Proceedings of the 4th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/72/2024.19364

Targeting HHLA2 Glycosylation via STT3A Knockout to Enhance NK Cell-Mediated Antitumor Activity in Breast Cancer

Jiawei Li

Huazhong University of Science and Technology, College of Life Sciences and Technology, Wuhan, 430074, China

1694488518@qq.com

Abstract. Breast cancer is one of the most prevalent and lethal cancers worldwide, with immune evasion posing a significant challenge to current therapeutic strategies. HHLA2, a recently identified immune checkpoint protein, plays a crucial role in tumor-mediated immune suppression, particularly through its interaction with NK cells. Aberrant glycosylation of HHLA2 in cancer cells contributes to immune escape by stabilizing its inhibitory effects. STT3A, an essential enzyme in the glycosylation process, is responsible for adding glycans to immune checkpoint proteins like HHLA2. This study investigates whether knocking out STT3A in MCF7 breast cancer cells using CRISPR-Cas9 technology can reduce HHLA2 glycosylation, thereby enhancing NK cell-mediated cytotoxicity. We employ a combination of in vitro assays (MTT for cell viability, mass spectrometry for glycosylation profiling) and in vivo xenograft models to assess tumor growth and immune response. The results of this study could provide valuable insights into developing novel cancer immunotherapies that target glycosylation-dependent immune evasion, offering a potential pathway to improve the prognosis for breast cancer patients.

Keywords: Breast cancer, HHLA2, Glycosylation, NK cells, STT3A, CRISPR-Cas9, Immunotherapy.

1. Introduction

Cancer immunotherapy has emerged as a transformative approach in oncology, with therapies designed to enhance the immune system's ability to recognize and destroy cancer cells. Among cancers, breast cancer remains one of the most common and deadly malignancies worldwide, affecting millions of women each year. It is a heterogeneous disease comprising various subtypes, including hormone receptor-positive, HER2-positive, and triple-negative breast cancer (TNBC), each with unique biological behaviors and therapeutic responses. Despite advances in treatments such as surgery, chemotherapy, radiation, and targeted therapies, many patients still experience recurrence and progression, underscoring the need for novel therapeutic strategies.

One of the key challenges in breast cancer therapy is immune evasion, which tumors can achieve through multiple mechanisms, including the manipulation of immune checkpoint proteins. HHLA2, a recently identified member of the B7 family, has garnered attention due to its role in immune evasion

© 2025 The Authors. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

DOI: 10.54254/2753-8818/72/2024.19364

across several cancers, including breast and colorectal cancers. HHLA2 interacts with inhibitory receptors such as KIR3DL3 on NK cells, leading to reduced immune responses against tumor cells.

Glycosylation, particularly N-glycosylation, is a critical post-translational modification that significantly affects the structure and function of immune checkpoint proteins like HHLA2. Aberrant glycosylation patterns in cancer cells contribute to immune escape by stabilizing immune checkpoint proteins and enhancing their inhibitory interactions with immune cells. STT3A, a key component of the oligosaccharyltransferase complex, catalyzes the addition of glycans to asparagine residues, playing a pivotal role in the glycosylation of proteins including HHLA2. By targeting STT3A, it is possible to reduce the glycosylation of HHLA2, potentially restoring immune recognition and enhancing the cytotoxicity of NK cells against tumor cells.

The rationale behind targeting HHLA2 glycosylation is further supported by findings that pharmacological inhibition of STT3A, using agents such as NGI-1, can suppress HHLA2 glycosylation. This suppression was shown to enhance NK cell-mediated antitumor responses, indicating a promising therapeutic strategy for overcoming immune evasion in cancers that express high levels of HHLA2. Previous studies have also identified a negative correlation between HHLA2 expression and patient prognosis, highlighting the potential of HHLA2 as a therapeutic target in cancer immunotherapy.

