学　位　論　文（要約）

Studies of roles of *NLRC5* expression in cancers on antigen presentation and host anti-cancer immunity

(癌の抗原提示とホストの抗癌免疫における *NLRC5* 発現の役割の研究)

２０２４年３月

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[Background and Objectives] NLRC5, recognized within the NLR family as CARD Domain Containing 5 and alternatively termed the Class I transactivator (CITA), plays a pivotal role as a transcriptional activator for MHC class I genes and their associated molecules. These molecules are essential for the presentation of antigens to CD8+ T cells, which are integral components of the body's anti-cancer defense mechanisms. Previous research, primarily through data mining approaches, has pinpointed NLRC5 as a potential mechanism through which cancers can evade the immune response. However, the precise function of NLRC5 in modulating the presentation of cancer antigens and its influence on the immune system's ability to combat cancer remains insufficiently elucidated through experimental studies. In our research, we delve into the impact of NLRC5 expression on cancer, specifically its role in regulating antigen presentation, bolstering host anti-cancer immunity, and determining the effectiveness of immune checkpoint blockade therapies, such as anti-PD-1 treatments. Our objective is to deepen the understanding of how NLRC5 interacts with cancerous cells and the immune system, with the goal of contributing to the development of innovative cancer therapies.

[Materials and methods] To elucidate the role of NLRC5 in mediating host anti-cancer immunity, we engineered a genomic deletion of Nlrc5 within mouse melanoma B16F10 and mouse breast cancer E0771 cell lines. Additionally, in pursuit of evaluating NLRC5 as a potential target for the innovation of new immunotherapeutic strategies, we pioneered a CRISPR/dCas9-TET1cd based targeted demethylation and activation (TDMa) system. This system was designed to augment the expression of Nlrc5/NLRC5 in both mouse melanoma B16F10 and human breast cancer MCF7 cell lines. To quantify the impact of Nlrc5 knockout or its activation on the expression levels of MHC class I and associated genes, we employed quantitative reverse transcription PCR (qRT-PCR) and RNA sequencing (RNA-Seq) analyses. Bisulfite sequencing was conducted to assess the methylation status of the Nlrc5 promoter, providing insights into epigenetic modifications. Flow cytometry analysis was utilized to evaluate the immunogenic properties of cancer cells post-genomic manipulation. Following these in vitro assessments, mouse cell lines underwent subcutaneous implantation to monitor tumor growth dynamics in a living organism. Further analyses, including flow cytometry and immunohistochemistry on the resultant tumors, were performed to delineate the characteristics of tumor-infiltrating lymphocytes (TILs) and the tumor cells themselves, providing a comprehensive understanding of the immune landscape and cancer cell behavior in vivo.

[**Results**]Our findings reveal that the targeted genomic deletion of Nlrc5 led to a significant reduction in the expression levels of MHC class I and associated genes in

both B16F10 and E0771 cells. Specifically, the absence of Nlrc5 accelerated tumor growth in E0771 models in vivo, attributing this phenomenon to the suppression of CD8+ T cell infiltration and activation mediated by MHC class I mechanisms. Conversely, due to inherently low MHC class I expression levels, the B16F10 model exhibited no growth alteration post-Nlrc5 deletion in the absence of external stimulation. Nonetheless, our investigations uncovered that Nlrc5 is crucial for the B16F10 tumor's responsiveness to anti-PD-1 therapy in transplantation experiments. These insights led us to conduct transcriptome screenings, from which we derived an optimized predictive model for melanoma's response to anti-PD-1 therapy.

Further explorations involved the application of a CRISPR/dCas9-based system for targeted demethylation and activation (TDM and TDMa) of the Nlrc5 promoter in both B16F10 and MCF7 cell lines. While the TDM approach alone yielded only a modest increase in MHC class I expression and tumor immunogenicity, the combined targeted demethylation and activation strategy (TDMa) significantly amplified MHC class I expression. RNA-Seq analysis corroborated the targeted and robust enhancement of Nlrc5 and subsequent MHC class I gene expression via the TDMa system. This augmentation was further evidenced by OT-I co-culture assays, indicating enhanced immunogenicity of B16F10 cells following Nlrc5's targeted modulation.

