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Identification of mechanisms related to the expression of *TMEM244* gene and its role in cutaneous T-cell lymphomas

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Elucidating the function of the transmembrane protein TMEM244 and its role in the development of lymphoid malignancies

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LIST OF SCIENTIFIC ABBREVIATIONS

CTCL - cutaneous T-cell lymphoma

FISH – fluorescence in situ hybridization

HDAC- histone deacetylase inhibitor

lncRNA- long non-coding RNA

MF - mycosis fungoides

PBMC – peripheral blood mononuclear cell

SS - Sézary Syndrome

TMEM- transmembrane protein

WHO- World Health Organisation

INTRODUCTION

Characteristics of cutaneous T-cell lymphomas

Cutaneous T-cell lymphomas (CTCLs) are a large, heterogeneous group of non-Hodgkin's lymphomas and constitute ~75% to 80% of all primary cutaneous lymphomas (Willemze et al., 2019). These lymphatic malignancies occur primarily in the skin, but can also involve the blood, lymph nodes, and/or internal organs in patients with advanced disease and are characterized by a cutaneous infiltration of malignant monoclonal T lymphocytes (Clark et al., 2011). Between 2000 and 2018 in the United States of America 14,942 individuals were diagnosed with CTCL, with an overall incidence rate of 8.55 per 1 000 000 cases and increase over the study period (annual percentage change of 0.61%) - findings that are consistent with recent studies from Europe (Cai et al., 2022, Dobos et al., 2021). CTCL is twice as common in men as in women and the incidence of CTCL increases with age, with an average onset between 50 and 60 years (Bradford et al., 2009, Willemze, 2003). Most cases occur in blacks, followed by non-Hispanic whites, Asian/Pacific Islanders, and Hispanic whites (Bradford et al., 2009). The most common subtype of CTCLs is mycosis fungoides (MF), characterized by an indolent curse in its early stages. Another type, Sézary syndrome (SS) is a very rare and much more aggressive variant. Other less common CTCL subtypes include adult T-cell leukemia/lymphoma, primary cutaneous CD30+ lymphoproliferative disorders, subcutaneous panniculitis-like T-cell lymphoma, primary cutaneous gamma/delta T-cell lymphoma, primary cutaneous aggressive epidermotropic CD8-positive T-cell lymphoma (provisional), primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoproliferative disorder (provisional), primary cutaneous acral CD8+ T-cell lymphoma (provisional), primary cutaneous peripheral T-cell lymphoma, not otherwise specified (NOS), extranodal NK/T-cell lymphoma and hydroa vacciniforme-like lymphoproliferative disorder (Kempf and Mitteldorf, 2021).

MF, the most common type of CTCLs, represents 44-62% of all cases. It occurs most frequently in older individuals, but can be diagnosed in younger individuals, including children (Pope et al., 2010, Virmani et al., 2017, Eklund et al., 2016). In the early stages of the disease, which can last for several years, MF presents as erythematous patches resembling inflammatory diseases such as psoriasis or dermatitis. In later stages, patches can progress into tumors and may infiltrate lymph nodes as well as internal organs (Kempf and Mitteldorf, 2021). Early skin lesions contain many inflammatory cells, including a majority of T-cells with a normal phenotype and a small population of T-cells with abnormal morphology and a malignant

phenotype. The infiltrate consists mainly of benign T-helper 1 cells, regulatory T-cells, and cytotoxic CD8+ T-cells, which to some extent appear to control malignant T-cells, but frequently lose the expression of other pan-T-cell antigens (Gjerdrum et al., 2007, Lee et al., 1999). Malignant T cells typically exhibit a mature CD4+ memory T-cell phenotype and are usually of clonal origin, lacking CD2, CD5, and/ or CD7 expression (Rosen and Querfeld, 2006). The prognosis in the patch and limited plaque stage is favorable with 5- and 10-year survival rates over 90% that reflects the indolent and slowly progressive course of the disease in most patients (Kempf and Mitteldorf, 2021).

