Expression and relationship of ECT2 with cell cycle proteins CDK1 and CyclinB1 on the Paclitaxel intervention in three negative breast cancer cells

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Expression and relationship of ECT2 with cell cycle proteins CDK1 and CyclinB1 on the Paclitaxel intervention in three negative breast cancer cells

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Abstract

Purpose To investigate the relationship of expression of ECT2 and cell cycle-related proteins CDK1 and CyclinB1 in triple-negative breast cancer cells (TNBC) after ECT2 overexpression and interference and after paclitaxel (PTX) therapy, and hope to provide some theoretical basis for the treatment of TNBC.

Methods ECT2 overexpression and interfering plasmid were applied to cultured TNBC HCC1806 cells and nude mouse transplantation tumor assays were performed, while PTX was added in the group, and Westen-blot detected the expression of ECT2, CDK1, and CyclinB1 proteins. The relationship between the ECT2 and CDK1 and CyclinB1 genes was analyzed by TCGA database.

Results In vitro experiments, the ECT2 overexpression group showed that ECT2 protein expression was higher than that of the control group before and after PTX treatment (P<0.05), and CDK1 and Cyclin B1 was similarly higher than the control group (P<0.05), but the three proteins after PTX treatment was lower than that before. The ECT2 interference group showed that the expression of ECT2 and CDK1 and Cyclin B1 was lower than that of the control group (P<0.05), especially after PTX treatment, the decrease of their expression was more significant. In vivo experiments, the expression of ECT2 was significantly higher in the overexpression group and the overexpression group with the addition of PTX than control group (P<0.05), and significantly lower in the PTX group, the interference group and the interference group with the addition of PTX than control group (P<0.05), and CyclinB1 protein expression was statistically different in the ECT2 overexpression group and interference group with the addition of PTX compared with the PTX control group (P<0.05). The results of the TCGA database analysis showed that there was a positive correlation between the ECT2 and CDK1 and CyclinB1 genes.

Conclusion ECT2 promotes TNBC cell progression by acting in the G2/M phase of the cell cycle, and it may form a positive feedback loop with Cyclin B1 to promote the progression of the cell cycle and accomplish cell proliferation under the regulation of CDK1. The overexpression of
ECT2 may cause TNBC resistance to PTX, and combination of anti-ECT2-targeted drugs and PTX may offer help in TNBC treatment.

Keywords  TNBC · ECT2 · CDK1 · Cyclin B1 · paclitaxel

Introduction

Breast cancer is a heterogeneous disease, and its different subtypes vary in response to treatment. Neoadjuvant therapy based on paclitaxel (PTX) analogs is an important means of breast cancer treatment today, and many patients benefit from it, yet the response to treatment is relatively poor for patients with TNBC, so specific predictors for different molecular subtypes of breast cancer are crucial (Ozcan. 2023). Breast cancer development is influenced by a variety of factors, among which the inter-regulatory effects of oncogenes and oncogenes in the cell cycle are important factors in the proliferation and progression of tumor cells. ECT2 promotes the proliferation of tumor cells and mainly acts in the G2/M phase of the cell cycle. In the previous experiments (Wang et al. 2020), we established an in vivo and vitro experimental model and carried out PTX treatment, the results showed that the ECT2 overexpression group before and after the PTX treatment the percentage of cell number in the G1 phase and G2/M phase and the change in the DNA content were significantly different from that of the control group, while the ECT2 interference group only had a significant difference in the G2/M phase compared with that of the control group. In this part, we further explored the mechanism of ECT2's effect on the growth and proliferation of TNBC cells by detecting the expression of ECT2 and cell cycle related factors CDK1 and CyclinB1 in the pre-test, and explored the relationship of related indexes after the treatment of PTX, so as to provide a certain theoretical basis for the development of breast cancer and targeted therapy.

Materials and methods

Vitro and vivo experiments
According to the previous experiments (Wang et al. 2020), Cell culture is carried out in vitro experiment, the frozen basaloid TNBC cell line HCC1806 (KeyGen Biotech, China) was quickly removed from liquid nitrogen and thawed at 37°C. The cells were then incubated in the medium until the cells grew exponentially, and followed by ECT2 overexpression and interference experiments (interference target sequence: 5′-GCTGAGCATTTCCCTTTCCATA-3′). The in vitro experiments were divided into ten groups, including the control, LV-negative control (LV-NC), LV-ECT2, sh-NC and sh-ECT2 groups, and PTX treatment in each group.