In this study, we hypothesize that knocking out STT3A in MCF7 breast cancer cells using CRISPR-Cas9 technology will reduce HHLA2 glycosylation, thereby increasing the susceptibility of these cells to NK cell-mediated killing. This hypothesis will be tested through a combination of in vitro and in vivo approaches, including MTT assays for cell viability, mass spectrometry for glycosylation profiling, and xenograft models for assessing tumor growth. The findings from this study could provide valuable insights into the development of novel cancer immunotherapies that target glycosylation-dependent immune evasion mechanisms.

2. Materials and Methods

2.1. Materials

- MCF7 breast cancer cell lines
- CRISPR-Cas9 plasmids targeting STT3A
- NK cells isolated from healthy donors
- NGI-1 (glycosylation inhibitor)
- Reagents: MTT assay kits, Western blotting reagents, mass spectrometry equipment

2.2. Methods

2.2.1. Cell Culture and Genetic Modification

MCF7 cells will be cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin[1]. STT3A will be knocked out using CRISPR-Cas9, and transfection efficiency will be confirmed by Western blotting and PCR. [2]

2.2.2. Glycosylation Analysis

Glycosylation levels of HHLA2 will be analyzed using mass spectrometry. Cells will be lysed, and HHLA2 will be immunoprecipitated using specific antibodies. The protein samples will be analyzed for glycan structures using mass spectrometry [3]

2.2.3. Cell Viability Assay (MTT)

Cell viability will be assessed using MTT assays post-STT3A knockout. NK cells will be co-cultured with MCF7 cells at different effector-to-target ratios (E:T ratios of 5:1, 10:1, and 20:1) for 24, 48, and 72 hours. Absorbance will be measured at 570 nm, and results will be statistically analyzed using a two-tailed Student's T-test (p-value < 0.05 considered significant) [4]

DOI: 10.54254/2753-8818/72/2024.19364

2.2.4. In Vivo Tumor Growth Assessment

STT3A-knockout MCF7 cells and control cells will be injected into immunodeficient mice to establish xenograft models. [5]Tumor growth will be monitored every 3 days using calipers, and tumor volume will be calculated.[6] Differences between groups will be analyzed using ANOVA [7]

2.2.5. Statistical Analysis

Data will be analyzed using a two-tailed Student's T-test for comparing two groups, and one-way ANOVA for multiple group comparisons. All experiments will be conducted with at least three replicates, and a p-value < 0.05 will be considered statistically significant.[7]

3. Results

3.1. Glycosylation levels of HHLA2

Possible Result 1: KO STT3A reduces HHLA2 glycosylation detected by Mass Spectrometry.

A reduction in HHLA2 glycosylation in KO STT3A cells compare to the control group indicates that STT3A is crucial for the N-glycosylation of HHLA2. This suggests that STT3A plays a role in the glycosylation pathway, and its knockout disrupts this process.

Possible Result 2: KO STT3A does not affect HHLA2 glycosylation compare to the control group detected by Mass Spectrometry, this suggests that STT3A may not be critical for the glycosylation of HHLA2, or there might be compensatory mechanisms in place.

3.2. Cells viability by MTT

Possible Result 3: KO STT3A co-cultured with NK Cells show decreased viability compare to the control group

(Lower Absorbance), suggests that STT3A-knockout MCF7 cells are more susceptible to NK cell-mediated cytotoxicity. This imply that the loss of STT3A enhances the ability of NK cells to kill KO STT3A.

Possible Result 4: KO STT3A co-cultured with NK Cells show similar viability with control group (Similar Absorbance), suggests that the STT3A knockout does not significantly affect NK cell-mediated cytotoxicity.

3.3. Tumor size change

Possible Result 5: KO STT3A decreases tumor size, if the tumors derived from STT3A-knockout MCF7 cells exhibit significantly reduced growth compared to control tumors, this suggests that knocking out STT3A impairs tumor progression in vivo.

Possible Result 6: KO STT3A does not decrease tumor size, if the tumor size in xenograft models does not differ significantly between STT3A-knockout cells and control cells, this suggests that the knockout of STT3A does not substantially impact tumor growth in vivo.