Until 2022, WHO classified SS as a more aggressive form of CTCL that accounts for only 5% of all cases (Prasad et al., 2016). The disease is presented with erythroderma, which involves more than 80% of the body, widespread red rash, and the presence of malignant lymphocytes (Sézary cells) in blood and lymph nodes (Spicknall, 2018). The malignant T-cell population in SS is characterized by great diversity, indicating a naïve/memory maturation phenotype (CD3+CD4+ and CD8-) (Roelens et al., 2017, Hristov et al., 2011). As in MF, the aberrant loss of pan-T-cell antigens, including CD2, CD3, CD4, CD5, CD7, and/or CD26 is observed (Boonk et al., 2016, Klemke et al., 2015). SS is characterized by a poor outcome, with a 5-year overall survival rate between 30 and 40% (Prasad et al., 2016). Recently, the overview of the upcoming 5th edition of WHO classification of hematolymphoid tumors was released. The autors indicate that although SS is closely related to MF, is in fact a distinct entity. Therefore, in order to emphasize its primary site of clinical presentation and consideration in dfifferential diagnosis, it was included in the section of mature T-cell and NK- cell leukemias (Alaggio et al., 2022).

Both MF and SS symptoms may include red rash and light or dark patches, a reddened rash, thickened red skin, tumors on the skin, or erythroderma (Larocca and Kupper, 2019). What's more, patients with MF or SS can develop a second lymphoma, such as Hodgkin lymphoma and a subtype of CTCLs lymphomatoid papulosis, as well as nonhematologic malignancies (Vakeva et al., 2000, Cengiz et al., 2017, Blazewicz et al., 2021).

Even though most early-stage MF patients have a good prognosis, the prognosis for advanced-stage MF and SS individuals remains poor (Agar et al., 2010). There are some treatments available depending on several factors such as a person's general health, age, and the stage of the disease, although none of them is curative. (Oka and Miyagaki, 2019). Patients with early-stage MF or SS may respond well to skin-directed therapies such as topical corticosteroid, mechlorethamine, and bexarotene, as well as ultraviolet phototherapy, total skin electron beam therapy, and localized radiotherapy (Trautinger et al., 2017). Patients with

refractory early-stage cutaneous and advanced-stage disease may require a combination of skin-directed and systemic therapies, such as immune-preserving treatments (retinoids, low-dose methotrexate, and low-dose etoposide) or immunostimulatory therapies (interferon) (Kamijo and Miyagaki, 2021). Although a variety of therapies are available, the duration of the clinical response is short (Hughes et al., 2015, Whittaker et al., 2016). What's more, there are many side effects of the therapy like fatigue, chills, blood clots, and infertility. In some cases, the only curative option in MF or SS is allogeneic stem cell transplantation in patients that meet the criteria (Virmani et al., 2015).

Several targeted therapies such as the use of brentuximab vedotin and mogamulizumab are being investigated now (Kim et al., 2015, Prince et al., 2017, Horwitz et al., 2014, Kim et al., 2018, Fuji et al., 2016, Duvic et al., 2015). However, the advances in novel therapies are hindered because of the rarity of disease occurrence as well as incomplete understating of the pathogenesis of CTCL. Because of that, there is a need to examine the mechanisms responsible for the development and progression of CTCL to improve the response rates and prolong disease remission.

Pathogenesis of CTCL

Many potential mechanisms, along with genetic mutations, association with chronic skin disorders, infectious etiology, and exposure to external factors have been studied in CTCL (Yamashita et al., 2012). Although genetic alterations and dysregulation of signaling pathways have been reported, the exact molecular mechanism of the pathogenesis is still unknown.

Genomic analyses have elucidated the complexity of the genetic landscape in CTCL, including a significant number of chromosomal abnormalities that mostly occur on chromosomes 8,10, and 17 (Choi et al., 2015, da Silva Almeida et al., 2015, Wang et al., 2015). Complex chromosomal structural rearrangements have been identified in over 65% of patient samples exhibiting at least one chromothripsis-like rearrangement. Somatic copy number variations (SCNVs) were favored over single nucleotide variants (SNVs) with 92% of all driver mutations arising from SCNVs (Choi et al., 2015). Among genes that are altered, there are those involved in TCR signaling pathways like *PLCG1*, *CARD11*, *CD28,* and *RLTPR* as well as those involved in NF-kB signaling like *PRKCB* and *CSNK1A1* (Park et al., 2017). Genes involved in immune surveillance (*CD58*, *RFXAP*), chromatin remodeling (*ARID1A*, *CTCF*, *BCOR*, *KDM6A*, *SMARCB1*, *TRRAP*), and T-cell activation and differentiation (*ZEB1*, *STAT5B*, *CD28, RARA*) have been also found to be mutated (Choi et al., 2015, Park et al., 2017). Additionally, affected genes include those involved in the TCR signaling pathway, like *CD28*, encoding

