 16α-hydroxyestrone, IHC Immunohistochemistry, qRT-PCR quantification real-time polymerase chain reaction, ELISA Enzyme-linked immunosorbent assay, CCK-8 Cell Counting Kit-8, DAMPs Damage-associated molecular patterns, LPS Lipopolysaccharide, OPLS-DA Orthogonal Partial Least Squares Discriminant Analysis, PBS Phosphate-buffered saline, CM Conditioned media, EMT Epithelial–mesenchymal transition.

Declarations

Ethics approval and consent to participate

This study was registered in the Chinese Clinical Trial Register on May 13, 2017 (No. ChiCTR-DOD-17011393), and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients. All the experiments that involved animals were approved and conducted under the oversight of the Animal Ethics Committee of Xuzhou Medical University and carried out in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

Funding

This study was supported by the Natural Science Foundation of China (No. 82173883, China); the Science and Technology Foundation of Xuzhou (No. KC21010, China); the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (No. 18KA350002, China); the Provincial Commission of Health and Family Planning in Jiangsu Province (No. H2017079, China) and the Science and Technology Planning Project of Jiangsu Province (No. BE2019636, China).

Authors’ contributions
XYZ, BZ and ZL provided study concept and design. HZ, JHX and YNZ collected and analyzed the data. HZ and JHX interpreted the data. HZ, JHX and YNZ performed the experiments. HZ, BZ, SQH and ZL collected the patients’ samples. XYZ, HZ and JHX wrote the main manuscript and HZ prepared all figures. All authors reviewed the manuscript.

Acknowledgements

We thank all volunteers in this study for their cooperation and thank the Department of Thyroid and Breast Surgery, Affiliated Hospital of Xuzhou Medical University for assisting in the collection of clinical samples from patients with breast cancer. We also thank the core facilities of the Key Laboratory of Immunology, Xuzhou Medical University for their technical support in flow cytometry.

References


34. Saad S, Gottlieb DJ, Bradstock KF, Overall CM and Bendall LJ. Cancer cell-associated fibronectin induces release of matrix metalloproteinase-2 from normal fibroblasts. Cancer Res. 2002; 62: 283-9. doi:


Tables

Table 1 The characteristics of the patients at baseline

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premenopausal healthy female</td>
<td>78</td>
<td>34.77 ± 4.59</td>
<td>23.35 ± 3.43</td>
</tr>
<tr>
<td>Premenopausal female patients with benign breast tumor</td>
<td>80</td>
<td>41.87 ± 6.06</td>
<td>24.27 ± 4.42</td>
</tr>
<tr>
<td>Premenopausal female patients with malignant breast tumor</td>
<td>80</td>
<td>44.95 ± 5.46</td>
<td>24.53 ± 3.59</td>
</tr>
</tbody>
</table>

A. 80 diagnosed with malignant breast cancer (luminal A) premenopausal female patients, 80 diagnosed with benign breast cancer premenopausal female patients, 78 premenopausal healthy female women
were recruited from the Affiliated Hospital of Xuzhou Medical University, Xuzhou, China.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female patients with malignant breast cancer</td>
<td>6</td>
<td>53.33 ± 1.03</td>
<td>24.45 ± 4.29</td>
</tr>
</tbody>
</table>

B. 6 premenopausal female patients with malignant breast cancer were also recruited to collect samples of cancerous tissues and para-cancerous tissues.

