Review of the Doctoral Dissertation

Title: *The molecular mechanism of PD-L1 overexpression in classical Hodgkin lymphoma (cHL)*

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Co-Supervisor: Dr n. med. Ryszard Konopinski

Overall Summary

This doctoral dissertation presents an extensive and well-designed study into the epigenetic and molecular regulation of PD-L1 expression in classical Hodgkin lymphoma (cHL), focusing on nuclear functions of PD-L1 and its interaction with chromatin remodeling complexes. The candidate investigates the hypothesis that PD-L1 not only functions on the cell surface to mediate immune escape but also translocates to the nucleus, where it interacts with epigenetic modifiers such as SWI/SNF complex and EZH2 (PRC2 complex) to regulate its own expression at the CD274 promoter locus, potentially contributing to positive feedback loops that sustain PD-L1 overexpression in cHL cells.

The dissertation is highly significant within the field of tumor immunology and epigenetics, especially considering incomplete responses to anti-PD-1/PD-L1 therapies in some patients with Hodgkin lymphoma. The candidate's investigation into nuclear PD-L1 functions and epigenetic regulation mechanisms offers novel insights and has the potential to inform new therapeutic strategies, particularly in therapy-resistant or relapsed cHL. The work is innovative, methodologically rigorous, and scientifically impactful, with the potential for clinical translation.

1. Substantive Assessment

A. Relevance and Originality of the Research Problem

The relevance of the research is undeniable given the central role of the PD-1/PD-L1 axis in cancer immunotherapy, particularly in cHL where PD-L1 overexpression is a well-documented hallmark of Hodgkin and Reed-Sternberg (HRS) cells. However, the mechanisms governing PD-L1 overexpression, especially beyond genomic amplification at 9p24.1, remain poorly understood. By examining nuclear localization of PD-L1 and its interaction with epigenetic modifiers, the candidate tackles a novel angle that could explain therapy resistance and variable response rates in cHL patients undergoing checkpoint blockade.

The originality lies in the exploration of nuclear PD-L1, a concept that is still emerging in the field. Previous literature has focused predominantly on membrane-bound PD-

L1. This thesis explores reverse signaling and nuclear PD-L1 functions in RNA processing and chromatin regulation, an underexplored area with significant therapeutic implications. Additionally, the study's use of chromatin immunoprecipitation (ChIP) and protein-protein interaction assays to map PD-L1's interactome adds further innovative value.

B. Evaluation of the Results and Their Significance for Science and Practice

The candidate's findings are comprehensive and robust, offering multiple levels of evidence to support the nuclear role of PD-L1. Notably, the discovery that PD-L1 interacts with SWI/SNF complex (BAF155) and EZH2 and co-localizes with histone modifications (H3K27Ac and H3K27me3) at the CD274 promoter, supports a positive feedback model of PD-L1 self-regulation. This has potential paradigm-shifting implications in understanding how tumor cells maintain immune evasion and how checkpoint blockade resistance might arise.

Furthermore, the mass spectrometry analysis identifying PD-L1's interaction with RNA processing proteins (e.g., FUS, SF3B1) suggests post-transcriptional roles, expanding PD-L1's known functions. These results may inform future drug development, including PD-L1 nuclear translocation inhibitors or targeting PD-L1 interactors to overcome resistance.

In practical terms, the study provides a rationale for combination therapies in cHL, integrating epigenetic drugs with immunotherapy. While EZH2 inhibition (EPZ-6438) did not reduce PD-L1 levels in L-1236 cells, the observed stability of PD-L1 expression suggests a redundant regulatory mechanism, underscoring the need for multifaceted therapeutic approaches.

C. Formal, Linguistic, and Stylistic Correctness

The dissertation is well-written, with a clear narrative and coherent progression through complex material. The scientific terminology is used accurately, and figures and tables are well-designed and properly referenced. The abstract succinctly conveys the main findings. Minor linguistic issues (e.g., subject-verb agreement, occasional punctuation errors) are rare and do not detract from readability. The logical organization of chapters ensures ease of comprehension, even for readers not specialized in molecular oncology.