TCR-associated enzymes (*PLCG1, PRKCQ, TNFAIP3*), and transcription factors (*NFKB2, STAT5B, ZEB1*) (Choi et al., 2015, Vaque et al., 2014). Other disrupted genes include those involved in DNA damage response (*TP53, POT1, ATM, BRAC1-2*), JAK-STAT signaling (*JAK3*, *STAT5B*), and chromatin modification (*ARID1A, TRRAP, DNMT3A, TET2*) (da Silva Almeida et al., 2015, Choi et al., 2015, Prasad et al., 2016, Ungewickell et al., 2015, Wang et al., 2015, Park et al., 2017, Patel et al., 2020).

Besides protein-coding genes, also long non-coding RNAs (lncRNAs) are deregulated in CTCL. LncRNAs are important regulators of cancer disease progression. Recent studies have examined the role of MALAT1 in CTCL patients. It has been shown that in CTCL MyLA cells, C-C motif chemokine ligand 21 (CCL21) activates mTOR leading to MALAT1 upregulation, with the observed surge in cell migration (Hong et al., 2019). Furthermore, MALAT1 was demonstrated to induce epithelial-mesenchymal transition (EMT) and cancer stem cell phenotype which is facilitated by the sponging of miR-124 by MALAT1 (Guo et al., 2022).

As epigenetic dysregulation is considered to be a hallmark feature of CTCL progression, multiple studies have been conducted to investigate the role of small, noncoding RNA molecules, microRNAs (miRNAs) in CTCL pathogenesis (da Silva Almeida et al., 2015, Ralfkiaer et al., 2011). Several onco-miRNAs have been identified, including miR-21, miR-199, miR-486, miR-214, and mir-155 (Lindahl et al., 2016, Ralfkiaer et al., 2014, Narducci et al., 2011, Litvinov et al., 2013). Although the molecular mechanism of miRNAs deregulation remains unclear, it may be related to JAK/STAT pathway as suggested in studies of miR-155 and miR-22 (Kopp et al., 2013, Sibbesen et al., 2015).

Both hypomethylation and hypermethylation signatures have been observed in CTCL pathogenesis. Genes involved in the methylation processes: *DNMT3A* (encoding methyltransferase), and *TET1/2* genes (encoding demethylases) are one of the most frequently affected, implying that genetic aberrations may underlie epigenetic dysregulation (Kiel et al., 2015, Choi et al., 2015, da Silva Almeida et al., 2015, Izykowska et al., 2017, Bastidas Torres et al., 2018). The role of histone deacetylase inhibitors (HDACs) in T-cells has been also extensively studied, as it was shown that many of them are important players in T-cell development and function (Stengel et al., 2015, Dovey et al., 2013, Yan et al., 2011, Dequiedt et al., 2005). Alterations in the structure, activity, or expression of HDACs can lead to dysregulation of gene transcription, resulting in a cancerous phenotype. In cells with aberrant HDACs expression, histone deacetylase inhibitors (HDACi) have emerged as a promising new class of drugs that have been demonstrated to induce cellular growth arrest, differentiation, and

apoptotic cell death in cancer cells of CTCL patients (Chun, 2015, Zhang et al., 2005, Marks et al., 2000).

In summary, it has been demonstrated that epigenetic modifications such as aberrant gene methylation and histone deacetylation are involved in the pathogenesis of CTCL. To fully understand the biology of CTCL, it is important to reveal the mechanism beyond the activation of genes and how it influences the disease pathogenesis. Therefore, in the first publication comprising this dissertation, we aimed to examine the epigenetic activation of the *TMEM244* gene in CTCL.