Nude mouse xenograft assay were performed in vivo experiments. Experiments included involved 36 four- to five-week-old nude mice with severe combined immunodeficiency. All animal experiments were approved by the Medical ethics committee of Shanxi Medical University. Nude mice were inoculated with cell suspensions. The tumors grew to 70 mm³ after 21 days, and Nude mice were randomized into 6 groups. The PTX, LV-ECT2-PTX and sh-ECT2-PTX groups received tail-vein injections of PTX at the dose of 6 mg/kg, and the control, LV-ECT2 and sh-ECT2 groups were intravenously injected with normal saline at the same dose and interval. After 21 days, the assay was completed and the mice were euthanized to harvest tumors.

**Westen-blot assay**

All collected tumor tissue samples for Westen-blot assay to detect the expression of ECT2 protein and cell cycle-related proteins CDK1 and CyclinB1, and the antibodies were purchased from the UK abcam company. Total tissue cellular proteins were extracted and the protein concentration was determined by BCA method, followed by gel electrophoresis experiments by SDS-PAGE. Protein samples were added sequentially, and the voltage was 80 V to start electrophoresis for 20 min, and then adjusted to 120 V to continue electrophoresis after bubbles appeared, until the bromophenol blue reached the bottom of the separator gel to stop the electrophoresis. Then transfer to the PVDF membrane, transfer the membrane at 200 mA for 100 min,
after that, wash and close to add diluted primary antibody (ECT2: 1:2000; CDK1: 1:10,000; CyclinB1: 1:2000) incubation and rinsing, add the secondary antibody, immerse the membrane in ECL luminescent solution, and finally machine exposure imaging to analyze the gray scale value.

**Bioinformatics analysis**

Visit the TIMER2.0 analysis website (http://timer.cistrome.org/) to analyze the relationship between ECT2 gene and CDK1, Cyclin B1 genes, the data were collected from the TCGA database, and 191 cases of basal-like invasive breast cancer were selected as the subjects of this study.

**Statistical analysis**

Statistical processing was completed using SPSS 20.0 data analysis software, t-test was used to analyze between two groups of independent data, and significant statistical differences were considered at \( P<0.05 \). The relationship between gene expression was analyzed using spearman correlation analysis.

**Results**

**ECT2 protein expression in ECT2 overexpression group and PTX therapy in vitro experiments**

Western blot test results showed that there was no significant difference in the expression of ECT2 between the control group and the NC group before and after addition of PTX treatment(\( P>0.05 \)). The expression of ECT2 was higher in the LV-ECT2 group than the control group (\( P<0.05 \)), and LV-ECT2-PTX group is also higher than that control-PTX group, but there was no difference between the LV-ECT2-PTX group and the control group (\( P>0.05 \)) (Fig. 1A and 1B).
Expression of cell cycle-related proteins CDK1 and Cyclin B1 in ECT2 overexpression group and PTX therapy in vitro experiments

Western blot test results showed that Before and after addition of PTX treatment, CDK1 and Cyclin B1 expression was significantly higher in the LV-ECT2 group than the corresponding control group ($P<0.05$), and expression of CDK1 protein in the LV-ECT2-PTX group was significantly lower than control group ($P<0.05$), but there was no difference in the comparison of Cyclin B1 ($P>0.05$) (Fig. 2A and 2B, 3A and 3B).
There was significant difference in the expression of ECT2 between the sh-ECT2 group and control group before and after addition of PTX treatment ($P<0.001$), but no difference between the NC group and the corresponding control group ($P>0.05$), as shown in Fig. 4A and 4B.

Expression of cell cycle-related proteins CDK1 and Cyclin B1 in ECT2
After silencing ECT2, the expression of CDK1 in the sh-ECT2 group was significantly lower than the corresponding control group before and after addition of PTX treatment \((P<0.05)\). And the expression of Cyclin B1 was lower in the sh-ECT2-PTX group than the control and control-PTX group \((P<0.05)\), but there was no significant difference between the sh-ECT2 group and control group \((P>0.05)\) (Fig. 5A and 5B, 6A and 6B).

