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Original Article

Electrochemotherapy inhibited breast cancer MDA-MB231 cell migration and invasion through PI3K/AKT signal pathway

Bing-Gang Zhou[1], Chang-Sheng Wei[2], Song Zhang[2], Zhi Zhang[2], Yi-Jun Shen[2], Jian Wang[3], Huan-Min Gao[3]

[1]Department of Tumour Surgery, The First People's Hospital of Yinchuan, Yinchuan 750002, Ningxia, China; [2]Ningxia Medical University, Yinchuan 750004, Ningxia, China; [3]Department of Neurology, Ningxia People's Hospital, Yinchuan 750002, Ningxia, China

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Abstract: Several clinical studies have showed effectiveness of electrochemotherapy (EChT) in malignant tumors metastases, however, the mechanism has not been known. Thus this study was to explore the mechanism of EChT on tumor migration and invasion. MTT assay was used to measure breast cancer MDA-MB231 cells growth inhibition induced by different EChT coulomb after 24 h. Transwell migration and matrigel invasion assays were performed to evaluate the migration and matrigel invasion respectively. Real-time RT-PCR and Western blot were used to evaluate the VEGF, MMP-2, and PTEN, AKT and p-AKT mRNA and protein expression in MDA-MB231 cells. RT-PCR showed the relative expression of VEGF and MMP-2 in 5C and 10C EChT groups compared with the control group (VEGF 0.591±0.038, MMP-2 0.601±0.045 in 5C EChT group; VEGF 0.453±0.003, MMP-2 0.410±0.064 in 10C EChT group, $P<0.01$ respectively). The relative gene expression of AKT reduced gradually, but PTEN increased. The expression of VEGF, MMP-2 and AKT intervened separately by 1C EChT and MK2206 (0.05 μmol/L), VEGF and MMP-2 in 3C, 5C and 10C EChT groups reduced gradually (VEGF 0.426±0.046, MMP-2 0.223±0.018 in 5C EChT group, $P<0.01$). P-AKT protein expression reduced, but PTEN increased. The total AKT protein expression was unchanged ($P>0.05$). At the same inhibitory rate of 1C EChT and MK2206 (0.05 μmol/L), the EChT group was more effective than MK2206 group in reducing the protein expression of VEGF, MMP-2 and p-AKT with increasing the protein expression of PTEN ($P<0.01$ respectively). EChT inhibited breast cancer cells migration and invasion through PI3K/AKT signal pathway.

Keywords: Electrochemical treatment (EChT), MDA-MB231, invasion, PI3K/AKT signal pathway

Introduction

Breast cancer accounts for 51% of the total cutaneous metastases [1]. Electrochemotherapy (EChT) is a complementary therapy for controlling cutaneous and subcutaneous metastases of malignancies [2-4]. Low intensity direct electric current is applied to a tumor through electrodes in the surrounding areas of the tumor body. EChT is promising to relieve tumor-associated symptoms and improve quality of life.

Recent meta-analysis showed an overall response rate for EChT of cutaneous metastases up to 75%, with a complete response rate of 47% [5]. This treatment is noted for its effectiveness and safe in anticancer and relieve pain with minimal invasiveness and lower cost [6-9].

The preliminary study indicated that EChT inhibited tumor invasion and metastasis by increasing cell permeability and enhancing drug uptake into tumour body [9-12], regulated the tumor cell cycle, and induced cell apoptosis [13-16]. However the molecular mechanisms of EChT on tumor invasion and metastasis have been unknown. Therefore the present study was to investigate the mechanism of the therapy in an in vitro model for human breast cancer.

Materials and methods

Cell line and cell culture

The human breast cancer cell line MDA-MB231 was obtained from the Beijing tumor cell bank (Beijing, China). MK2206 (MCE, NJ). RPMI1640 cell nutrient solution, fetal calf serum and pan-

creatin (Hyclone, UT), MTT kit (Nanjing KGI company, Jiangsu, China), Transwell cabins (Unique company, Beijing, China), Trizol reagents (Gibco, NY), reverse transcription kit and fluorescent reagent (Thermo, MA, USA), holoprotein extraction kit and BCA protein assay kit (Nanjing KGI company, Jiangsu China), rabbit anti-VEGF monoclonal antibody and mouse anti-MMP-2 monoclonal antibody (Abcam, Cambride, UK), mouse anti-PTEN monoclonal antibody, mouse anti-p-AKT polyclonal antibody, rabbit anti-AKT monoclonal antibody (Proteintech Group Inc, IL), ZAY-6BII electrochemical treatment device (Beijing University of Aeronautics and Astronautics, Beijing, China).