Putative roles of many infectious agents have been also investigated in CTCL. A significant incidence of skin infections in patients with CTCL with an association between pathogenic burden and disease severity has been reported (Bonin et al., 2010). *Staphylococcus aureus, Borrelia burgdorferi, Chlamydophila pneumoniae*, and dermatophytes were implicated as triggers or promoters of CTCL (Nedoszytko et al., 2018, Abrams et al., 2001, Ponzoni et al., 2011, Tothova et al., 2006, Nguyen et al., 2008, Talpur et al., 2008). *S.aureus* has not only been shown to act as a superantigen and stimulate the proliferation of malignant T- cells but also to effectively eliminate cytotoxic T-cell-mediated killing of malignant cells, therefore allowing immune evasion and continuing the growth of malignant CTCL cells (Krejsgaard et al., 2014, Blumel et al., 2020). Moreover, the role in triggering CTCL of certain viruses such as Human T-cell leukemia/lymphotropic virus type 1 (HTLV-1), HTLV-2, HIV, Epstein-Barr virus, Human Herpesvirus (HHV)-6, HHV-7, and HHV-8 has been investigated (Sakamoto et al., 2006, Poiesz et al., 2000, Wilkins et al., 2006, Novelli et al., 2009, Kreuter et al., 2008, Erkek et al., 2001, Ponti et al., 2008). However, despite many studies, the role of infectious agents in the pathogenesis of CTCLs remains controversial.

To systematically review the novel insight into molecular heterogeneity of malignant cells in CTCL in the fourth publication of this dissertation we analyzed previous RNA sequencing and single-cell RNA sequencing studies and highlighted novel molecular changes, differentially expressed genes, and CTCL-related processes.

TMEMs - characteristics, and role in cancer

The transmembrane protein (TMEM) family is a very heterogeneous group of proteins, constituting approximately 30% of the proteome (Carpenter et al., 2008, Babcock and Li, 2014). To be regarded as TMEM members, proteins must contain at least one putative transmembrane segment that spans completely or partially through biological membranes (Guo et al., 2019). The name of TMEM proteins comes either from the nature of the protein or from the lack of

information about its possible structure and function,. In most cases, they can be renamed and reclassified, after further characterization, into more specific categories related to their function (Marx et al., 2020). The proteins of this family are predicted to be components of cellular membranes, such as cytoplasmic membranes, mitochondrial membranes, lysosomes, and Golgi apparatus. TMEMs are present in many cell types playing important physiological functions such as protein glycosylation (TMEM165), immunity response (TMEM173), autophagy regulation (TMEM41B), or smooth muscle contraction (TMEM16A) (Foulquier et al., 2012, Ishikawa and Barber, 2008, Moretti et al., 2018, Thomas-Gatewood et al., 2011).

Differential regulation of the expression of some TMEM genes has been linked to several types of cancer such as lung cancer (TMEM48), hepatic cancer (TMEM7), colorectal cancer (TMEM25) or lymphomas (TMEM176) (Qiao et al., 2016, Xu et al., 2019, Hrasovec et al., 2013, Zuccolo et al., 2013). TMEMs have been demonstrated to be involved in cancer development (TMEM43, TMEM116, TMEM14A) suggesting that this family is a significant group in cancer research (Jiang et al., 2017, Zhang et al., 2021, Zhang et al., 2022). Functionally, several TMEM family members have been described as tumor suppressors while others as oncogenes. Some TMEMs described as candidate tumor suppressors were found to be downregulated in tumor tissues compared to healthy ones (TMEM25, TMEM7, TMEM97) (Doolan et al., 2009, Zhou et al., 2007, Ramalho-Carvalho et al., 2018). On the other hand, many TMEMs were found to be upregulated in cancers. Some are involved in tumor invasion and metastasis (TMEM48, TMEM16A, TMEM88, TMEM206) while others have been associated with poor prognosis (TMEM45B, TMEM106C, TMEM2) (Qiao et al., 2016, Liu et al., 2012, Zhang et al., 2015, Zhao et al., 2019, Hu et al., 2016, Duan et al., 2021, Kudo et al., 2020). Although mutagenic changes have been mostly associated with cancer growth and drug resistance, epigenetic modifications and the tumor microenvironment have also been connected to chemoresistance. Such examples are TMEM45A involved in the chemoresistance of breast and liver cancers and TMEM205 involved in resistance to cisplatin in epidermoid carcinoma (Flamant et al., 2012, Shen et al., 2010). Other TMEMs have also been associated with tumor growth, however, their mechanisms of action are still unknown for example TMEM140, upregulated in tumor tissue compared to normal tissue (Li et al., 2015).