**Table 2** MRM parameter settings for the LC-MS/MS analytical method

<table>
<thead>
<tr>
<th></th>
<th>Precursor ions (m/z)</th>
<th>Daughter ions (m/z)</th>
<th>Dwell Time (ms)</th>
<th>DP (V)</th>
<th>CE (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>504.100</td>
<td>171.100</td>
<td>20</td>
<td>140.000</td>
<td>48.000</td>
</tr>
<tr>
<td>E2</td>
<td>506.100</td>
<td>171.200</td>
<td>20</td>
<td>130.000</td>
<td>47.000</td>
</tr>
<tr>
<td>2-MeOE1</td>
<td>534.100</td>
<td>171.100</td>
<td>20</td>
<td>70.000</td>
<td>60.000</td>
</tr>
<tr>
<td>4-MeOE1</td>
<td>534.100</td>
<td>171.200</td>
<td>20</td>
<td>100.000</td>
<td>45.000</td>
</tr>
<tr>
<td>2-MeOE2</td>
<td>536.100</td>
<td>171.100</td>
<td>20</td>
<td>80.000</td>
<td>45.000</td>
</tr>
<tr>
<td>4-MeOE2</td>
<td>536.100</td>
<td>171.100</td>
<td>20</td>
<td>80.000</td>
<td>45.000</td>
</tr>
<tr>
<td>2-OHE1</td>
<td>753.300</td>
<td>170.100</td>
<td>20</td>
<td>60.000</td>
<td>78.000</td>
</tr>
<tr>
<td>4-OHE1</td>
<td>753.300</td>
<td>170.100</td>
<td>20</td>
<td>50.000</td>
<td>80.000</td>
</tr>
<tr>
<td>2-OHE2</td>
<td>755.300</td>
<td>170.100</td>
<td>20</td>
<td>90.000</td>
<td>73.000</td>
</tr>
<tr>
<td>4-OHE2</td>
<td>755.300</td>
<td>170.100</td>
<td>20</td>
<td>50.000</td>
<td>95.000</td>
</tr>
<tr>
<td>16α-OHE1</td>
<td>520.100</td>
<td>171.200</td>
<td>20</td>
<td>60.000</td>
<td>53.000</td>
</tr>
<tr>
<td>d5-E2</td>
<td>511.200</td>
<td>171.200</td>
<td>20</td>
<td>80.000</td>
<td>40.000</td>
</tr>
<tr>
<td>E3</td>
<td>522.200</td>
<td>171.100</td>
<td>20</td>
<td>90.000</td>
<td>46.000</td>
</tr>
<tr>
<td>16-epiE3</td>
<td>522.000</td>
<td>171.000</td>
<td>20</td>
<td>80.000</td>
<td>50.000</td>
</tr>
<tr>
<td>17-epiE3</td>
<td>522.000</td>
<td>171.000</td>
<td>20</td>
<td>80.000</td>
<td>50.000</td>
</tr>
<tr>
<td>3-MeOE1</td>
<td>534.000</td>
<td>171.000</td>
<td>20</td>
<td>40.000</td>
<td>41.000</td>
</tr>
</tbody>
</table>
Table 3 Primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>GAAGATGCTGGTTCCCTGCC AGCGTGCAGTTCAGTGATCG</td>
</tr>
<tr>
<td>Nlrp3</td>
<td>GCGATCAACAGGAGAGACCTT TCCACTCCTCTTTCAATGCTGT</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>TCGCTTTTCTGCTCTTCCACA GGCATCTGCGCTCTACCATCT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GCTGCACTTTGGAGTGATCG ATGAGGTACAGGCCCTCTGA</td>
</tr>
<tr>
<td>IL-6</td>
<td>ATGAGGAGACTTGCGCTGGTGAA CTCTGGCTTTGCCCTCACTACTCTC</td>
</tr>
<tr>
<td>CCL-5</td>
<td>CCCTCGCTGTCATCCTCATTG TCGGGTGACAAAGACGACTGC</td>
</tr>
<tr>
<td>CCL-2</td>
<td>CTCATAGCAGCCACCTTTCCATTCC ACACCTCTAGGCTGGCTATCTTTA</td>
</tr>
<tr>
<td>IL-18</td>
<td>TCTGACTGTAGAGATAATGCACCCCATACCTCTAGGCTGGCTATCTTTA</td>
</tr>
<tr>
<td>MMP2</td>
<td>CACCTACACCAAGAACCCTCCGTCTG GTGCCAAGGTCAATGTCAGGAGAG</td>
</tr>
<tr>
<td>MMP9</td>
<td>AGTCCACCCTTTGGCTCTTCCC TCTCTGCCACCCGAGTGTAACC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCATCAACGACCCCCCTTC AGCCCCAGCCTTCTCCA</td>
</tr>
</tbody>
</table>

A. Primer sequences for qRT-PCR (human)
<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>TCCAGGATGAGGACATGAGCACGAACGTCACACACCAGCAGGTTA</td>
</tr>
<tr>
<td>Nlrp3</td>
<td>GCCGTCTACGTCTTTTCTCTTTTCCATCCGCAGCCAGTGAACAGAG</td>
</tr>
<tr>
<td>MMP2</td>
<td>ACCATGCAGAAGCCAAGATGTGAGGTCCAGGTGTTGGAACAGAG</td>
</tr>
<tr>
<td>MMP9</td>
<td>AGTCCACCTTTGTGCTCTTTCCC TCTCTGCACACCGAGTGAACACC</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGCCCTTGCTTGACCAGCAGCAGCTTCTCT</td>
</tr>
</tbody>
</table>

B. Primer sequences for qRT-PCR (mouse)

**Figures**

![Diagram a](image1)
![Diagram b](image2)
![Diagram c](image3)
![Diagram d](image4)
Oestrogen homeostasis is disrupted in patients with breast cancer

