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The effects of HHLA2 overexpression in MCF-7 breast cancer cells on HHLA2 protein glycosylation, and on MCF-7 cell viability and apoptosis

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Abstract:

Purpose: Since HHLA2 protein is overexpressed in BC tumors and its glycosylation promotes immune system escape and accelerates tumor progression, this paper aimed to investigate whether HHLA2 protein induces its glycosylation and improves the survival and reduces apoptosis of MCF-7 breast cancer cells. This paper hopes that this inquiry will lead to the development of new therapies to control the overexpressed HHLA2 protein in solid tumors, particularly breast cancer, to inhibit breast cancer progression. Methods: Measure glycosylation by mass shift on western blot, measure viability by MTT assay, and apoptosis by Annexin V/PI FACs. Positive control is Taxol, negative control is the blank control group. Possible results: There are four possible outcomes: (1) HHLA2 overexpression promotes self-glycosylation, increases MCF-7 breast cancer cell survival, and reduces apoptosis. (2) HHLA2 overexpression does not promote self-glycosylation but increases MCF-7 breast cancer cell survival and reduces apoptosis. (3) HHLA2 overexpression does not promote self-glycosylation and does not increase MCF-7 breast cancer cell survival, or reduce apoptosis. (4) HHLA2 overexpression promotes self-glycosylation but does not increase MCF-7 breast cancer cell survival and reduce apoptosis. Conclusion: The observed phenomenon will be useful for future immunotherapy against HHLA2 for breast cancer.

Keywords: MCF-7 breast cancer cells, overexpression of HHLA2, HHLA2 glycosylation.

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1. Introduction

Breast cancer (BC) is currently the leading cause of death from malignant tumors among women worldwide.

In 2022, breast cancer is responsible for 670,000 deaths worldwide. In 157 out of 185 countries, the most common female cancer is breast cancer. In China, the incidence of breast cancer in women in 2022 will be 511,700 per 100,000 people, second only to lung cancer. It is urgent to reduce the incidence of breast cancer and explore effective treatment options. [1]

Immunotherapy is one of the current mainstays of treatment for cancers such as breast cancer, but cancers activate other pathways to evade the immune system. One of the key pathways to suppress is the HHLA2/receptor. In most of cancers, the expression of HHLA2 is higher than that of PD-L1. Inferred from recent studies, HHLA2 and PD-L1 are not correlated in cancer, suggesting that the mechanisms by which the immune system suppresses HHLA2 and PD-L1 are completely independent. Therefore, the HHLA2 receptor may be a potential new blocking pathway to improve cancer immunotherapy. [2]

Some studies have found that the co-repressor molecule HHLA2 is highly expressed in breast cancer tissues, suggesting that HHLA2 may affect the development of breast cancer [3].

Human endogenous retroviral subfamily H long terminal repeat-associated protein 2 (HHLA2) is also known as B7-H7. Like other B7 family members, HHLA2 exerts immunomodulatory effects through its receptor or interacting molecules. And promotes T cell proliferation, T cell differentiation, and NK cell activation through specific mechanisms. And the immunosuppressive function of HHLA2-KIR3DL3 predominated. In addition, HHLA2 does not interact with the CD28 family or other known members of the B7 family, and it can be speculated that the mechanism of action of HHLA2 may be complementary to that of other members of the B7 family. [4][5].

At the same time, we believe that abnormal protein glycosylation is a hallmark of tumorigenesis and progression, and evidence suggests that glycosylation may influence sustained proliferative signaling, resistance to cell death, regulation of cellular energy, pro-tumor inflammation and immune evasion. [5]. It has been mentioned that N-glycosylation of HHLA2 proteins regulated by STT3A mediates immunosuppression of NK cells by HHLA2 proteins, thus promoting the progression of CRC. [5]. Since the HHLA2 protein is similarly overexpressed and similarly glycosylated in MCF-7 breast cancer cells, it is inferred that overexpressed HHLA2 in MCF-7 breast cancer cells also promotes BC progression through certain pathways.

Hypothesis:

I predict that overexpression of HHLA2 in MCF-7 breast cancer cells leads to increased glycosylation increased viability and decreased apoptosis in MCF-7 cells.

2. Methods

HHLA2 overexpressing human breast cancer cell line (MCF-7), DMEM medium, and fetal bovine serum were purchased. A cell proliferation kit (MTT) was purchased. FACS Annexin V/PI Fluorescence Double Staining Apoptosis Detection Kit was purchased.

