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Apr 17, 2023

Prof. dr hab. Ryszard Słomski
Przewodniczacy komisji doktorskiej
Instytut Genetyki Czlowieka
Polska Akademia Nauk, Poznan

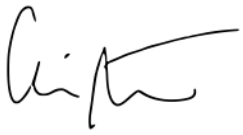
Szanowny Panie Profesorze

W załączeniu przesyłam recenzje pracy doktorskiej p. mgr. Karoliny Rassek.

Moja ocena tej pracy jest bardzo wysoka i dlatego też mam zaszczyt przedłożyć Wysokiej Radzie Instytutu Genetyki Człowieka Polskiej Akademii Nauk WNIOSEK O DOPUSZCZENIE mgr. Karoliny Rassek do dalszych etapów przewodu doktorskiego, gdyż „Rozprawa doktorska spełnia warunki określone w art.13 ust.1 ustawy z dnia 14 marca 2003 ro stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki (Dz. U. Nr. 65, poz. 595, z późniejszymi zmianami).

Zwracam się również do Wysokiej Rady o WYRÓŻNIENIE przedstawionej mi do recenzji pracy doktorskiej.

Z wyrazami szacunku,



Prof. Robert Gniadecki, MD, Ph.D., D.M.Sci.

Review of the PhD thesis "Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas" by Karolina Rassek

Supervisor: prof. Grzegorz Przybylski, MD, PhD
Co-supervisor: Katarzyna Iżykowska, PhD
Institute of Human Genetics, Polish Academy of Science

INTRODUCTORY COMMENTS

The PhD dissertation is based on four published articles, comprising 3 original papers and one review article:

1. Iżykowska K., Rassek K., Żurawek M., Nowicka K., Paczkowska J., Ziółkowska-Suchanek I., Podralska M., Dzikiewicz-Krawczyk A., Joks M., Olek-Hrab K., Giefing M., Przybylski G.K. Hypomethylation of the promoter region drives ectopic expression of TMEM244 in Sézary cells. Journal of Cellular and Molecular Medicine, 2020 Aug 14; 24(18):10970-10977.
2. Rassek K., Iżykowska K., Żurawek M., Pieniawska M., Nowicka K., Zhao X., Przybylski G.K. TMEM244 is a long non-coding RNA necessary for CTCL cell growth. International Journal of Molecular Sciences, 2023, 24(4), 3531.
3. Rassek K., Iżykowska K., Żurawek M, Nowicka K., Joks M., Olek-Hrab K., Olszewska B., Sokołowska- Wojdyło M., Biernat W., Nowicki R.J., Przybylski G.K. TMEM244 Gene Expression as a Potential Blood Diagnostic Marker Distinguishing Sézary Syndrome from Mycosis Fungoides and Benign Erythroderma. Journal of Investigative Dermatology, 2023, 143, 344-347.
4. Rassek K., Iżykowska K. Single-Cell Heterogeneity of Cutaneous T-Cell Lymphomas Revealed Using RNA-Seq Technologies. Cancers, 2020, 12(8), 2129. (review)

All journals are well established in the field of experimental medicine, oncology and dermatology and are placed in the upper quartile regarding the impact factor in the field. KR is the first author in three papers and second author in one paper. Paper 3 is a Letter to Editor, but should be classified as a research paper, because J Invest Dermatol (the top journal in the field of experimental dermatology and skin science) has a policy to publish concise, definitive reports in the letter format. Those papers would easily qualify as full papers in other, comparable journals. The number of publications and the quality of the journals fulfill the customary requirements to PhD theses in most European and American universities.

According to the statements of co-authorship, KR contributed sufficiently to all manuscript to include them in her thesis. She was responsible for study design, practical laboratory work, as well as drafting the manuscript. The list of techniques performed by KR are listed for each paper.

The thesis itself comprises the four above mentioned manuscripts, preceded by an Introduction and followed by conclusions and general abstract. The thesis is written in fluent, idiomatic English using appropriate scientific terminology.

The main objective of this thesis was to investigate the role of the transmembrane protein coding gene (TMEM244) in cutaneous T-cell lymphoma (CTCL). The idea builds on the original findings of the supervisor of the thesis who was the first to demonstrate that TMEM244 is ectopically expressed in malignant cells from patients with Sezary's syndrome (SS). The specific aims covered in this thesis were to identify the mechanism responsible for TMEM244 expression in CTCL, to understand the biological role of TMEM244, and to investigate the potential usefulness of TMEM244 in the diagnosis of CTCL. The major conclusions are that TMEM244 is expressed as lncRNA (but not as protein) and the transcription is regulated by specific DNA demethylation of the promoter. TMEM244 seems to play a role in the regulation of the growth of CTCL cell lines. Moreover, TMEM244 seems to be a promising marker of SS.