**Fig. 5** CDK1 protein expression level in ECT2 interference group. A Western blot analysis of CDK1 expression in ECT2 interference group and PTX treatment. B Histogram show Comparison of relative expression levels of CDK1 protein in each group. *VS. control, \(P<0.05\); #VS. control, \(P<0.001\); &VS. control-PTX, \(P<0.05\)

**Fig. 6** Cyclin B1 protein expression level in ECT2 interference group. A Western blot analysis of Cyclin B1 expression in ECT2 interference group and PTX treatment. B Histogram show Comparison of relative expression levels of Cyclin B1 protein in each group. *VS. control, \(P>0.05\); #VS. control, \(P<0.001\); &VS. control-PTX, \(P<0.05\)
Expression of ECT2 protein in the overexpression and interference groups and PTX therapy in vivo experiments

Western-blot test results showed that the expression of ECT2 was significantly lower in the PTX treatment group than in the control group \((P<0.05)\), and its was significantly higher in the ECT2 overexpression group than that control group \((P<0.05)\), and there was a significant difference between the LV-ECT2-PTX and control-PTX \((P<0.05)\). The expression of ECT2 in the sh-ECT2 and sh-ECT2-PTX groups was significantly lower than that in the control group \((P<0.001, P<0.05)\) (Fig. 7A and 7B).

Expression of cell cycle related proteins CDK1 and Cyclin B1 in the overexpression and interference groups of ECT2 and PTX therapy in vivo experiments

Results from Western-blot assay showed that the expression of CDK1 protein were significantly lower in PTX and sh-ECT2-PTX groups than that control group \((P<0.05)\), and there were no significant difference between the other groups and the control group \((P>0.05)\). There was no significant difference in Cyclin B1 protein expression in LV-ECT2 group and sh-ECT2 group compared with the control group \((P>0.05)\), but both LV-ECT2-PTX and sh-ECT2-PTX were significantly different.

Figure 7: Expression of ECT2 protein in the ECT2 overexpression and interference groups and PTX therapy. A Western blot analysis of ECT2 expression in ECT2 overexpression and interference groups and PTX therapy. B Histogram show Comparison of relative expression levels of ECT2 protein in each group. * VS. control, \(P<0.05\); **VS. control-PTX, \(P<0.05\); # VS. control, \(P<0.001\); ## VS. control-PTX, \(P<0.05\)
from the PTX group. \( P<0.05 \) (Fig. 8A, 8B and 8C).

**Fig. 8** Expression of CDK1 and CyclinB1 protein in the ECT2 overexpression and interference groups and PTX therapy. **A** Western blot analysis of CDK1 and CyclinB1 expression in ECT2 overexpression and interference groups and PTX therapy. **B** Histogram show Comparison of relative expression levels of CDK1 protein in each group. *VS. control, \( P>0.05 \); **VS. control-PTX, \( P>0.05 \); # VS. control, \( P>0.05 \); ## VS. control-PTX, \( P<0.05 \). **C** Histogram show Comparison of relative expression levels of CDK1 protein in each group. *VS. control, \( P>0.05 \); **VS. control-PTX, \( P<0.05 \); # VS. control, \( P>0.05 \); ## VS. control-PTX, \( P<0.05 \).

**TIMER2.0** analysis tool to analyze the relationship between ECT2 gene and cell cycle-related genes CDK1 and Cyclin B1

We selected 191 cases of basal-like type invasive breast cancer cases to detect the relationship between ECT2 and the expression of CDK1 and Cyclin B1, and the results showed that there was a positive correlation between ECT2 and the expression of both CDK1 and Cyclin B1 \( (P<0.001) \), as shown in Fig. 9A and 9B.
Cell proliferation is one of the important features of cellular life activities, and the proliferation of tumor cells is a new organism formed by the abnormal proliferation of cells in the organism, and the dysregulation and disorder of the cell cycle in the process of this proliferation is the main reason leading to the unlimited proliferation of tumor cells (Pack et al. 2019). In the process of cell cycle progression, whether the G1/S phase or G2/M phase is dysregulated or disordered, it will lead to uncontrolled cell proliferation or apoptosis (Frade et al. 2015).