(a) The concentrations of 15 oestrogens in serum samples from human patients in the Normal (78 premenopausal healthy women), Benign (80 premenopausal benign breast cancer patients), and Malignant (80 premenopausal malignant breast cancer patients) groups were measured by LC–MS/MS. The results are analysed by Student's t-test and presented as the means ± SEMs, *P<0.05, **P<0.01, ***P<0.001 vs. the Normal group. ##P<0.01 vs. the Benign group. (b) The concentrations of 15 oestrogens in samples of human para-cancerous tissues and cancerous tissues (paired 6 patients) were measured by LC–MS/MS. The results are analysed by Student's t-test and presented as the means ± SEMs, *P<0.05, **P<0.01 vs. para-cancerous tissues. (c) An OPLS-DA score plot showing the oestrogen metabolic profile of the Normal (green), Benign (blue) and Malignant (red) groups. (d) An OPLS-DA score plot showing the oestrogen metabolic profile of samples of human para-cancerous tissues (green) and cancerous tissues (blue).
Mammary levels of hydroxylated oestrogen are positively correlated with the levels of inflammation in TME.

(a) Schematic illustration of subcutaneously implanted oestradiol sustained-release tablets in MMTV-PyMT mice. (b) The concentrations of 15 oestrogens in the breast tissues of MMTV-PyMT mice from each group were measured by LC–MS/MS. The results are analysed by Student’s t-test and presented as the means ± SEMs, n=6, *P<0.05, **P<0.01, ***P<0.001 vs. the Control (MMTV-PyMT) group. (c) Flow cytometry analysis of the tumor macrophage surface marker CD11b⁺, F4/80⁺ in MMTV-PyMT mice. The
data are analysed by Student’s t-test and presented as the mean ± SEMs, n=8, **P<0.01 vs. the Control (MMTV-PyMT) group. (d) CD68 expression in tumors of MMTV-PyMT mice was analysed by IHC. (e) Tumor growth of MMTV-PyMT mice in each gene-positive group. The data are analysed by Student’s t-test and presented as the mean ± SEMs, n=6, ****P<0.0001 vs Control (MMTV-PyMT) group. (f) Sections of tumors from MMTV-PyMT mice were stained with H&E and subjected to histological analysis.

Figure 3

Hydroxylated oestrogen activates TAMs to mediate breast tumor progression and metastasis

(a) Effects of E2 and 2-OHE2 or 4-OHE2 on the migratory capacity of MCF-7 cells. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3. (b) Effects of E2 and 2-OHE2 or 4-OHE2 on the proliferative capacity of MCF-7 cells. The data are analysed by Student’s t-test and presented as the mean
± SEM, n=3; **P<0.01 compared to the control. (c) Schematic diagram of MCF-7 or MDA-MB-231+THP-1 cell co-culture model. (d) Effects of E2 and 2-OHE2 or 4-OHE2 on the migratory capacity of cells in the MCF-7+THP-1 cell co-culture model. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3; *P<0.05, **P<0.01 compared to the control group. (e) Effects of E2 and 2-OHE2 or 4-OHE2 on the proliferative capacity of MCF-7 in the MCF-7+THP-1 cell co-culture model. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3; *P<0.05, **P<0.01 compared to the control group. (f) Effects of E2 and 2-OHE2 or 4-OHE2 on the migration capacity of MCF-7 in the MCF-7+THP-1 cell co-culture model. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3; *P<0.05, **P<0.01, ***P<0.001 compared to the control group.

Figure 4

Hydroxylated oestrogen activates TAMs to mediate breast tumor progression and metastasis

(a) Schematic illustration of the establishment of xenograft tumors in four-week-old female BALB/C-NU/NU (nude) mice. (b-c) Effect of oestrogen on the induction of xenografted breast cancer tumors. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=6; **P<0.01, ***P<0.001 compared to the Control group. (d) Sections of xenograft tumors derived from MCF-7 cells in mice treated with oestrogen or tumors derived from MCF-7+THP-1 cells in mice treated with oestrogen were stained with H&E and subjected to histological analysis. (e) F4/80 expression in tumors derived from MCF-7 cells in mice treated with oestrogen or in tumors derived from MCF-7+THP-1 cells in mice treated with oestrogen was analysed by IHC. Representative images are shown.
The NLRP3 inflammasome is activated in mammary TAMs and functions as an oestrogen sensor.