Cell culture and electrode making

Human breast cancer cell MDA-MB231 was cultured in RPMI1640 cell nutrient solution containing 10% fetal calf serum, penicillin 100 μL/mL, streptomycin 100 μL/mL, in 37°C, 5% CO_2, 100% Humidity incubator, solution was changed every day, pancreatin digested and subculture when adherent cells were observed.

The cover of 24 holes plate was drilled vertically with two holes at every corresponding hole location, with an interval of 1 cm between two platinum electrodes. Remains were fixed in each hole. Electrodes were dipped into the culture medium just above the cell layer.

Cell treatment in vitro

The same amount of cells which in logarithmic growth phase were cultured in 6-hole plate after treated with 0.25% pancreatin digestion. Control and treatment groups were randomly divided. The control groups were 0C and the treatment groups were divided into 5 groups according to different power: 1C, 3C, 5C, 10C, and 15C, each was repeated 6 holes, measurement results averaged. Adherent cells filled up the bottom of the hole, The control groups without current, the treatment groups were gradually boosted to the current stably at l0 mA within voltage 5-8 V. Adjusted the current slowly back to zero. Cells were continued at 37°C, 5% CO_2 incubator for 6 h or 24 h.

Cell growth inhibition studies

Transfer 200 μL cells to 96-hole plate after EChT treatment with different coulomb dose (1C, 3C, 5C, 10C, 15C) for 6 h and 24 h, and add MTT solution (5 mg/mL) 20 μL to each well at the same time, continue to incubate 4 h at 37°C MTT solution before to stop reaction, supernatant off. Added 150 μL DMSO to each holes and oscillated 10 min. 570 nm wavelengths, the absorbance of each hole was measured by enzyme labeling instrument, enzyme-linked absorbance (A) data were obtained.

Inhibition Ratio = (control group A-experimental group A)/control group A × 100%

The absorbance D (570) value that MK2206 of different concentrations affect on human breast cancer cell line MDA-MB231 of 24 h each hole was measured by the same way.

Transwell migration assays

The control group was 0C and the treatment groups were treated with 5C EChT; cells were treated with 0.25% pancreatin digestion and have an inoculation in a cabin with 25 μL matrigel glue. Added 200 μL (1×10^5/cabin) cell suspension on above cabinet, add 500 μL RPMI1640 cell nutrient solution containing 10% fetal calf serum in blow cabinet, Cells were continued in 37°C, 5% CO_2 incubator for 24 h cultivation. The cabinets were proceed with 4% formalin fixation 15 min, then dyed with gentian violet 15 min, cotton swabs were used to remove the cells off which could not go though filter membrane, selected 10 fields of vision in light microscope randomly, counted number of cells in each field of vision.

Transwell invasion assays

There is no matrigel glue on transwell cabinet; other experimental procedures were the same with transwell migration assays.

The experimental group and EChT treatment

The experimental group was divided into negative control group, MK2206 positive control group and different coulomb dose EChT treatment group. MK2206 positive group was at same concentration with EChT 1C dose of cell inhibition rate of 10%. Fluorescence quantitative RT-PCR selected EChT intervene power 3C, 5C (because this method is accurate to detect from the mRNA molecule level). Western blot selected EChT treatment coulomb dose 1C, 3C, 5C, 10C; MK2206 intervene concentration was

Table 1. Real-time fluorescence quantitative RT-PCR primer sequence

Gene	Upstream primer (5'→3')	Downstream primer (5'→3')	Fragment Length (bp)
VEGF	TTCTGAGTTGCCCAGGAGAC	TGGTTTCAATGGTGTGAGGA	173
MMP-2	TATGGCTTCTGCCCTGAGAC	CACACCACATCTTTCCGTCA	142
PTEN	AGACCATAACCCACCACAGC	CACCAGTTCGTCCCTTTCC	125
AKT	GCCCAACACCTTCATCATC	TCCTCCTCCTGCTTCTTGAG	141
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC	258

Table 2. Variation of cell inhibition rate after different EChT in 24 h (n=9, $\bar{x}\pm s$) (EChT power: Coulomb C)

Groups	Inhibition rate (%)
Control	0.00±0.36
1C	10.18±0.46[a]
3C	25.04±0.78[a]
5C	65.13±0.62[a]
10C	89.42±0.84[a]
15C	95.23±0.73[a]

a: P<0.05, Compared with the control group.