ECT2 protein is a type of GTP Enzyme activated protein, which can bind to GTP enzyme and promote cell division and cell motility, through the previous study (Wang et al. 2020), we supposed that the proliferation of TNBC cells were promoted by ECT2 mainly occurs in the G2/M phase. In order to further investigate the role played by ECT2 proteins in TNBC, we conducted another study on the expression profile of ECT2 proteins. In the ECT2 overexpression group, we found that ECT2 expression was reduced after the addition of PTX, but there was no significant difference in comparison with the LV-ECT2 group and control. The analysis of one of the reason

**Fig.9** Analysis of the correlation relationship between ECT2 and the cell cycle-related factor CDK1 and CyclinB1 in the TCGA database. **A** The line graph show a positive correlation between ECT2 and CDK1 gene, $P<0.001$. **B** The line graph show a positive correlation between ECT2 and CyclinB1 gene, $P<0.001$. 

**Discussion**

Cell proliferation is one of the important features of cellular life activities, and the proliferation of tumor cells is a new organism formed by the abnormal proliferation of cells in the organism, and the dysregulation and disorder of the cell cycle in the process of this proliferation is the main reason leading to the unlimited proliferation of tumor cells (Pack et al. 2019). In the process of cell cycle progression, whether the G1/S phase or G2/M phase is dysregulated or disordered, it will lead to uncontrolled cell proliferation or apoptosis (Frade et al. 2015).

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may be that PTX inhibits the growth of tumor cells, leading to a decrease in the expression of ECT2, and the second is that overexpression of ECT2 leads to resistance of tumor cells to PTX, while the hypothesis that PTX directly acts on ECT2 leading to a decrease can not be established, and further research is needed. In the interference group, ECT2 protein expression was significantly reduced after ECT2 was interfered, and the decrease of ECT2 protein expression was very obvious after PTX treatment. From the results of the in vivo experiments, it can be seen that ECT2 expression had no significant difference in the ECT2 overexpression group after addition of PTX treatment compared with the control group, whereas the expression of ECT2 was significantly reduced in the control group by the addition of PTX drug treatment. Similarly, ECT2 protein expression was reduced after the ECT2 interference, and the reduction was even more pronounced after the addition of PTX drug. Although the reduction was more significant after the addition of PTX in the interference group, it is not clear whether or how much inhibition effect of PTX on ECT2 in this process. However, from the above results, we can infer that ECT2 is involved in the division of tumor cells in the G2/M phase, providing a boost to cell proliferation. And we believe that the application of ECT2-targeted inhibitors in combination with PTX for the treatment of TNBC should have better efficacy.

Most studies have shown that ECT2 is phosphorylated during its involvement in cell division and is associated with the expression of other proteins (Justilien et al. 2011; Yang et al. 2020; Tsuda et al. 2022), including the proteins CDK1 and Cyclin B1, which are closely related to the G2/M phase of the cell cycle (Cook et al. 2011). Therefore, we examined the expression of both proteins in tumor tissues to explore the relationship between ECT2 and the two. The results from vitro experiments, it was found that the expression of CDK1 and Cyclin B1 in ECT2 overexpression group was higher than that in the control group before PTX treatment, and the expression of both was reduced after PTX treatment, but both of them were higher than the control group after PTX treatment. The expression of CDK1 and Cyclin B1 in the ECT2 interference group was significantly decreased. Especially after PTX treatment, the reduction of protein expression was more obvious. Both showed completely opposite trends in the
ECT2 overexpression and interference groups. Accordingly, we hypothesized that the expression of ECT2 has a positive feedback effect on the expression of cell cycle-related proteins CDK1 and Cyclin B1. However, the results of the in vivo experiments are not what we thought they were. CDK1 expression was only significant in the ECT2-interfering group after the addition of PTX compared with the control group, and Cyclin B1 also showed differences only after adding PTX to each group. It is evident that ECT2 interference or overexpression did not cause a feedback effect on the expression levels of CDK1 and Cyclin B1. We analyzed that the ECT2 gene may not be an upstream gene of CDK1 and Cyclin B1, but may be a downstream regulatory gene of both. Because in G2/M phase, CDK1 is the main regulatory protein, and Cyclin B1 is the main regulatory protein in M phase, the binding of CDK1 and Cyclin B1 can phosphorylate the substrate proteins, which leads to chromosome condensation and nuclear membrane disassembly, and the activity of phosphorylated proteins is increased in this process, which leads to the continuous division of the cell (Barbiero et al. 2022; Maryu and Yang 2022; Chen et al. 2022), and one of these phosphorylated proteins may probably be the ECT2 proteins.