SPECIFIC COMMENTS

Thesis- Introduction - p. 7-15

In this introductory part, KR provides background information on the classification and clinical aspects of CTCL, reviews current views on CTCL pathogenesis, and discusses the need for diagnostic markers in CTCL. A separate section is dedicated to the background information on TMEM genes and proteins and their role in cancer.

The Introduction is well-written and gives a sufficient background for the reader to understand the aims of the thesis. Especially, the section on the pathogenesis of MF/SS is well-drafted and shows a good understanding of the current views on the role of oncogenic mutations. Mutations of the genes involved in the epigenetic regulation of chromatin (methylation, histone acetylation) and the role of non-coding RNAs are highlighted, as those mechanisms have relevance for the interpretation of the findings of this thesis.

The section on TMEM is very clear and provides enough context to the reader who is not familiar with this gene family. I appreciate that KR provided historical background to her thesis, describing how her research group developed the research hypothesis. It is laudable that this research is not just a random investigation of one of many genes potentially involved in CTCL but is a result of careful thought and foundation studies of Prof. Przybylski.

The weakest part of the introduction is the review of potential diagnostic biomarkers in CTCL. KR described extensively the current state of knowledge of the available and potentially novel markers and this part was done very well. However, I would welcome some more discussion on the clinical need for the markers (diagnostic versus prognostic) and the potential pitfalls regarding the clinical application. The questions of specificity and sensitivity are not trivial in the diagnosis of rare diseases. Bayesian statistical modeling is especially useful to show formidable challenges of discriminatory power of diagnostic tests for low prevalent conditions (such as SS).

Hypothesis and Specific Objectives p. 16

The research hypothesis was that "specific activation of TMEM244 expression in Sézary cells contributes to the lymphoma development and can be used as a diagnostic biomarker." The term "specific expression" is somewhat vague because I believe that showing that expression is specific for SS is a part of diagnostic biomarker study rather than the fact established beforehand. Also, by reading the thesis the specificity of expression was mainly in relation to blood cells, rather than isolated

neoplastic cells. My interpretation of the aim is that TMEM244 “contributes to the development of CTCL, is selectively expressed in the blood in SS patients, and can be used as a diagnostic biomarker.”

Below the research hypothesis, KR lists 6 separate aims of the study. Although technically correct, the multiplicity of the aims dilutes the direction of the study. A much better description of the aims is given in the Summary, where those six aims are synthesized into three: identification of the mechanism responsible for TMEM244 activation in CTCL, establishing the function and the coding potential of TMEM244, and to examine TMEM244 expression in a subpopulation of blood cells of healthy individuals and to address whether its expression may serve as a diagnostic tool for SS.

Nevertheless, the hypothesis and study aims, as they are, are perfectly logical and provide a coherent structure for the experimental work.

Paper 1: Hypomethylation of the promoter region drives ectopic expression of *TMEM244* in Sézary cells

This paper shows that TMEM244 is specifically expressed in malignant cells in SS patients, in SS cell lines. Moreover, the clever CRISPR approach was used to show the role of TMEM244 promoter demethylation as a main mechanism regulating the ectopic expression of TMEM244 in SS.
CTCL samples were obtained from CD4+ fraction of peripheral blood from 5 patients with SS and 3 patients with MF. Established, well-characterized SS cell lines were used. Additional control material was taken from healthy individuals and from patients with non-CTCL hematological malignancies. The overall design is very careful; however, I miss clinical information regarding the patients and the samples. As blood involvement varies largely between patients, it would be very useful to know the clinical stage of the patients, the peripheral blood SS cell count (B stage), and the presence of any concomitant treatment at the time of sample collection (in particular administration of treatments which may affect methylation status of the genes). I presume that in a high-level genetic lab, those samples were sequenced at some point (WGS or WES) and I would like to know the tumor cell fraction for the samples. This information is crucial for MF because blood involvement in MF is usually absent (or only detected by molecular methods). I also wonder why MF cell lines and MF cells purified from the skin were not included as a control. Low TMEM244 in MF might be caused by an absence of malignant cells in the sample rather than an inherent lack of expression in the lymphoma.

The experimental, molecular part was very well done, and I have no major comments. In conclusion, I agree that the main aims of the paper were achieved (i.e. showing TMEM expression in SS via promoter demethylation mechanism, no expression in normal cells), but the secondary aim (selectivity of TMEM244 expression in SS) would need some further experimental support.

Paper 2: TMEM244 is a long non-coding RNA necessary for CTCL cell growth.

In this study the authors showed that TMEM244 is expressed as a long non-coding RNA that plays a role in the growth of CTCL cell lines (indicating its role in vivo). The experiments are very well designed. Expression of TMEM244 was measured with qRT-PCR in SeAx and HH cells (SS lines) and for control purposes, in non-CTCL cell lines and in Jurkat cell lines with induced TMEM244 overexpression. With subcellular fractionation followed by qRT-PCR and RNA-FISH, they confirmed localization of TMEM244 transcript in the cytoplasm and to a lower degree, in the nucleus For functional assays, the cells were

transduced with shRNAs targeting its transcript and found lower proliferation rate and slight elevation of apoptosis in cells with inhibited TMEM244.