2.1 . Cell Culture

In the DMEM medium containing 10% FBS fetal bovine serum, add 20U insulin/100ML culture medium. Stabilize the cell state by standing at 37℃ for 1-2 hours in a CO2 incubator. 1:2 passaging, add 1-2 ml of 0.25% trypsin, and put it in the incubator at 37℃ to digest for 10-15 minutes, and terminate the digestion when most of the cells rounded and fell off. After centrifugation (1000rpm, 5min), the supernatant was discarded and resuspended with 1-3mL of complete medium. The cells were then resuspended in a fresh medium. The cells were incubated for 72 hours for subsequent experiments.

The cells were divided into two groups, one was the experimental group of MCF-7 cells treated with paclitaxel the other was the blank control group of untreated MCF-7 cells.

2.2 . MTT assay

Quantitative determination of cell viability by MTT.

Using the MTT kit, plates were incubated in 96-well plates for 24 h and then MTT was added. 20ul MTT was added to each well at a typical concentration of 5 mg/mL (05 g + 100 ml PBS). After the addition of 20ul MTT per well, the plates were placed in an incubator at 37°C for 4 hours. Measurements were carried out under an enzyme marker and absorbance values were measured at 570 nm to estimate the number of surviving cells.

Three independent experiments were repeated for each treatment condition. All the test groups with paclitaxel addition were compared with the blank control group. P-values were calculated by paired t-test.

2.3 . Western Blot assay

Glycosylation of HHLA2 was analyzed using Western Blot.

Total protein was extracted from cell samples and protein concentration was determined by BCA method. The membrane was then heated with 20 μg of Protein Sampling Buffer at 95 °C for 5 min and wet-transferred onto a

nitrocellulose membrane after gel electrophoresis on 10% SDS-PAGE, the membrane was stained with Coomassie brilliant blue and closed with 5% milk for 1 h at room temperature (~25 °C). After the addition of one, the membrane was incubated at 4 °C for 2 h. The membrane was washed, secondary antibody was added and incubated at room temperature for 2 h. The membrane was then washed and the colour developer was added. Detection of target gene-specific protein components expressed in separated proteins.

Three independent experiments were repeated for each treatment condition. All positive groups with paclitaxel addition were compared with the blank control group. p values were calculated by paired t-test.

2.4 . FACS Annexin V/PI assay

Induction of apoptosis. Centrifuge 300g cells for 5 minutes, discard the supernatant, collect the cells, gently resuspend the cells with PBS and count them. Centrifuge to collect 1-5 x 10^5 cells and discard the supernatant. Wash the cells once with PBS, centrifuge, and discard the supernatant. Resuspend cells by adding 500μL of 1XBindingBuffer per sample. Add 5μLAnnexinV-EGFP staining solution and 5μLPI staining solution. Gently vortex to mix and incubate for 15 minutes at room temperature away from light. The PI assay was performed immediately.

Three independent experiments were repeated for each treatment condition. All positive groups to which paclitaxel was added were compared with the blank control group. P values were calculated by paired t-test.

2.5 . Statistical analysis

The experimental group of MCF-7 cells treated with Taxol was compared with the blank control group of untreated MCF-7 cells in all three assays. These comparisons included MCF-7 cell activity, HHLA2 glycosylation in MCF-7 cells, and apoptosis of MCF-7 cells. Each assay was done three times for each experimental group, making n=3, and two-sided t-tests were performed at a 95% confidence level to assess whether the differences between the experimental and control groups in terms of viability, glycosylation, and apoptosis were statistically significant. Otherwise, the null hypothesis that HHLA2 overexpression does not enhance MCF-7 cell viability, does not increase HHLA2 glycosylation, and does not decrease MCF-7 cell apoptosis cannot be rejected.

3. Results

Table 1: Combination of Possible Results (CR)

Combination Result # (CR#)				
	HHLA2 overexpression increases MCF-7 viability by MTT	HHLA2 overexpression increases its glycosylation by western blot mass shift	HHLA2 overexpression decreases apoptosis by FACS Annexin V/PI	Support of hypothesis
1	+	+	+	Full
2	+	+	-	Partial
3	+	-	+	Partial
4	-	+	+	Partial
5	-	-	+	Partial
6	-	+	-	Partial
7	+	-	-	Partial
8	-	-	-	Fully Contradicts

Note. "+" represents a positive result of "HHLA2 overexpression affects this indicator", and "-" represents a negative result of "HHLA2 overexpression does not affect this indicator".

CR1: In the MCF-7 cell line in vitro, the blank control group without paclitaxel treatment showed increased survival and decreased apoptosis compared to the experimental group treated with paclitaxel. And increased glycosylation of HHLA2. This completely supported my hypothesis.

CR2: In the MCF-7 cell line in vitro, the blank control group without paclitaxel treatment showed increased survival and unreduced apoptosis compared to the experimental group treated with paclitaxel. And increased glycosylation of HHLA2. Partially supported my hypothesis.

