The Regulatory Mechanism of EpCAM N-Glycosylation-Mediated MAPK and PI3K/Akt Pathways on Epithelial-Mesenchymal Transition in Breast Cancer Cells
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Introduction
Breast cancer is one of the most common malignant tumors clinically, accounting for about 30% of new cancers in women, and it is the major cause of death in female cancer patients (1). Statistics show that the probability of female patients dying from breast cancer is about 14% every year in China (2). Surgical resection, radiotherapy, chemotherapy, and other adjuvant therapies are the main methods for the treatment of breast cancer, but there are still shortcomings such as poor treatment effect, high toxicity, and high recurrence, and high metastasis rate (3,4). Therefore, finding new treatment methods for breast cancer is of great significance in improving the survival rate and the prognosis of patients.

Epithelial-mesenchymal transition (EMT) plays an important role in biological processes such as tumor invasion and metastasis (5). The EMT is mainly regulated by a variety of transcription factors, such as Slug, which is involved in the transformation of epithelial cells into mesenchymal cells (6). During EMT, epithelial cells express E-cadherin and hinder the polarity of epithelial cells, ultimately reducing the adhesion function of epithelial cells (7). There is also evidence that the up-regulated expression of N-cadherin and Vimentin can enhance the ability of cell migration and invasion (8).

The tumor is a cellular disease, and changes in cellular glycoproteins also play an important role in the progression of cancer. Epithelial cell adhesion molecules (EpCAM), also known as epithelial cancer molecules (EpCAM), also known as epithelial cell adhesion molecules, play an important role in the progression of cancer. EpCAM is mainly composed of the N-terminal extracellular domain, transmembrane domain, and C-terminal intracellular domain. Park et al. (2020) (10) suggested that EpCAM showed a trend of high expression in patients with postoperative recurrence and poor prognosis of liver cancer. Yang et al. (2020) (11) pointed out EpCAM in colon cancer cells and discovered that cell proliferation and colony formation were significantly reduced. Thus, it is proved that...
EpCAM participates in and regulates the process of cancers. Glycosylation is also involved in the occurrence, development, and metastasis of cancers and can be used for early diagnosis and prognosis prediction of diseases (12). Abnormal glycosyltransferases are involved in the formation of multiple types of tumor antigens, can act as ligands for cell adhesion molecules, and participate in the adhesion of cancer cells or vascular endothelial cells selectively (13). Studies have confirmed that the N-terminal extracellular domain of EpCAM contains 3 N-glycosylation sites (14). However, there is relatively little evidence of whether EpCAM N-glycosylation can be involved in the EMT of breast cancer.

Therefore, this work was intended to explore the regulatory mechanism of EpCAM N-glycosylation modification on EMT in breast cancer cells. 2 types of breast cancer cells were selected and transiently transfected with EpCAM N-glycosylation mutant plasmids (Mutant-EpCAM group), to detect the effects of EpCAM N-glycosylation mutations on cell proliferation, clonality, apoptosis, EMT marker molecular proteins, and mitogen-activated protein kinase (MAPK)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathways. It was aimed to provide research data for in-depth understanding of the molecular mechanism of the breast cancer process and the search for novel therapeutic targets for breast cancer.

Materials and methods

Experimental materials

Breast cancer cells MCF-7 and MDA-MB-231 were purchased from ATCC in the United States. DMEM medium and RPMI-1640 medium were from Gibco Corporation, the United States. EpCAM N-glycosylation mutant plasmids, empty vector control plasmids, and strains were purchased from Guangzhou RiboBio Co., Ltd. Lipofectamine 2000 kits were from Thermo Fisher Scientific Inc., the United States. PrimeScript™ RT reagent Kits with gDNA Eraser (Perfect Real Time) kits and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kits were produced by Takara Bio (Beijing) Inc. Rabbit monoclonal antibody EpCAM, rabbit monoclonal E-cadherin, rabbit monoclonal Vimentin, rabbit monoclonal Caspase-3, rabbit monoclonal Bcl-2, rabbit monoclonal Bax, rabbit monoclonal p38, rabbit monoclonal p-p38, rabbit monoclonal PI3K, rabbit monoclonal p-PI3K, rabbit monoclonal Akt, rabbit polyclonal antibody p-Akt, primary protein antibody of mouse monoclonal β-actin, and the secondary antibody of rabbit anti-human IgG H&L (HRP) were produced by Abcam PLC., the United Kingdom.