3.4. Combination of Possible Results (CR)

Table 1. Possible combination of results

Possible observation	KO STT3a reduces HHLA2 glycosylation detected by mass spectrometry	KO STT3a decreases cell viability by MTT	KO STT3a decreases tumor size	Support of hypothesis
CR1	+	+	+	Full
CR2	+	+	-	Partial
CR3	+	-	+	Partial
CR4	-	+	+	Partial
CR5	-	-	+	Partial

DOI: 10.54254/2753-8818/72/2024.19364

Table 1. (continued).

CR6	-	+	-	Partial
CR7	-	-	+	Partial
CR8	-	-	-	Fully Contradicts

Note. "+" represents decreasing the tumor size as hypothesis pathway. "-" represents a opposite result of one step in the hypothesis pathway.

4. Discussion

Previous studies have uncovered the molecular basis for targeting HHLA2 in immunotherapy for colorectal cancer, demonstrating that the glycosylation of HHLA2, regulated by the glycosyltransferase STT3A, is crucial for its stability, membrane localization, and ability to promote immune evasion by suppressing NK cell responses.[8,9]However, little has been done to explore the impact of knocking out STT3A using CRISPR-Cas9 to influence HHLA2 glycosylation, subsequently decreasing cell viability and ultimately reducing tumor size.

Building on these findings, our study aims to further validate this therapeutic strategy in a different cancer type, breast cancer, to broaden the applicability of targeting glycosylation pathways in cancer immunotherapy. By employing a genetic knockout approach, we directly disrupt the glycosylation process of HHLA2 in MCF7 breast cancer cells, providing a more definitive understanding of how STT3A influences immune interactions and tumor progression. This study integrates in vitro and in vivo models, using mass spectrometry, cell viability assays, and xenograft experiments, to comprehensively assess the effects of STT3A knockout. This approach could reveal new pathways for enhancing cancer immunotherapy, potentially offering more sustained therapeutic effects than current methods that focus on checkpoint inhibition.

Possible Result1, the case where STT3A knockout reduces HHLA2 glycosylation, decreases cell viability, and decreases tumor size (corresponding to CR1), the data supports the hypothesis that STT3A is crucial for glycosylation and affects both immune interactions and tumor growth. This result indicates that STT3A plays a significant role in these processes, with its knockout leading to enhanced immune-mediated tumor clearance.

Possible Result 2: STT3A knockout reduces HHLA2 glycosylation, decreases cell viability, but does not decrease tumor size (CR2). The reduction in glycosylation enhances NK cell-mediated cytotoxicity, leading to lower cell viability, but tumor growth is maintained due to compensatory pathways such as Wnt/β-catenin or JAK/STAT, which support tumor survival. This result confirms that while STT3A-driven glycosylation is critical for HHLA2's immune evasion, other mechanisms compensate to ensure continued tumor growth despite the decreased viability.

Possible Result 3: STT3A knockout reduces HHLA2 glycosylation, does not affect cell viability, but still decreases tumor size (CR3). The reduced glycosylation impairs HHLA2's ability to evade immune surveillance, leading to enhanced NK cell-mediated tumor clearance and a reduction in tumor size. The unaffected cell viability suggests that while glycosylation is not essential for cell survival, it plays a key role in tumor-immune interactions, particularly in promoting immune evasion.

Possible Result 4: STT3A knockout does not affect HHLA2 glycosylation, decreases cell viability, and decreases tumor size (CR4). The effects on both cell viability and tumor size indicate that STT3A influences other critical pathways beyond glycosylation. This suggests that proteins such as EGFR or PD-L1, which are involved in tumor survival and immune evasion, may be regulated by STT3A. The reduction in tumor size confirms STT3A's broader role in supporting tumor progression through mechanisms unrelated to HHLA2 glycosylation.

Possible Result 5: STT3A knockout does not affect HHLA2 glycosylation or cell viability but still decreases tumor size (CR5). The reduction in tumor size, despite no changes in glycosylation or viability, points to alternative mechanisms of tumor growth regulation. This could involve changes in the tumor microenvironment, such as reduced angiogenesis or altered immune cell infiltration, which diminish the

DOI: 10.54254/2753-8818/72/2024.19364

tumor's ability to sustain its growth independently of NK cell-mediated cytotoxicity or glycosylation changes.