When B16F10 cells treated with the TDMa system were implanted in vivo within C57BL/6 mice, a marked inhibition of tumor growth was observed, underscoring the necessity of an intact adaptive immune system for tumor suppression, as no significant growth differences were noted in immunodeficient NUDE mice. Notably, the activation and infiltration of CD8+ T cells were specifically enhanced in B16F10 tumors, a response not mirrored in CD4+ T cells. Immunohistochemical analyses further demonstrated that the targeted manipulation of Nlrc5 not only increased the overall number of tumor-infiltrating lymphocytes but also concentrated CD8+ T cells at the tumor core.

Moreover, our study illustrated that the targeted demethylation and activation of Nlrc5 heightened the sensitivity of B16F10 tumors to anti-PD-1 therapy. This effect was paralleled by a specific enhancement in CD8+ T cell infiltration and activation, as opposed to no observed change in CD4+ T cell responses, highlighting a nuanced interplay between targeted genetic modifications, immune cell dynamics, and therapeutic responsiveness.

[**Discussion**] NLRC5 is prominently expressed in cells integral to the immune system, prompting extensive research into its role as a transactivator of MHC class I within this context. However, the function of NLRC5 in the capacity of MHC class I transactivation within cancer cells remains significantly underexplored. Given the

critical importance of MHC class I as a target in cancer therapy, our investigation zeroes in on this crucial molecule to regulate its expression. Through methodologies encompassing both genomic deletion and targeted demethylation and activation, we meticulously delineate the role of Nlrc5 in modulating immunogenicity via MHC class I, thereby augmenting host anti-cancer immunity in melanoma and breast cancer contexts.

Our approach to the genomic deletion of Nlrc5, aligning with prior studies utilizing Nlrc5 knockout models, demonstrates that the absence of Nlrc5 leads to a decrease in MHC class I expression, irrespective of mIFN-γ stimulation. This study pioneers the exploration of Nlrc5's impact on the MHC class I antigen presentation pathway within cancerous contexts. Conversely, the application of the TDMa system for targeted demethylation and activation of Nlrc5/NLRC5 in B16F10 and MCF7 cell lines further substantiates that Nlrc5 activation significantly bolsters MHC class I expression and consequently, the host's anti-cancer immunity. Moreover, our findings reveal that this targeted activation strategy propels CD8+ T cells towards the tumor core, a phenomenon previously associated with improved prognostic outcomes yet lacking in mechanistic clarity. We elucidate that the upregulation of surface MHC class I in cancer cells is a pivotal factor driving the infiltration of CD8+ T cells into the tumor's epicenter. Additionally, our research illustrates that the targeted demethylation and activation of Nlrc5 primes B16F10 cells for a heightened response to anti-PD-1 therapy. This observation aligns with prior studies indicating a correlation between surface MHC class I levels, NLRC5 expression, and favorable responses to immune checkpoint blockade therapies, although detailed mechanisms behind these correlations have remained elusive until now. Our results compellingly demonstrate that CD8+ T cell-mediated anti-cancer immunity is not only specifically but also synergistically activated by the combination of anti-PD-1 treatment and targeted manipulation of Nlrc5 expression. These insights underscore NLRC5's potential as a strategic therapeutic target to significantly amplify the effectiveness of anti-PD-1 treatments, offering a nuanced approach to enhancing immune-mediated cancer therapy outcomes.

[**Conclusion**] In conclusion, our research deepened our understanding of the role of Nlrc5 in host anti-cancer immunity. Our strategy to induce and genotyping genomic deletion is solid and can be applied to other genes and cell lines. We show that the CRISPR/dCas9 mediated targeted demethylation and activation strategy of Nlrc5 is a viable and novel immunotherapy approach to enhance cancer immunogenicity. For future study, optimization of delivery system to effectively induce targeted demethylation and activation in vivo could contribute to development of novel immunotherapy.