Research Paper

Effect of Caffeic Acid on Nitrophenylethyl Ester through Regulating EGFR/STAT3/Akt Pathway Related Proteins on Triple Negative Breast Cancer

WEI HUANG, FANGFANG PENG[1], YUNLIN YU[2] AND HAIWEI LIU[2]*

Department of Surgery, [1]Department of Internal Medicine, Maternal and Child Health Hospital of Yichang, [2]Department of General Surgery, Affiliated Renhe Hospital of China Three Gorges University, Yichang, Hubei Province 443000, China

The objective of this study is to examine the impact of caffeic acid on triple negative breast cancer by regulating epidermal growth factor receptor/signal transduction and activator of transcription 3/protein kinase B pathway related proteins. The blank control group cells were immortalized human normal breast epithelial cell line MCF-10A, without any treatment, and were cultured routinely; the cells in the triple negative breast cancer group were human triple negative breast cancer MDA-MB-231 cells, which were routinely cultured without any treatment; in caffeic acid p-nitrophenyl ethyl ester group, human triple negative breast cancer MDA-MB-231 cells were added with caffeic acid p-nitrophenyl ethyl ester 10 µg/ml. After continue to incubate in the incubator for 48 h. The proliferation activity and the number of cell migration of caffeic acid on nitrophenylethyl ester group were lower compared to the triple negative breast cancer group. The number of cell invasion of caffeic acid on nitrophenylethyl ester group was lower in comparison to the triple negative breast cancer group, and the apoptosis rate exhibited a statistically significant increase in comparison to the triple negative breast cancer group. The levels of epidermal growth factor receptor, signal transduction and activator of transcription 3, protein kinase B, messenger ribonucleic acid, and protein were found to be significantly lower in the caffeic acid group compared to the triple negative breast cancer group. The expression levels of matrix metalloproteinase-2, matrix metalloproteinase-9 and vascular endothelial growth factor-A protein in the caffeic acid nitrophenethyl group were lower in comparison to the triple negative breast cancer group. Caffeic acid p-nitrophenyl ethyl ester can reduce the activity of triple negative breast cancer cells, inhibit the growth, migration and invasion of triple negative breast cancer cells, and induce apoptosis of colorectal cancer cells. The mechanism potentially exhibits a correlation with the regulation of the epidermal growth factor receptor/signal transduction and activator of transcription 3/protein kinase B signaling pathway.

Key words: Caffeic acid, p-nitrophenylethyl ester, epidermal growth factor receptor, signal transduction, protein kinase B, triple negative breast cancer

Breast Cancer (BRCA), is the prevailing form of cancer among women. With the acceleration of the pace of modern life and the continuous increase of life pressure, the prevalence of BrCa is progressively rising annually, posing a significant threat to the overall well-being of women, both in terms of their physical and psychological health[1]. BRCA can be divided into three types; estrogen receptor positive BRCA, estrogen receptor negative BRCA and triple negative BRCA (Triple Negative Breast Cancer (TNBC))[2]. NBC accounts for about 15 %-20 % of all pathological types of BRCA. It is difficult to cure completely because of its easy metastasis and recurrence. It has gradually become a difficult problem in clinical treatment, with the characteristics of poor targeting, high refractory

***Address for correspondence**
E-mail: xunjike605007267@163.com

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degree, poor prognosis and so on[3]. In-depth study of the pathogenesis and etiology of TNBC and looking for new treatment targets have important clinical significance for improving patients' clinical symptoms, slowing down the progression of cancer and improving patient's quality of life. The Epidermal Growth Factor Receptor (EGFR) is a transmembrane glycoprotein possessing Protein Tyrosine Kinase (PTK) activity. It has the ability to interact with the Epidermal Growth Factor (EGF) and form either homodimers or heterodimers with kinase activity[4]. Overexpression of EGFR can stimulate the enhancement of a variety of biological functions by inducing downstream intracellular signal transduction pathways such as Phosphatidylinositol 3 Kinase/Protein Kinase B (PI3K/AKT) and c-JUN N-Terminal Kinase/ Signal Transducer and Transcriptional Activator (JNK/STAT), such as promoting cell growth, preventing apoptosis, accelerating invasion and angiogenesis[5]. Caffeic acid phenethyl ester represents a prominent constituent of propolis, exhibiting significant biological activity. It has been found that caffeic acid phenethyl ester can effectively block EGFR signal transduction pathway to inhibit tumor cell migration and angiogenesis[6]. Caffeic acid p-nitrophenlethyl ester, a synthesized derivative of caffeic acid phenlethyl ester, exhibits enhanced pharmacological properties compared to its precursor, encompassing antioxidant, anti-inflammatory, anti-cancer, and various other biological effects. The objective of this study was to investigate the impact of caffeic acid p-nitrophenylethyl ester on TNBC by regulating proteins related to EGFR/STAT3/Akt pathway; the intention was to offer a novel perspective for the therapeutic approach to TNBC in a clinical setting.