Table 3. Variation of cell inhibition rate after different concentration of MK2206 in 24 h (n=9, $\bar{x}\pm s$) (MK2206: μmol/L)

Control	0.00±0.40
0.05 μmol/L	11.02±0.65[a]
0.1 μmol/L	17.85±0.69[a]
0.2 μmol/L	19.84±0.18[a]
0.5 μmol/L	26.31±0.41[a]
1.0 μmol/L	33.41±1.10[a]

a: *P*<0.05, compared with the control group.

0.05 μmol/L. Blank control group mixed in PBS of equal volume without current.

RNA isolation and RT-PCR

Total cellular RNA was extracted from cultured MDA-MB231 cells after 24 h. Trizol reagents 500 μl added in the blank control group, EChT treatment group of different coulomb dose (3C, 5C), and MK2206 positive control group (final concentration 0.05 μmol/L). Nanodrop spectrophotometer detects RNA purity. D (260)/D (280) in 1.8 to 2.0 was used for the test. Reverse transcription and amplification reaction on the basis of thermo reverse transcription and fluorescence Mix Instructions. PCR reaction primer sequence was shown in **Table 1**. GAPDH as internal reference.

Western blot analysis

Abstract total protein of the blank control group of intervene MDA-MB231 cell line 24 h, MK2206 positive control group (final concentration 0.05 μmol/L), and EChT treatment group of different coulomb dose (1C, 3C, 5C, 10C). Measure protein concentration in each group according to BCA protein assay kit instructions. 30 μg protein in each group, a total 20 μl sample-loading electrophoresis and transmembrane. VEGF and MMP-2 factor constant-current transfer membrane in 300 mA for 110 min, other 3 factors constant-current transmembrane for 50 min. After seal membrane with 5% skimmed milk powder 1 h, incubate VEGF, MMP-2, PTEN, AKT, p-AKT antibody (VEGF antibody concentration 1:800, other antibody concentration 1:2000, GAPDH concentration 1:800). Incubate in 4°C overnight. PBST washed the membrane (TBST for p-AKT) 5 times in 25 min. Goat anti-rabbit and goat anti-mouse IgG antibody of concentration 1:6000 incubated 60 min in room temperature, washed the membrane again and exposure the film.

Statistical analysis

Data was represented as $\bar{x}\pm s$. SPSS 17.0 Statistical Software was used. ANOVA was used for multivariate comparison.

Results

Variation of cell inhibition rate

The comparisons between each EChT treatment groups and blank control group were statistically significantly (*P*<0.05, **Table 2**). The comparisons between each MK2206 effect groups and blank control group were statistically significantly (*P*<0.05, **Table 3**). No Significant differences was found between EChT treatment 1C group and MK2206 effect 0.05 μmol/L group (*P*>0.05).

EChT Transwell experiment

Transwell invasion experiment showed MDA-MB231 cells were treated with 5C EChT, the number of invasion cells in each high power field was (361±13) to (99±7), compared with

Table 4. Relative expression levels of each gene after EChT different coulomb dose and different concentration MK2206 in 24 h (n=9, $\bar{x}\pm s$)

Group	VEGF	MMP-2	PTEN	AKT
Control group	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
MK2206 group	0.601±0.028[a]	0.541±0.043[a]	2.565±0.070[a]	0.610±0.018[a]
EChT 3C group	0.620±0.081[a]	0.581±0.025[a]	1.310±0.059[a]	0.633±0.042[a]
EChT 5C group	0.413±0.024[a]	0.389±0.072[a]	1.605±0.019[a]	0.575±0.083[a]

a: P<0.05, Compared with the control group.

the control group (*P*<0.01). This result suggested that EChT could inhibit the breast cancer cell ability of invasion.

Transwell migration experiment showed cells were treated with 5C EChT, the number of migration cells in each high power field was (376±14) to (129±11), compared with control group (*P*<0.01). This result suggested that EChT could inhibit the breast cancer cell ability of migration.