According to the literature, ECT2 has different phosphorylation sites in different tumors, and the common sites in lung cancer are Thr341 and T790 (Cook et al. 2011; Kosibaty et al. 2019), and T359D was found to be the phosphorylation site in gastric cancer (Chen et al. 2017), and other sites related to human cells, such as T153A, K195M, and R176A, which are closely related to cell division and proliferation (Gómez-Cavazos et al. 2020). However, the phosphorylation sites of ECT2 in breast cancer are not known, and further studies are needed to discover and confirm them. Regardless of the phosphorylation site of ECT2 in breast cancer, we believe that ECT2 may be a downstream regulator of CDK1 and Cyclin B1 as described above.

PTX acts tumor inhibition mainly through the G2/M phase of the cell cycle, a certain dose of PTX can cause the accumulation of microtubule proteins, which leads to the blockage of spindle formation in mitosis and cell cycle arrest, and a high dose of PTX is able to cause the cell cycle to stop in the G2/M phase, leading to a large number of cell deaths (Yoo et al. 2013; Torres et al. 1998). Although we are not able
to determine whether or how much ECT2 plays a certain role in the action of PTX in the process of tumor cells, one thing is for sure, both ECT2 and PTX have a certain effect on the expression of cell cycle-related proteins CDK1 and Cyclin B1, but the specific mechanism of action needs to be studied in depth.

In order to prove the relationship between ECT2 and CDK1 and Cyclin B1, we applied the TIMER2.0 analysis tool to analyze the correlation between the ECT2 gene and CDK1 and Cyclin B1 genes in the TCGA database. Since there is no separate TNBC type in the database, we chose basal-like breast cancer as the research object, and the results showed a positive correlation between the ECT2 and CDK1 and Cyclin B1 expression, indicating that all three play a role in promoting cell division and proliferation during cell cycle progression. Due to ECT2 may be regulated by CDK1 (Tsuda et al. 2022) a positive feedback loop between the three may also be formed to promote cell division, because CDK1 itself does not have kinase activity, and it needs to be activated by binding to Cyclin B1 in order to fulfill its regulatory role (Sanchez et al. 2003; Levasseur et al. 2019). This regulatory role between ECT2 and the two may be an important component in the formation of the tumor microenvironment, which is one of the main causes of poor tumor treatment, and thus we hypothesized that ECT2 may be a potential target leading to drug resistance in patients.

**Conclusions**

In summary, ECT2 is involved in the progression of the G2/M phase of the cell cycle in promoting the proliferation of TNBC cells and may be regulated by CDK1, which together with Cyclin B1 forms a positive feedback loop to promote cell cycle progression, and accomplishing cell proliferation. Tumor cells with ECT2 overexpression have an attenuated PTX inhibitory effect, which may be one of the reasons for TNBC's resistance to PTX. The inhibitory effect of tumor cells after ECT2 interference was significantly enhanced by the addition of PTX, so we speculate that if PTX and anti-ECT2-targeted drugs can be applied in the future for the treatment of
breast cancer, the effect should be better, which may provide new ideas for the treatment of TNBC.

**Author contributions** HW designed article, data analysis, article writing and version approval. XL performed experiments and data collection. HW performed experiments and designed figures. JS analyzed data collection. HZ designed and revised article. All authors contributed to the article and approved the manuscript.

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**Data availability** The corresponding author will respond to reasonable requests for the datasets used in the current work.

**Conflict of interest** All authors declared that there was no conflict of interest.

**Ethics approval** The studies involving cell lines were approved by Medical Ethics Committee of Shanxi Medical University (No.: 2018034). The studies were conducted in accordance with the local legislation and institutional requirements.

**Consent to participate** Not applicable.

**Consent to publish** Not applicable.

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