MATERIALS AND METHODS

Materials and reagents:

MCF-10A and MDA-MB-231 cells were from the American Type Culture Collection (ATCC) cell bank. P-nitrophenyl ethyl caffeate was synthesized and analyzed as described in previous study[6]. EGFR, STAT3, Akt, messenger Ribonucleic Acid (mRNA) primers and Beta (β)-actin primers (Sigma company, United States of America (USA)); quantitative Polymerase Chain Reaction (qPCR) detection kit, Cell Counting Kit-8 (CCK-8) detection kit, Immunohistochemically (IHC) sheep anti-rabbit second antibody kit, Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) apoptosis kit (Shanghai Biyuntian Co., Ltd.); Transwell chamber (Corning Company, USA); artificial reconstructed basement membrane glue (Matrigel) from BD company of the United States. EGFR, matrix Metalloproteinase (MMP)-2, STAT3, Akt, matrix MMP-9, Vascular Endothelial Growth Factor A (VEGFA), β-actin-antibody (Abcam Biotechnology Co., Ltd.).

Methods:

Cell culture and treatment: Following resuscitation, the cells were incubated with Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10 % fetal bovine serum, and subsequently incubated in a constant temperature incubator (37°, 5 % Carbon dioxide (CO_2)). Set blank control group, TNBC group and caffeic acid p-nitrophenylethyl ester group. The cells in the blank control group were immortalized human normal breast epithelial cell line MCF-10A without any treatment, and the cells in the TNBC group were human BRCA MDA-MB-231 cells without any treatment, and the MDA-MB-231 cells in the caffeic acid p-nitrophenyl ethyl ester group were cultured in the incubator for 48 h after adding 10 μg/ml of caffeic acid p-nitrophenyl ethyl ester. Each experiment was repeated 6 times.

Western blot: Adjust the cell density to 1×10^6 cells/well and inoculate them into 6-well plates, incubate them in a constant temperature incubator (37°, 5 % CO_2), then homogenize them into 10 % homogenate by adding cellular protein lysis buffer at 4° and then centrifuge them and take the supernatant for examination. Bicinchoninic Acid Assay (BCA) method was used to determine the protein concentration, gel was prepared, electrophoresed for 90 min, gel was cut, membrane was rotated for 90 min, milk was sealed, washed, and then sequentially incubated with primary and secondary antibodies, developed, and Bio-Rad Image Lab software was used to analyze the results.

qPCR: Adjust the cell density to 1×10^6 cells/well inoculated in 6-well plates and cultured in a constant temperature incubator (37°, 5 % CO_2), MDA-MB-231 cells were cultured in the incubator after adding p-nitrophenylethyl caffeic acid 10 μg/ml for 48 h, following the instructions of the TRIzol

reagent, Then RNA was reversely transcribed into cDNA. We performed the amplification reactions as per the instructions provided with the Tras Start Top Green qPCR Super mix. After the reaction was completed, a software program was used to calculate relative mRNA expression.

CCK-8: Human BRCA MDA-MB-231 cells were cultured in incubator for 48 h after adding 10 µg/ml caffeic acid p-nitrophenyl ethyl ester, 10 µl of CCK-8 is added to each sample and incubated for 4 h. Absorbance at 450 nm is measured by a spectrophotometer. The quantified result is equal to (Optical Density (OD) measurement of the experimental group-OD measurement of the control group)/(negative control-mock).