Breast cancer cell culture

Breast cancer cells MCF-7 were placed in a DMEM medium containing 10% foetal bovine serum (FBS), 100 kU/L penicillin, and 100 mg/L streptomycin. MDA-MB-231 cells were placed in an RPMI-1640 medium containing 10% FBS, 100 kU/L penicillin, and 100 mg/L streptomycin. These were cultured at 37 ℃ with 5% carbon dioxide. When the cells were at about 80% confluence, they were observed under an inverted microscope and digested with 0.25% ethylene diamine tetraacetic acid-containing trypsin for subculture. Cells in the logarithmic growth phase were used for cytological function experiments.

Construction of EpCAM N-glycosylation mutein breast cancer cell line

Breast cancer cells were seeded in a 6-well plate at a density of 1.5x10^6 cells/well, and the cells were transfected when the cell confluence reached about 70%-80%. The EpCAM N-glycosylation mutant plasmids and empty vector control plasmids were extracted, then transfected according to the instructions of the lipofectamine 2000 kit. These were placed in a cell incubator for 6 hours and then replaced with a complete medium for continued culturing.

Immunofluorescence localization

The glass slides loaded with breast cancer cells MCF-7 and MDA-MB-231 were taken out and washed 3 times with phosphate buffer solution (PBS). 4% paraformaldehyde was dropped for a 15-minute fixation. They have washed 3 times with PBS again, 0.1% Triton-X-100 solution was added dropwise to put aside for 10 min. They washed 3 times with PBS once again, the residual solution on the slides was blotted with filter paper. The immunostaining blocking solution was added dropwise for blocking
for 1 hour. Afterward, solution on the slides was blotted with filter paper again, and the diluted primary antibody EpCAM antibody (1:100) was added dropwise. They were placed in a wet box and incubated overnight at 4 °C. After these slides were washed 3 times with PBS and then blotted with filter paper, the diluted and fluorescently labeled secondary antibody (1:100) was added dropwise. Incubated for 1 hour at room temperature, they were washed 3 times with PBS and blotted with filter paper again. The cells were soaked completely with diamidino-phenyl-indole (DAPI) added dropwise and incubated at room temperature for 5 min. After the slides were washed with PBS for 3 times and blotted dry, an appropriate amount of anti-fluorescence quencher was added dropwise. The cells under fluorescent staining were observed and photographed using an inverted fluorescence microscope.

**Methyl thiazolyl tetrazolium (MTT) detection of cell proliferation**

The breast cancer cells of each group 24 hours after transfection were taken, digested with 0.25% trypsin and made into a single-cell suspension; 20 μL cells of 1×10^4 cells/well concentration were inoculated into a 96-well cell culture plate. The remaining culture volume was supplemented to 100 μL with RPMI-1640 cell culture medium containing 10% FBS, and the culture was continued for 72 hours. 20 μL of MTT medium was added, and the routine culture was continued. The transfected breast cancer cells in the logarithmic growth phase were taken as the internal reference gene, the relative expression levels of mRNA in the EMT-related markers were calculated, including that in E-cadherin, N-cadherin, and Vimentin in cells in each group. Table 1 presents the quantitative primer information.

**Plate cloning experiment**

The breast cancer cells of each group were taken 24 hours after transfection, digested with 0.25% trypsin and made into the single-cell suspension. The cells were seeded in a 6-well cell culture plate at a concentration of 1×10^3 cells/well. Pigment-epithelium-derived-factor recombinant protein was added, and the routine culture was continued. The medium was replaced at 3-day intervals. After 10 days, the cell clones were observed, the original medium was discarded, and the cells were rinsed, fixed, and stained. Finally, the clones with more than 50 cells were observed and counted under the microscope.