CR3: In the MCF-7 cell line in vitro, the blank control group without paclitaxel treatment showed increased survival and decreased apoptosis compared to the experimen-

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tal group treated with paclitaxel. And no increase in glycosylation of HHLA2. Partially supported my hypothesis.
CR4: In the MCF-7 cell line in vitro, the blank control group without paclitaxel treatment did not show an increase in survival but a decrease in apoptosis compared to the experimental group treated with paclitaxel. And increased glycosylation of HHLA2. Partially supported my hypothesis.
CR5: In the MCF-7 cell line in vitro, the blank control group without paclitaxel treatment did not have increased survival but decreased apoptosis compared to the experimental group treated with paclitaxel. And glycosylation of HHLA2 was not increased. Partially supported my hypothesis.
CR6: In the MCF-7 cell line in vitro, there was no increase in survival and no decrease in apoptosis in the blank control group without paclitaxel treatment compared to the experimental group treated with paclitaxel. However, glycosylation of HHLA2 was increased. Partially supported my hypothesis.
CR7: In the MCF-7 cell line in vitro, the blank control group without paclitaxel treatment showed an increase in survival but not a decrease in apoptosis compared to the experimental group treated with paclitaxel. And glycosylation of HHLA2 was not increased. Partially supported my hypothesis.
CR8: In the MCF-7 cell line in vitro, survival did not increase, and apoptosis did not decrease in the blank control group without paclitaxel treatment compared to the experimental group treated with paclitaxel. And there was no increase in glycosylation of HHLA2. This does not support my hypothesis at all.

4. Discussion

CR1: My hypothesis is fully supported. After treatment with paclitaxel, MCF-7 showed reduced cell viability, suppressed cell proliferation inhibition, and a significant increase in late apoptosis. Increased glycosylation of HHLA2 was also detected. Overexpression of HHLA2 protein in MCF-7 cells may increase the survival of MCF-7 cells by inhibiting T cell-mediated tumor immune escape.
CR2: Partially supports the hypothesis. Overexpression of HHLA2 had no significant effect on MCF-7 cell apoptosis under the conditions tested. It may be due to MCF-7 apoptosis through other pathways such as ferroptosis genes. Induction of iron death with S ferroptosis genes is needed to further test the conjecture.
CR3: Partial support for the hypothesis: HHLA2 glycosylation was not increased, suggesting that overexpression of HHLA2 does not have a positive feedback effect on its glycosylation. Possibly HHLA2 glycosylation is not caused by its overexpression but by gene mutations or ST-T3A.
CR4: Partially supports the hypothesis. Possibly MCF-7 cell proliferation is regulated by miR-367. Overexpression of HHLA2 is not a major factor.
CR5: Partially supports the hypothesis. It is hypothesized that HHLA2 overexpression has a more significant effect on MCF-7 apoptosis than on survival. It might be possible to verify the conjecture by testing cells that normally express HHLA2.
CR6: Partially support the hypothesis. The survival rate and apoptosis level of MCF-7 cells remained the same as that of the control group, suggesting that HHLA2 overexpression may not affect T cells, etc.
CR7: Partially support the hypothesis. The paclitaxel-treated experimental group may have produced Taxol resistance and does not reduce apoptosis.
CR8: A completely unsupported hypothesis. The experimental and control groups have consistent levels of cell viability, apoptosis, and glycosylation of HHLA2 protein and are not affected by HHLA2 overexpression. If so, the experiment needs to be redesigned.
Qi Li et al. suggested that immune-related pathways are involved in iron death and HHLA2, and they found that HHLA2 is differentially expressed in breast cancer and may be involved in iron death during the disease. [7] This means that overexpression of HHLA2 is associated with both viability and apoptosis of MCF-7 cells. It promotes the cellular activity of MCF-7 and also reduces apoptosis in this cell. This is consistent with possible results 1 and 5 in the article.
The positive feedback effect of HHLA2 overexpression on its glycosylation would be confirmed, if HHLA2 downregulates tumor suppressor genes by inhibiting T cells and NK cells, among others, thus allowing tumor immune evasion. If overexpression of HHLA2 enhances its glycosylation, then the aberrant protein would amplify the polar association effect, resulting in an increasing amount of glycosylated HHLA2, which promotes the immune escape of cancer cells and facilitates the progression of BC.
The results were then combined to give an overview of the effects of overexpression of HHLA2 on MCF-7 cells.

5. Conclusion

In conclusion, this study investigated the effect of HHLA2 on its glycosylation and MCF-7 breast cancer cells. The results of the study will indicate whether HHLA2 overexpression can induce auto-glycosylation. It will also indicate whether HHLA2 overexpression increases MCF-7 survival and decreases apoptosis in MCF-7 cells, thereby

affecting breast cancer progression. The observed phenomenon will contribute to future immunotherapy updates against HHLA2 in breast cancer.

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