2. Methodological Assessment

A. Choice of Literature and Ability to Use Literature Sources

The candidate demonstrates excellent command over the relevant literature, with comprehensive citations of both foundational studies and recent advancements in PD-L1 biology, SWI/SNF complex functions, and cHL pathogenesis. The references are current (primarily post-2015), and the candidate engages critically with the literature, particularly in the discussion section, where she compares her findings with similar regulatory phenomena in other cancers (e.g., breast, prostate, bladder cancers). This reflects a mature understanding of the broader scientific landscape.

Notably, the candidate correctly situates her findings within ongoing debates about epigenetic regulation and immune resistance**,** indicating her ability to synthesize information and identify gaps in current knowledge.

B. Accuracy in Formulating Problems and Hypotheses

The problem formulation is precise: how PD-L1 is regulated at the chromatin level in cHL, and whether nuclear PD-L1 interacts with epigenetic machinery to promote its own overexpression. The objectives are clearly delineated:

1. To assess CD274 promoter regulation by chromatin remodelers and histone marks;
2. To investigate nuclear PD-L1 interactors and their potential regulatory roles.

Both objectives are clearly addressed through well-aligned experimental designs, with results fully supporting or refining the hypotheses. The candidate also anticipates alternative explanations and discusses limitations (e.g., potential copy number variation effects on ChIP results), demonstrating scientific rigor.

C. Relevance of the Chosen Methods and Research Tools and Ability to Apply Them

The candidate employs a diverse array of well-established and advanced molecular techniques, all appropriate for the research questions. These include:

- Western blotting for protein expression validation.
- qRT-PCR for transcript quantification.
- Flow cytometry for surface protein analysis.
- Immunoprecipitation and Mass Spectrometry for interactome mapping.
- Chromatin Immunoprecipitation (ChIP-qPCR) for DNA-protein interaction studies.
- Yeast Two-Hybrid System (Y2HS) for protein-protein interaction validation.
- MTT assays for cell viability under EZH2 inhibition.

Methodological details are exhaustively described, ensuring reproducibility. The use of proper controls (e.g., IgG in IP, DMSO in MTT assays) and technical replicates confirms the candidate's proficiency. Additionally, statistical analyses (2^–ΔΔCT for qPCR, triplicates for MTT assays) are appropriate and adequately described. The candidate demonstrates excellent technical command, especially in ChIP-qPCR data interpretation, where she identifies positional co-localization of histone marks and chromatin remodelers at PD-L1 promoter sites.

D. Correctness of the Paper's Organization and Content Structure

The dissertation is organized into logical, well-structured sections**:** Introduction, Objectives, Materials and Methods, Results, Discussion, Conclusion, References**,** with lists of figures and tables**.** The methods section is especially comprehensive, reflecting meticulous planning and thorough execution. The results section is

enriched by clear visuals, including diagrams of PD-L1 interactomes and positional maps of chromatin binding, which greatly aid interpretation.

The discussion section skillfully integrates primary data with literature, offering insightful interpretations, and proposing follow-up studies, such as long-term EZH2 inhibition models or investigating other regulatory complexes. The conclusion succinctly recaps findings and contextualizes their impact.

3. Final Conclusion

The dissertation meets and exceeds the standards specified in the Acts on academic degrees and titles, demonstrating scientific maturity, original thinking, and technical excellence. The research findings represent a notable advance in the understanding of PD-L1 regulation in classical Hodgkin lymphoma, and have potential translational implications for immunotherapy-resistant cancers.

Recommendation

The doctoral dissertation submitted for my review meets the conditions specified in Article 13 of the Act of March 14, 2003 on academic degrees and titles and on degrees and titles in the arts (Journal of Laws No. 65, item 595, as amended) in connection with Article 179(1) of the Act of July 3, 2018 – Provisions introducing the Acts on Higher Education and Science (Journal of Laws of 2018, item 1669, as amended).

Accordingly, I have the honor and pleasure to recommend to the Scientific Council of the National Institute of Oncology – National Research Institute in Warsaw that Ms. Hummaira Sadaf be admitted to the subsequent stages of the doctoral procedure.

The most important merits of this dissertation include:

- Originality of topic and novel insights into nuclear PD-L1 functions.
- High methodological rigor and command of molecular techniques.
- Significant potential for clinical translation in cHL therapy.
- Research conducted at a renowned center with the highest degree of referral capability

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