**Reverse transcription-polymerase chain reaction (RT-PCR) experiment**

The Trizol method was used for the extraction of total RNA from breast cancer cells, and the concentration and purity of total RNA were determined by an ultraviolet spectrophotometer, asA260/280 was required to be in the range of 1.8–2.0. The instructions of the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) were followed to perform reverse transcription of cDNA, and RT-PCR detection was made according to the instructions of the SYBR® Premix Ex Taq TM kit. The reaction program of RT-PCR was set as 95 °C for 30 seconds pre-denaturation, 95 °C for 15 s, 55 °C for 30 s, as well as 72 °C for 30 s (40 cycles); the fluorescence signals were collected. β-actin gene was taken as the internal reference gene, the relative expression levels of mRNA in the EMT-related markers were calculated, including that in E-cadherin, N-cadherin, and Vimentin in cells in each group. Table 1 presents the quantitative primer information.

**Western Blot experiment**

The transfected breast cancer cells in the logarithmic growth phase were taken from each group, and RIPA lysate was added for the extraction of protein from the cells. The BCA protein quantification kit was used to quantify the extracted protein, and the protein content was calculated.
according to the standard curve. 50 μg of the protein was sampled for electrophoresis, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out after boiling and denaturation. After PVGF transfer membrane treatment, the cells were placed in a blocking solution containing 5% nonfat milk powder blocking treatment. Proteins were diluted with 1% nonfat milk at the following ratios. The EMT marker proteins were diluted as E-cadherin (1:50000), N-cadherin (1:20000), and Vimentin (1:5000); for apoptosis marker proteins, Caspase-3 (1:5000), Bcl-2 (1:1000), and Bax (1:10000); for MAPK/PI3K/Akt pathway marker proteins, p38 (1:5000), p-p38 (1:1000), PI3K (1:1000), p-PI3K (1:1000), Akt (1:10000), and p-Akt (1:1000); primary antibody of internal reference gene β-actin (1:10000). They were incubated overnight at 4 ℃; the primary antibody was eluted with Tris Buffered Saline Tween. The fluorescently labeled rabbit anti-human IgG H&L (HRP) secondary antibody diluted at 1:20000 was added, then they were incubated at 37 ℃ for 1 hour. They were washed 3 times with PBS, and the protein bands were visualized with DAB chemiluminescent reagent.

Statistical analysis
SPSS 20.0 was utilized for differential analysis. Shapiro-Wilk was used to test whether the data conformed to a normal distribution, and the Bartlett test was adopted for the homogeneity test of variances. Measurement data conforming to normal distribution were expressed as mean ± standard deviation (±s). The comparisons among multiple groups were performed by one-way analysis of variance, while the comparisons of means between two groups were performed by SNK-q test. When P<0.05, a difference was considered to be statistically significant.

Results

Cellular localization of EpCAM and EpCAM N-glycosylated muteins
Immunofluorescence staining was performed only for the localization of EpCAM and EpCAM N-glycosylated mutant proteins in breast cancer cells MCF-7 and MDA-MB-231, as the results are shown in Figure 1. There was no significant difference shown in the expression localization of EpCAM in breast cancer cells MCF-7 and MDA-MB-231 between the normal control and the mutant groups. This indicated that EpCAM N-glycosylation mutations did not change the localization of EpCAM in plasmids.

The effect of EpCAM N-glycosylation on the proliferation of breast cancer cells
Normal breast cancer cells MCF-7 and MDA-MB-231 were taken as controls, the MTT method was adopted for the detection and comparison in cell proliferation activity after transfection with empty vector plasmids and EpCAM N-glycosylation mutant plasmids. As shown in Figure 2, the OD of MCF-7 and MDA-MB-231 cells increased with the increase of cellular culture time in the control group and blank plasmid transfection group, and no significant difference was shown in OD between the two groups (P>0.05). However, the OD of the Mutant-EpCAM group was greatly lower than that of the control group and blank plasmid group (P<0.05).

The effect of EpCAM N-glycosylation mutation on the clonality of breast cancer cells
Plate clone formation assay was to detect the effect of EpCAM N-glycosylation mutation on the clonality of breast cancer cells, as the results were presented in Figure 3. There was no significant difference in the number of clones in MCF-7 and MDA-MB-231 cells between the control group and the blank plasmid
group ($P>0.05$). However, the number of cell clones in the Mutant-EpCAM group was lower significantly than that in control and blank plasmid groups ($P<0.05$).