Possible Result 6: STT3A knockout does not affect HHLA2 glycosylation, decreases cell viability, but does not decrease tumor size (CR6). The decrease in cell viability suggests that STT3A influences key survival pathways, yet the lack of impact on tumor size indicates compensatory mechanisms are at play. Pathways such as autophagy or stress responses, like the unfolded protein response (UPR), may be activated to help the tumor cells withstand the loss of viability and sustain growth. These compensatory mechanisms enable the tumor to adapt to cellular stress, preventing a reduction in tumor size despite reduced cell viability

Possible Result 7: STT3A knockout reduces HHLA2 glycosylation but does not affect cell viability or tumor size (CR7). This highlights that while STT3A is crucial for HHLA2 glycosylation, this specific modification alone is not sufficient to significantly influence cell viability or tumor progression in this model. This may be due to other glycosylation-independent pathways being dominant in regulating cell survival and tumor growth. For instance, signaling cascades like MAPK or PI3K/AKT might remain unaffected by glycosylation changes and continue driving tumor progression, thus overriding the influence of reduced HHLA2 glycosylation

Possible result 8: Finally, if STT3A knockout shows no significant impact on HHLA2 glycosylation, cell viability, or tumor size (CR8), it suggests that STT3A might not play a crucial role in these processes in the studied context. This indicates that its effects could be minimal or that other factors might be overriding its impact.

5. Conclusion

This study investigates the potential of targeting HHLA2 glycosylation by knocking out STT3A in MCF7 breast cancer cells to enhance NK cell-mediated cytotoxicity. The combination of CRISPR-Cas9 gene editing, mass spectrometry for glycosylation profiling, MTT assays for cell viability, and in vivo xenograft models will comprehensively assess the impact of STT3A knockout. We hypothesize that STT3A knockout will reduce HHLA2 glycosylation, increase NK cell-mediated cytotoxicity, and decrease tumor size. Should the results validate this hypothesis, they would highlight the significance of STT3A and glycosylation in modulating immune responses and tumor progression. This research could provide valuable insights into developing novel immunotherapeutic strategies targeting glycosylation-dependent immune evasion mechanisms, potentially offering new avenues for improving cancer treatment outcomes.

References

[1] Vantangoli, M.M., et al., MCF-7 Human Breast Cancer Cells Form Differentiated Microtissues in Scaffold-Free Hydrogels. PLoS One, 2015. 10(8): p. e0135426.

[2] Xu, C., CRISPR/Cas9-mediated knockout strategies for enhancing immunotherapy in breast cancer. Naunyn Schmiedebergs Arch Pharmacol, 2024.

[3] Liu, H., et al., Mass spectrometry-based analysis of glycoproteins and its clinical applications in cancer biomarker discovery. Clin Proteomics, 2014. 11(1): p. 14.

[4] Kumar, P., A. Nagarajan, and P.D. Uchil, Analysis of Cell Viability by the MTT Assay. Cold Spring Harb Protoc, 2018. 2018(6).

[5] Morton, C.L. and P.J. Houghton, Establishment of human tumor xenografts in immunodeficient mice. Nat Protoc, 2007. 2(2): p. 247-50.

[6] Van Court, B., et al., Measurement of mouse head and neck tumors by automated analysis of CBCT images. Sci Rep, 2023. 13(1): p. 12033.

[7] Mishra, P., et al., Application of student's t-test, analysis of variance, and covariance. Ann Card Anaesth, 2019. 22(4): p. 407-411.

[8] Zhang, D., et al., Pharmacological suppression of HHLA2 glycosylation restores anti-tumor immunity in colorectal cancer. Cancer Lett, 2024. 589: p. 216819.

DOI: 10.54254/2753-8818/72/2024.19364

[9] Li, W., et al., Unlocking the potential of HHLA2: identifying functional immune infiltrating cells in the tumor microenvironment and predicting clinical outcomes in laryngeal squamous cell carcinoma. Cancer Immunol Immunother, 2024. 73(10): p. 207.