(a-c) Changes in the protein expression levels of NLRP3 and ASC in THP-1 cells in different treatment groups. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, *P<0.05, **P<0.01, ***P<0.001 compared to the Control group. (d) IL-1β contents in the cell supernatants. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3; *P<0.05, **P<0.01 compared to the control group. (e) The mRNA levels of IL-1β in THP-1 cells in different treatment groups. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3; *P<0.05, **P<0.01 compared to the control group. (f-h) Changes in the protein expression levels of NLRP3 and ASC in THP-1 cells after virus infection. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, *P<0.05, ***P<0.001 compared to the CON group; #P<0.05 ##P<0.01 compared to the CON+4-OHE2 group. (i) The mRNA levels of IL-1β in THP-1 cells after virus infection. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3; *P<0.05 compared to the control group; ##P<0.01 compared to the CON+4-OHE2 group.
Figure 6

The NLRP3 inflammasome is activated in mammary TAMs and functions as an oestrogen sensor

(a) The effect of 4-OHE2 on the inflammatory microenvironment after NLRP3 gene expression was knocked down in co-cultured THP-1 cells. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, **P<0.01 vs. the CON group; ##P<0.01 vs. the CON+4-OHE2 group. (b) Effects of 4-OHE2 on the proliferative capacity of MCF-7 cells after NLRP3 gene expression was knocked down in co-
cultured THP-1 cells. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, **P<0.01 vs. the CON group; ###P<0.001 vs. the CON+4-OHE2 group. (c-e) The protein expression of MMP2 and MMP9 in MCF-7 cells in the co-culture system. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, *P<0.05, **P<0.01, ***P<0.001 vs. the CON group; ###P<0.01 compared with the CON+4-OHE2 group. (f) The expression of Nlrp3 after treatment with siNlrp3 in THP-1 cells. (g-i) After knockdown of the NLRP3 in THP-1 cells, the protein expression of MMP2 and MMP9 in MCF-7 cells was measured. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, *P<0.05, **P<0.01 vs. the Control group. (f) The expression of Nlrp3 after treatment with siNlrp3 in THP-1 cells. (g-i) After knockdown of the NLRP3 in THP-1 cells, the protein expression of MMP2 and MMP9 in MCF-7 cells was measured. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, *P<0.05, **P<0.01 vs. the Control group; #P<0.01 compared with the CON+4-OHE2 group. (j-k) The mRNA levels of MMP2 and MMP9 in MCF-7 cells in the co-culture system. The data are presented as the mean ± SEM, n=3, *P<0.05, **P<0.01 vs. the Control group. (l-m) The mRNA levels of MMP2 and MMP9 in MCF-7 cells in the co-culture system after NLRP3 was knocked. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, *P<0.05, ***P<0.001 vs. the CON group. ##P<0.01 vs. the CON+4-OHE2 group.
Abnormal hydroxylated oestrogen levels mediate activation of the inflammasome signalling pathway in mammary TAMs

(a) NLRP3 expression in the tumors of the xenograft BALB/C-NU/NU (nude) mouse model was analysed by IHC. (b) The protein levels of NLRP3, ASC, MMP9 and MMP2 in tumors of the xenograft BALB/C-NU/NU (nude) mouse model. (c-f) The mRNA levels of NLRP3, IL-1β, MMP2 and MMP9 in tumors of the xenograft BALB/C-NU/NU (nude) mouse model. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=6, **P<0.01, ***P<0.001 compared to the Control group. (g) NLRP3 expression in the tumors of MMTV-PyMT mice was analysed by IHC. (h) The protein levels of NLRP3, ASC, MMP2 and MMP9 in tumors of MMTV-PyMT mice. (i-l) The mRNA levels of NLRP3, IL-1β, MMP2 and MMP9 in tumors of MMTV-PyMT mice.
MMP9 in tumors from MMTV-PyMT mice. (i-l) The mRNA levels of NLRP3, IL-1β, MMP2 and MMP9 in tumors of MMTV-PyMT mice. The data are analysed by Student’s t-test and presented as the mean ± SEM, n=3, *P<0.05, **P<0.01 compared to the Control group (MMTV-PyMT).

Figure 8
Validation of the association between hydroxylated oestrogen and breast cancer microenvironment based on clinical data

(a-c) Expression levels of CD163, NLRP3 and MMP9 during breast disease progression. The data are analysed by Student’s t-test and expressed as the mean ± SEM, **P<0.01, ***P<0.001 compared with the Normal group. (d-f) Overall survival curve. K-M Plotter was used to analyse the role of CD163, NLRP3 and MMP9 in ER⁺ breast cancer prognosis. (g) Summary diagram.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.tif
- Additionalfile2.tif
- Additionalfile3.tif
- Additionalfile4.tif
- Additionalfile5.tif
- Additionalfile6.tif
• Additionalfile7.tif
• Additionalfile8.tif
• Additionalfile9.tif
• Additionalfile10.tif
• Additionalfile11.tif
• Additionalfile12.tif
• Additionalfile13.tif