![Figure 2. MTT proliferation results after MCF-7 and MDA-MB-231 cells were transfected with different plasmids. Note: Compared with that of the control group, $^aP<0.05$; compared with that of the blank plasmid group, $^bP<0.05$.](image)

The effect of EpCAM N-glycosylation mutation on the apoptosis level of breast cancer cells

The expression levels of apoptosis-related proteins Caspase-3, Bcl-2, and Bax were detected through western blot in each group of cells, and the results are presented in Figure 4. There was not a significant difference in the expression levels of Caspase-3, Bcl-2, and Bax in MCF-7 and MDA-MB-231 cells between the control and the blank plasmid group after transfection ($P>0.05$). The protein levels of Caspase-3 and Bax in cells transfected with EpCAM N-glycosylation mutant plasmids were remarkably higher than those in control group and blank plasmid group ($P<0.05$). The protein expression level of Bcl-2 was highly lower than that in the control and blank plasmid groups ($P<0.05$).

![Figure 4. Expression of apoptosis proteins in MCF-7 and MDA-MB-231 cells transfected with different plasmids. Note: (A) showed the western blot strips, (B) showed the relative expression level of Caspase-3, (C) showed the relative expression level of Bcl-2, and (D) showed the relative expression level of Bax. $^aP<0.05$ in comparison with those in control group, while $^bP<0.05$ compared with blank plasmid group.](image)

Effect of EpCAM N-glycosylation mutation on mRNA levels of EMT-related marker proteins in breast cancer cells

RT-PCR was conducted to detect the mRNA expression levels of EMT-related marker proteins E-cadherin, N-cadherin, and Vimentin in each group. As shown in Figure 5, no significant difference was found in the mRNA levels of E-cadherin, N-cadherin, and Vimentin in MCF-7 and MDA-MB-231 cells between the control group and the blank plasmid group after transfection ($P>0.05$). The mRNA expression level of E-cadherin in cells was significantly higher in the Mutant-EpCAM group than that in the control group and blank plasmid group ($P<0.05$). The mRNA expression levels of N-cadherin and Vimentin were much lower than those in the control and the blank plasmid groups ($P<0.05$).
The effect of EpCAM N-glycosylation on the protein level of EMT-related marker proteins in breast cancer cells

The protein expression levels of EpCAM and EMT-related marker proteins E-cadherin, N-cadherin, as well as Vimentin were detected by western blotting in cells of each group, which are shown in Figure 6. Not a significant difference was observed in the protein expression levels of EpCAM, E-cadherin, N-cadherin, and Vimentin in MCF-7 and MDA-MB-231 cells between control and the blank plasmid groups after transfection ($P>0.05$). The expression level of E-cadherin protein in the Mutant-EpCAM group was notably higher than that in both the control group and blank plasmid group ($P<0.05$). In contrast, the protein levels of EpCAM, N-cadherin, and Vimentin were lower obviously than that in the control and blank plasmid groups ($P<0.05$).

The effect of EpCAM N-glycosylation on protein levels of MAPK and PI3K/Akt pathways in breast cancer cells

Western blotting was utilized for detecting the protein expression levels of MAPK and PI3K/Akt pathways-related marker proteins p38, p-p38, PI3K, p-PI3K, Akt, and p-Akt in each group of cells. The results were shown in Figure 7, from which no significant difference was shown in the expression levels of p38, p-p38, PI3K, p-PI3K, Akt, and p-Akt proteins in MCF-7 and MDA-MB-231 cells after transfection in both control group and blank plasmid group ($P>0.05$). The protein levels of p-p38, p-PI3K, and p-Akt were lower significantly in the Mutant-EpCAM group than those in the control and blank plasmid groups ($P<0.05$).
Discussion