Transwell: The MDA-MB-231 cells were added with resveratrol 15 µg/ml and cultured for 48 h, each group was adjusted to have a cell density of 5×105 cells/well inoculated in the upper chamber of the Transwell, in the lower chamber, medium containing 10 % fetal bovine serum was added. The control group was given the same amount of DMEM culture medium, fixed, stained, and then microscopically count the purple stained perforated cells and calculate the cell migration ability. For the detection of cell invasion ability, the upper chamber of the Transwell was first covered with Matrigel in an ultra-clean bench, and the subsequent steps were the same as that of cell migration.

Statistical method:

Analysis and processing of data were carried out using the Statistical Package for the Social Sciences (SPSS) 22.0 statistical software, and the measurement information was expressed by (x±s), and the comparison was made by t-test. In comparison to the blank control group, $^{a}p<0.05$ and in comparison to the TNBC group, $^{b}p<0.05$.

RESULTS AND DISCUSSION

The TNBC group exhibited elevated cell proliferative activity and cell migration compared to the blank control group, whereas the caffeic acid p-nitrophenyl ethyl ester group demonstrated reduced levels of these activities in comparison to the TNBC group as shown in Table 1.

The number of cell invasion in the TNBC group was higher compared to the blank control group, the number of cell invasion in the caffeic acid p-nitrophenyl ethyl ester group was lower compared to the TNBC group, and the overall apoptosis rate was higher compared to the TNBC group as shown in Table 2.

The expression levels of EGFR, STAT3, and Akt mRNA were comparatively elevated in the TNBC group compared to the blank control group, while the relative expression of EGFR, STAT3 and Akt mRNA in the caffeic acid p-nitrophenyl ethyl ester group was lower compared to the TNBC group as shown in Table 3.

TABLE 1: EFFECT OF CAFFEIC ACID p-NITROPHENLETHYL ESTER ON PROLIFERATION AND MIGRATION OF TNBC CELLS

Group	n	Cell proliferative activity	Number of cell migration
Blank control	6	0.10±0.01	73.25±15.70
TNBC	6	0.22±0.04[a]	142.74±24.34[a]
Caffeic acid p-nitrophenylethyl ester	6	0.15±0.03[b]	106.48±17.44[b]
F		25.154	19.022
p		0.000	0.000

Note: Comparison to the blank control group, $^{a}p<0.05$ and comparison to the TNBC group, $^{b}p<0.05$

TABLE 2: EFFECT OF CAFFEIC ACID P-NITROPHENLETHYL ESTER ON INVASION AND APOPTOSIS OF TNBC CELLS

Group	n	Overall apoptosis rate (%)	Number of cell invasion
Blank control	6	9.97±1.23	51.72±10.31
TNBC	6	9.51±1.13[a]	121.05±20.26[a]
Caffeic acid p-nitrophenylethyl ester	6	31.34±4.25[b]	93.12±16.35[b]
F		134.293	26.157
p		0.000	0.000

Note: Comparison to the blank control group, $^{a}p<0.05$ and comparison to the TNBC group, $^{b}p<0.05$

TABLE 3: EFFECT OF CAFFEIC ACID P-NITROPHENLETHYL ESTER ON THE EGFR, STAT3 AND AKT mRNA IN TNBC CELLS

Group	n	EGFR mRNA	STAT3 mRNA	Akt mRNA
Blank control	6	1.24±0.32	0.83±0.35	0.77±0.09
TNBC	6	2.47±0.54[a]	2.59±0.67[a]	2.05±0.46[a]
Caffeic acid p-nitrophenylethyl ester	6	1.65±0.40[b]	1.76±0.43[b]	1.32±0.35[b]
F		12.744	18.451	21.687
p		0.000	0.000	0.000

Note: Comparison to the blank control group, [a]p<0.05 and comparison to the TNBC group, [b]p<0.05

The protein expression levels of EGFR, STAT3 and Akt in the TNBC group were higher compared to the blank control group, while EGFR, STAT3 and Akt in the caffeic acid p-nitrophenyl ethyl ester group were lower compared to the TNBC group as shown in Table 4.

The protein expression levels of MMP-2, MMP-9, and VEGFA were found to be lower in the group treated with caffeic acid p-nitrophenyl ethyl ester compared to the group with TNBC as shown in Table 5.