RT-PCR detect the expression levels of VEGF, MMP-2, PTEN and AKT gene

Compared with the control group, as the increase of EChT coulomb dose, the relative expression levels of VEGF and MMP-2 in 3C and 5C EChT groups were reduced gradually, the relative gene expression level of AKT was also reduced gradually, but that of PTEN of PI3K/AKT signal pathway was increased gradually. The expression levels of VEGF, MMP-2 and AKT in the cells intervened separately by 1C EChT and 0.05 μmol/L, MK2206 with no significant difference at the same inhibition rate (*P*>0.05), but increases the expression level of PTEN (*P*<0.05, **Table 4**).

Western blot detect the protein expression of VEGF, MMP-2, PTEN, AKT and p-AKT gene

When compared with the control group, as the increase of EChT treatment coulomb dose, the protein expression of VEGF and MMP-2 reduced gradually. The protein expression of p-AKT also reduced gradually, but PTEN increased gradually. The total AKT protein expression was unchanged obviously. At the same inhibition rate of 1C EChT and MK2206 (0.05 μmol/L), the EChT group was more effective than MK2206 group in reducing the protein expression of VEGF, MMP-2 and increasing the protein expression of PTEN, and MK2206 group more effective in reducing the protein expression of p-AKT. However, the total AKT protein expression of the two groups was no significantly different (*P*> 0.05).

Discussion

Tumor metastasis is a multistep process, which includes detachment of cancer cells from primary tumor, migration, adhesion, and invasion into the blood or lymphatic vessels. Recurrent metastasis of tumor is one of the most intractable problems in clinical cancer treatments.

It is well known that VEGF and MMP-2 could regulate the vascularization and tumor metastasis as prognostic indicator for a variety of cancer. The digest of tumor extracellular matrix make it premise condition for cancer invasion and metastasis [17, 18]. Degradation of basal membrane is the key procedure for tumor invasion and metastasis induced by MMP-2 and MMP-9 [19]. VEGF and MMP-2 are highly expressed in aggressive breast tumors with poor clinical outcome [20]. The inhibition of VEGF and MMP-2 expressions is a critical step in the prevention of cancer metastasis [21, 22]. In 2005, Kim and colleagues [23] discovered the activation of PI3K/AKT signal pathway as one of the main mechanisms of tumor cells anti-apoptotic. VEGF and MMP-2 expressions were mediated by the PI3K/Akt pathway [24, 25]. PI3K is a lipid kinase that controls multiple cellular processes through AKT activation. AKT activation leads to cancer invasion and metastasis by stimulating the secretion of MMPs [26, 27]. Under the influence of the internal and external factors, the starting of PI3K/AKT signal pathway induces cell proliferation, differentiation and avoid apoptosis, playing an important role in the malignant behavior of multidrug resistance to chemotherapeutic drugs and the recurrent metastatic of tumor cells [28-31].

PI3K/AKT signal pathway and recurrent metastasis of breast cancer are closed related [31]. Variety of growth factors of breast cancer changed via PI3K/AKT signal pathway. After chemotherapy, the AKT phosphorylation level increased and activated PI3K/AKT signal pathway, and further activating downstream factors, lead to the multidrug resistance and recur-

rent metastasis. Moreover, PI3K inhibitor would lead to a reduction in MMP-2 activation, cell migration and cell invasion [32-34].

The present study showed that at the same inhibition rate, in EChT groups, the expression levels of VEGF and MMP-2 were reduced gradually compared with AKT inhibitor MK2206 positive control group, and expression levels of VEGF and MMP-2 were reduced as the increase of EChT treatment coulomb dose, showed significant dose-dependence and with strong ability to inhibit cell migration and invasion; and favorite expressions of PTEN in PI3K/AKT signal pathway. Western blotting results showed that increasing trend of expression of PTEN protein and further inhibited the activating of AKT, correspond to decreasing expression of p-AKT protein as the increase of EChT treatment coulomb dose.

In conclusion, the present study demonstrated that EChT decreased the invasive ability of MDA-MB-231 breast cancer cells via PI3K/AKT signal pathway. These results provide new insights into molecular mechanisms involved in the anti-invasive activity of EChT in breast cancer cells. EChT might be a potentially useful anti-invasive agent for cutaneous and subcutaneous metastases of different malignancies.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Huan-Min Gao, Department of Neurology, Ningxia Hui Autonomous Region People's Hospital, Northwest University for Nationalities, Yinchuan 750002, Ningxia, China. Tel: +86-18695111531; Fax: +86-951-5920302; E-mail: gaohuanmin@126.com

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