Breast cancer is a very common malignant tumor worldwide, and it is the major cause of death in postmenopausal women, accounting for about 23% of cancer-related deaths (15). The incidence of breast cancer is increasing year by year and getting younger nowadays. Therefore, finding diagnostic markers and therapeutic targets for breast cancer is greatly significant for reducing mortality and improving prognosis. EMT is a kind of cancer cell-related process with stem cell-like characteristics, which plays an important role in embryonic development and tumor phenotypic metastasis (16). The main feature of EMT is the loss of epithelial cell polarity and cell adhesion, which induces and promotes the motility and invasion of cancer cells (17). However, studies have proved that changes in protein glycosylation are involved in regulating the EMT process of cancer cells (18). Protein glycosylation is common and important in protein post-translational processing and modification and participates in the regulation of cell proliferation, differentiation, and immune responses (19). EpCAM is a transmembrane glycoprotein as well as a class of oncogenes, and it shows a trend of high expression in breast cancer cells (20). Studies have suggested that EpCAM exhibits a hyperglycosylated state in cancer tissues (21). There are 3 glycosylation sites in the N-terminal extracellular domain of EpCAM, namely N74, N111, and N198. Removing the glycosylation sites will shorten the half-life period of EpCAM significantly, indicating that these sites are important in maintaining the function of EpCAM (22). The mechanism of EpCAM N-glycosylation mutation on EMT in breast cancer cells was analyzed in this work.

The immunofluorescence technique was utilized to detect the expression localization of EpCAM in breast cancer cells after EpCAM N-glycosylation mutation. The results suggested that N-glycosylation mutation did not affect the expression localization of EpCAM in breast cancer cells. The results of the MTT cell proliferation assay and western blotting assay showed that the proliferation activity of breast cancer cells was markedly decreased after EpCAM N-glycosylation mutation. The expression of EpCAM protein was decreased, the expressions of apoptosis proteins Caspase-3 and Bax were increased, and the expression of Bcl-2 was decreased. It indicated that EpCAM N-glycosylation mutation reduced the expression of EpCAM in breast cancer cells effectively, enhanced the apoptosis ability of breast cancer cells, and inhibited cell proliferation.

E-cadherin, N-cadherin, and Vimentin are EMT marker molecules (23). In this work, RT-PCR and western blotting techniques were used for the detection of the expression changes of E-cadherin, N-cadherin, and Vimentin in breast cancer cells after transient transfection. The expression of E-cadherin was up-regulated, while the expressions of N-cadherin and Vimentin were down-regulated, compared to those of normal cells. It indicated that EpCAM N-glycosylation could play a role in regulating the occurrence of EMT in breast cancer cells. PI3K/Akt is an important autophagic regulatory pathway, and this activated pathway can participate in the regulation of breast cancer, ovarian cancer, endometrial cancer, and other diseases (24-26). The MAPK pathway can be activated by growth factors, transcription factors, ligands of G protein-coupled receptors, and more (27). Abnormal activation of the P38 MAPK pathway can promote the expression of various cancer cell-related proteins and promote the resistance of cancer cells to chemotherapeutic drugs (28). This work was intended to probe whether MAPK and PI3K/Akt pathways were involved in the process of EpCAM M-glycosylation on the occurrence of EMT in breast cancer cells. Therefore, the expression levels of MAPK and PI3K/Akt pathway-related proteins PI3K, Akt, and p38 in breast cancer cells were detected after transient transfection. The phosphorylation levels of PI3K, Akt, and p38 were highly reduced after EpCAM M-glycosylation compared with those of the normal control cells. It was suggested that EpCAM M-glycosylation could inhibit the activation of MAPK and PI3K/Akt pathways in breast cancer cells, and then regulate the EMT process of cells. Such a finding was similar to the research results of Luo et al. (2018) (29).

Conclusion

This was to research the mechanism of regulating EMT in breast cancer cells by EpCAM N-glycosylation mediated by MAPK and PI3K/Akt pathways. 2 types of breast cancer cells were used as the research objects transiently transfected with EpCAM N-glycosylation mutant plasmids. The
changes in cell proliferation and apoptosis, EMT-related marker molecules, as well as MAPK and PI3K/Akt pathways-related proteins were analyzed. The results proved that EpCAM N-glycosylation could inhibit the proliferation of breast cancer cells, promote cell apoptosis, and hinder the occurrence of cellular EMT. The EpCAM N-glycosylation mainly acted by inhibiting the activation of MAPK and PI3K/Akt pathways. The mechanism of EpCAM N-glycosylation on the EMT process of breast cancer was analyzed using cell models in vitro merely. In the future, animal models of breast cancer need to be prepared for studying the regulation of survival, recurrence, and metastasis. The research results could provide an experimental basis for finding early diagnostic markers and new therapeutic targets for breast cancer.

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Authors’ contribution
This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents.

Interest conflict
There are no conflicts of interest.

References