Nevertheless, the majority of TMEM proteins remain partially described and their mechanisms of action is unclear. A better characterization of such proteins could help in understanding their effect on cancer cells and open the way to the discovery of new drug targets and more efficient therapies. Previous results of prof. Przybylski group indicated the association between SS and the ectopic *TMEM244* expression suggesting its participation in the

development of this lymphoma (Izykowska et al., 2017). In line with these findings and due to a lack of information concerning the function of *TMEM244,* in the second publication of this dissertation, we have undertaken basic research aimed to understand the function of this gene.

Novel potential markers of CTCL

Because of a clinical and histologic resemblance to benign inflammatory skin diseases and the complex diagnostic criteria of CTCL patients, there has been a search for a simple diagnostic marker that would differentiate this lymphoma. Earlier gene expression studies have identified gene signatures in the skin and blood of patients with MF and SS and proposed their value as diagnostic markers. Studies have shown several genes being overexpressed in CTCL such as PLS3, TWIST1, and GATA6 (Nebozhyn et al., 2006, van Doorn et al., 2004). GATA6 overexpression was correlated with the induction of CD137L expression, which promotes the proliferation, survival, and migration of SS T cells and CTCL cell lines (Mehdi et al., 2021, Wong et al., 2015). On the other hand, FOXO1A was found to be downregulated in T-cells derived from SS patients, resulting in the loss of mechanisms controlling the cell cycle, cell death, cell metabolism, and oxidative stress (van Doorn et al., 2004). RNA sequencing analysis was also performed for CTCL samples. Using single-cell RNA sequencing and flow cytometry, a cluster of 5 genes (S100A4, S100A10, IL7R, CCR7, and CXCR4) that was highly expressed by most malignant cells has been identified (Buus et al., 2018). Another analysis demonstrated a 17-gene expression signature that was common between highly proliferative tumor cells in five CTCL patients: *ACTG1, ANP32B, ATP5C1, DUT, HMGN1, HN1, NPM1, NUSAP1, PCNA, PPA1, PPIA, PSMB2, RAN, RANBP1, SET, SMC4,* and *STMN1*. Interestingly, *PCNA, ATP5C1,* and *NUSAP1* genes overlap with the expression of *TOX*, a previously reported marker for identifying malignant lymphocytes in CTCL (Gaydosik et al., 2019). Recently, a new study revealed 86 different driver genes mutated in CTCL, with 19 genes not previously associated with this disease and two not previously associated with any cancer. Although many driver genes are shared between MF and SS, some of them are entity-unique such as tumor suppressor gene *TP53* which was deleted in 87% of SS samples but in only 7% of MF samples (Park et al., 2021).

Also, the diagnostic potential of miRNA profiling in CTCLs was examined. It was suggested that miRNAs have the potential to differentiate CTCLs from healthy skin or benign skin diseases (Ralfkiaer et al., 2011, Marstrand et al., 2014, Shen et al., 2018). It was shown that miR-26a, miR-222, miR-181a, and miR-146a could not only be used to differentiate tumors from reactive MF but also seem to be involved in cancer progression (Manso et al., 2018). It

was also shown that plasma miR-155, miR-203, and miR-205 could be used as biomarkers of tumor burden and response to therapy (Dusilkova et al., 2017).

The establishment of more accurate and easier diagnostic methods is crucial to improve the management of patients suspected of CTCL. This challenge was undertaken in the third publication comprising this dissertation.

HYPOTHESIS AND SPECIFIC OBJECTIVES

The main aim of this dissertation was to extend the current knowledge about the biological function of the transmembrane protein gene *TMEM244,* and the role of its activation in the development of lymphoid malignancies. The risen hypothesis was that specific activation of *TMEM244* expression in Sézary cells contributes to the lymphoma development, and can be used as a diagnostic biomarker.

To gain new knowledge about *TMEM244,* and to test this hypothesis, the following specific aims were formulated:

1. Analysis of *TMEM244* expression in CTCL samples.
2. Identification of the molecular mechanism responsible for the activation of *TMEM244* transcription through analysis of the promoter region methylation status.
3. Identification of a protein encoded by the *TMEM244* gene.
4. Determination of the expression level of *TMEM244* in different subpopulations of peripheral blood mononuclear cells from healthy individuals.
5. Determination of the effect of *TMEM244* knockdown in T cell lines.
6. Evaluation of the usefulness of *TMEM244* expression in the diagnosis of Sézary syndrome.