BRCA is a malignant tumor caused by uncontrolled proliferation of breast epithelial cells. During the initial phase of the disease, patients often show symptoms such as enlargement of axillary lymph nodes, nipple discharge, breast mass and so on. During the advanced stage, the potential for cancer cells to spread to distant sites and cause multiple organ lesions poses a direct threat to the life and safety of patients[7]. TNBC, comprising approximately 20 % of all BRCA cases, is characterized by the absence of estrogen receptor, progesterone receptor, and human EGFR2. Due to its elevated rates of metastasis and recurrence, TNBC exhibits a poor prognosis, resulting in a low 5 y survival rate[8]. Due to the distinctive molecular phenotype exhibited by TNBC, patients diagnosed with this subtype are unresponsive to both endocrine and molecular targeted therapeutic approaches[9]. At present, the primary therapeutic approach for individuals diagnosed with TNBC involves the utilization of local surgical resection and systemic chemotherapy. Nevertheless, the efficacy of traditional postoperative adjuvant radiotherapy is suboptimal, resulting in a notable prevalence of metastasis and recurrence among patients. Therefore, TNBC has emerged as the most formidable subtype of BRCA. Research has demonstrated a significant correlation between activated carcinogenic molecules and the advancement of tumors and their subsequent spread to other parts of the body. Moreover, the deliberate eradication of these carcinogenic agents has been found to effectively decelerate the pace at which tumor metastasis occurs[10]. Therefore, it is urgent to study drugs that can inhibit activated carcinogenic molecules in order to reduce tumor metastasis.

Numerous studies have demonstrated a strong correlation between the aberrant activation of proteins, such as EGFR, and the progression and metastasis of TNBC, survivin and epithelial-mesenchymal transformation-related proteins[11]. EGFR is a transmembrane glycoprotein with PTK activity, which plays an important role in tumor formation and development, and gradually becomes the key target of tumor targeted therapy[5]. EGFR exhibits overexpression in over 50 % of patients with TNBC and assumes a crucial regulatory function in the processes of proliferation, migration, angiogenesis, and anti-apoptotic defense mechanisms within TNBC cells, and EGFR can activate and mediate downstream signal transduction pathways, including intracellular signal transduction pathways such as PI3K/AKT and JNK/STAT[12]. Numerous studies have substantiated the presence of aberrant EGFR expression across diverse malignant neoplasms, whereby the activation of EGFR molecules effectively triggers the PI3K/Akt and JNK/STAT signaling cascades, thereby substantiating their role in facilitating tumor metastasis[13].

The PI3K/AKT signaling pathway is extensively employed in the human body as a signal transduction pathway, exerting a crucial influence on the regulation of various cellular processes such as cell proliferation, differentiation, migration, and apoptosis. Additionally, this pathway actively participates in angiogenesis, facilitates the conversion of epithelial cells into mesenchymal

cells, and significantly impacts chemotherapy resistance, among numerous other biological processes[14]. AKT serves as both the direct target gene and downstream effector of PI3K, making it a crucial component in PI3K signaling. The phosphorylation of AKT can be employed as a reliable indicator of PI3K activity. Furthermore, AKT phosphorylation exerts regulatory control over downstream proteins, thereby modulating fundamental cellular functions[15]. Research has substantiated that the excessive activation of the PI3K/AKT signaling pathway assumes a significant role in governing the proliferation, survival, and invasiveness of endometrial cancer cells, BRCA cells, and gastric cancer cells[16]. Furthermore, empirical research has demonstrated that the JAK/STAT signal transduction pathway plays a significant role in facilitating tumor cell growth and metastasis, while concurrently impeding the anti-tumor immune response. Additionally, the Interleukin-6 (IL-6)/JAK/STAT3 pathway has been found to exhibit a strong correlation with the proliferation of Cluster of Differentiation (CD) 44^+ CD24-stem cell-like BRCA cells[17]. Basal-like tumors exhibit an enrichment of $CD44^+$ CD24-stem cell-like BRCA cells, and their biological functions are very similar to those of TNBC[18]. In addition, In the investigation of patients diagnosed with metastatic TNBC, IHC analysis of TNBC specimens revealed that 40.4 % of the patients exhibited moderate to high levels of pSTAT3 protein expression[19].