My only critical comment pertains to the conclusions about the role of TMEM244 in cell growth. The authors wrote that: “our results showed that TMEM244 expression is necessary for the growth of CTCL cells.” I believe that this conclusion was too strong. The growth assays were very basic (counting of GFP+ cells over time) and were not based on any direct measurement of proliferation (e.g. by flow cytometry, BrdU assays, etc). Flow cytometry (done here to assess apoptosis) would be ideal because the proliferation rate could be quantitatively correlated to GFP expression. Even by cell counting, some proliferation was still happening even in cells with TMEM244 inhibition. Finally, it would also be interesting to see the effect of TMEM244 overexpression. Besides that, the research is extremely strong for the molecular part and the results are novel and significantly increase the understanding of the mechanism of regulation of TMEM244 in SS cells.

Paper 3: TMEM244 Gene Expression as a Potential Blood Diagnostic Marker Distinguishing Sézary Syndrome from Mycosis Fungoides and Benign Erythroderma

This study is a further development on Paper 1, which indicated selective expression of TMEM244 in SS, but not in MF in peripheral blood. Here, the scope of examined samples was enlarged and included 8 patients with benign erythroderma and 3 new MF samples. The CD4/CD8 ratios were provided, which partially addresses the critical comment for Paper 1 that clinical information on B staging was missing. This paper confirmed that the peripheral blood of SS had a much higher expression of TMEM244 than MF and benign erythroderma. Moreover, TRBV10-1—J2-7—C2 rearrangement was determined and found only in one SS patient, which the authors interpreted as evidence of higher sensitivity of TMEM244 than TCR clonality analysis in SS diagnosis.

As a preliminary, proof-of-concept study, the paper is excellent. My comments here are more coarse-grained and relate to the overall design. The comparison in this study were between cases of obvious SS (CD4/CD8 >10, erythroderma) to MF without blood involvement and benign erythroderma. Those cases do not require additional biomarkers. The authors prove the concept, but not the utility. This is not a deficiency, the paper should be interpreted in the context of how it had been designed (which is a proof of concept study), but conclusions that “expression in the blood can be used to distinguish SS from MF and non-malignant erythroderma, therefore has the potential to improve the disease diagnosis” does not truly reflect the purpose of this work. Second, I do not understand the comparison with TRBV10-1—J2-7—C2 rearrangement. In a clinical setting, TCR β rearrangements are done via PCR amplification of CDR3 region, demonstrating the dominant amplicon, and measuring its length via capillary electrophoresis. TRBV10-1 is one of many possible rearrangements - and actually, the most common segment to rearrange is TRBV20-1 (I happen to know that because we have analyzed TRBV rearrangement patterns across different T-cell lymphomas [Blood Adv. 2022;6:2334]). The conclusion that “TMEM244 has higher sensitivity than TCR clonality analysis in SS diagnosis” is probably incorrect and drawn on the basis on non-standard TCR rearrangement test. It is a lesser point, because comparisons to TCR rearrangements were not even the purpose of this paper.

Paper 4: Single-Cell Heterogeneity of Cutaneous T-Cell Lymphomas Revealed Using RNA-Seq Technologies

This is a narrative review summarizing available scRNAseq data. It is well written and I do not have any significant comments. I read it with interest. This review provides context to the original research in this thesis, especially in relation to the significance of non-coding RNAs in CTCL.

CONCLUSIONS AND RECOMMENDATION

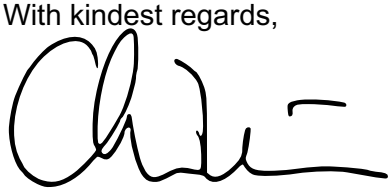
It was a pleasure to read and evaluate this thesis. **The work was well-designed, followed logical thought, and was innovative.** The strength of the thesis was the molecular part: showing the expression of TMEM244, proving its function as lncRNA, and explaining the basis of its dysregulation. As such, this work is an excellent starting point for more translational studies on patients with CTCL.

Some of the critical comments listed above were triggered by the high novelty of the experiments rather than the inherent deficiencies of the work. As a clinician scientist, I would welcome a more clinically relevant design of the biomarker studies, but I also fully understand that this PhD was primarily a basic research project, and its aim was to investigate the mechanisms and not to introduce a new diagnostic method. I am also aware that the availability of samples from CTCL is a problem due to the low prevalence of the disease. In such cutting-edge research, compromises were unavoidable.

KR contributed vastly to the project, both conceptually and by performing experiments.

In my assessment, this thesis fulfills and surpasses the usual requirements for the PhD degree and can be ranked at the top international level. I also recommend considering this thesis for an award.

With kindest regards,



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