The findings indicated that the relative expressions of EGFR, STAT3, Akt mRNA and protein in caffeic acid p-nitrophenylethyl ester group were lower than those in TNBC group. It is suggested that caffeic acid p-nitrophenyl ethyl ester can reduce the protein expression of EGFR, STAT3 and Akt in TNBC cells. In addition, the results of this study also found that the cell proliferation activity and the number of cell migration in the caffeic acid p-nitrophenyl ethyl ester group were lower compared to the TNBC group. The number of cell invasion in caffeic acid p-nitrophenyl ethyl ester group was lower compared to the TNBC group, and the overall apoptosis rate in caffeic acid p-nitrophenylethyl ester group was higher compared to the TNBC group. The findings propose that caffeic acid p-nitrophenyl ethyl ester can inhibit the metastasis of TNBC by regulating EGFR/STAT3/Akt signal pathway. Caffeic acid phenethyl ester is a bioactive natural ingredient extracted from propolis. Studies have found that caffeic acid phenethyl ester can effectively block EGFR signal transduction pathway to inhibit tumor cell migration and angiogenesis, and it has inhibitory activity against prostate cancer, BRCA, colon cancer and other cancers[20]. Caffeic acid p-nitrophenl ethyl ester is a derivative of caffeic acid phenethyl ester synthesized in the laboratory. It exhibits superior pharmacological properties compared to caffeic acid phenethyl ester, encompassing antioxidant, anti-inflammatory, anti-cancer, and various other biological effects[21].

The findings indicated that the protein expression levels of MMP-2, MMP-9, and VEGFA were comparatively reduced in the group treated with caffeic acid p-nitrophenylethyl ester, in comparison to the group with TNBC. MMP-2 and MMP-9, belonging to the matrix metalloproteinases family, exert significant influence on angiogenesis; wound healing, and tumor invasion. Furthermore, the investigation reveals the involvement of VEGFA in angiogenesis and metastasis[22]. Tumor cells can promote angiogenesis and digest extracellular matrix by secreting VEGFA and metalloproteinases to provide conditions for tumor metastasis.

TABLE 4: EFFECT OF CAFFEIC ACID p-NITROPHENYLETHYL ESTER ON PROTEIN EXPRESSION OF EGFR, STAT3 AND AKT IN TNBC CELLS

Group	n	EGFR	STAT3	Akt
Blank control	6	0.28±0.04	0.30±0.03	0.33±0.14
TNBC	6	0.86±0.28[a]	0.76±0.11[a]	0.78±0.12[a]
Caffeic acid p-nitrophenylethyl ester	6	0.57±0.08[b]	0.52±0.09[b]	0.44±0.14[b]
F		15.521	45.156	18.481
p		0.000	0.000	0.000

Note: Comparison to the blank control group, [a]$p<0.05$ and comparison to the TNBC group, [b]$p<0.05$

TABLE 5: EFFECT OF CAFFEIC ACID P-NITROPHENYLETHYL ESTER ON PROTEIN EXPRESSION OF MMP-2, MMP-9 AND VEGFA IN TNBC CELLS

Group	n	MMP-2	MMP-9	VEGFA
Blank control	6	0.33±0.17	0.37±0.09	0.31±0.03
TNBC	6	0.84±0.18[a]	0.86±0.16[a]	0.64±0.03[a]
Caffeic acid p-nitrophenylethyl ester	6	0.35±0.14[b]	0.43±0.14[b]	0.49±0.04[b]
F		15.521	45.156	144.529
p		0.000	0.000	0.000

Note: Comparison to the blank control group, [a]p<0.05 and comparison to the TNBC group, [b]p<0.05

Numerous academic studies have unequivocally validated the efficacy of inhibiting the expression of MMP-2, MMP-9, and VEGFA in impeding tumor growth, metastasis, and angiogenesis[23]. Therefore, p-nitrophenyl ethyl caffeine may inhibit the growth and metastasis of BRCA by inhibiting MMP-2, MMP-9 and VEGFA. The expression of these proteins is related to EGFR/STAT3/Akt signal pathway[24].

To sum up, caffeic acid p-nitrophenyl ethyl ester can reduce the viability of TNBC cells, inhibit the growth and metastasis of TNBC cells, and induce apoptosis of colorectal cancer cells. The mechanism potentially exhibits a correlation with the regulation of the EGFR/STAT3/Akt signal pathway.

Conflict of interests:

The authors declared no conflict of interests.

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