1st ARTICLE ENTITLED:

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

Katarzyna Iżykowska, **Karolina Rassek,** Magdalena Żurawek, Karina Nowicka, Julia Paczkowska, Iwona Ziółkowska-Suchanek, Marta Podralska, Agnieszka Dzikiewicz-Krawczyk, Monika Joks, Karolina Olek-Hrab, Maciej Giefing, Grzegorz Krzysztof Przybylski

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Background: SS is characterized by many complex changes in the genome, transcriptome, and epigenome (Choi et al., 2015, da Silva Almeida et al., 2015, Wang et al., 2015). Epigenetic dysregulation appears to play an important role in the development and progression of SS, as SS cells are characterized by extensive changes in DNA methylation (van Doorn et al., 2016). However, the role of hypomethylation in the regulation of gene expression in SS has not been extensively studied. The study of Wong et al. was the first to show that the genome of CD4+ SS T-cells is significantly hypomethylated compared to CD4+ T-cells of healthy donors, and that promoter-specific hypomethylation is associated with overexpression of *PLS3*, *TWIST1,* and *GATA6* (Wong et al., 2015). Prof. Przybylski group revealed ectopic expression of *transmembrane protein 244* (*TMEM244)* gene in SS patients. This aberrantly activated expression was detected by RNA sequencing in nine SS patients and confirmed by qRT-PCR on additional group of SS patients. Here we addressed the hypothesis that, in SS, DNA hypomethylation is involved in the upregulation of *TMEM244* expression that is only trace in normal lymphocytes.

Results: First, we have evaluated *TMEM244* expression using qRT-PCR in different T- and B-cell lymphoma and leukemia patients, in mononuclear cells of healthy individuals, as well as in T-cell lymphoma (TCL) and T-cell acute lymphoblastic leukemia (T-ALL) cell lines. In line with our previous results, *TMEM244* expression was detected in all SS patients (n=5), SS-derived cell lines: SeAx, Hut78, HH and T-cell derived Hodgkin lymphoma HDLM2 cell line. In contrast, there was no *TMEM244* expression in mononuclear cells from healthy donors (n=5) and the T-ALL Jurkat cell line. Further, to evaluate whether *TMEM244* expression is regulated by DNA methylation, three CpG dinucleotides in the TMEM244 promoter region were analyzed (genomic position: chr6:130,182,479-130,182,514; GRCh37/hg19). As determined

by bisulfite DNA pyrosequencing in all samples without *TMEM244* expression promoter was methylated in 85-89% (MV=86%), while in samples expressing *TMEM244* methylation of the promoter was 2-68% (MV=44%). Overall, the percentage of promoter DNA methylation was inversely proportional to the level of *TMEM244* expression (R=-.7813; P<.00001). In addition to methylation analysis, we used the CRISPR system for targeted demethylation in T-cell line Jurkat, to prove the mechanism of transcription activation of *TMEM244*. Transduction of Jurkat cell line with catalytically inactive Cas9 protein (dCas9) fused to TET1 active domain resulted in marked demethylation of *TMEM244* promoter with the use of 2 single guide RNAs (sgRNAs) (25% for sg5 and 30% for sg6). A less prominent effect was observed with the use of a mixture of those sgRNAs (10%), as compared to cells transduced with TET1 inactive domain or 2 non-targeting controls. Activation of *TMEM244* expression in modified cells was negatively correlated with the level of promoter demethylation (R=-0.4766, p< .0002). Taken together, it proved that epigenetic modification in the form of DNA methylation is responsible for *TMEM244* expression.

Conclusions: In this study, we have shown for the first time that methylation is a key regulatory mechanism of *TMEM244* expression. A negative correlation between *TMEM244* expression and methylation was confirmed in hematological malignancies and T-cell lines. Therefore, CRISPR-dCas9-induced demethylation of the *TMEM244* promoter supports the hypothesis of epigenetic regulation of *TMEM244* expression.

KR contribution to this article

- patient samples preparation: density gradient centrifugation of peripheral blood mononuclear cells and $CD4^+$ T cells sorting
- cell lines culturing and viral transductions
- most DNA and RNA isolations
- reverse transcriptions and qRT-PCRs
- most Western Blot experiments
- expression data analyses
- reviewing and preparing the final manuscript version.

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ORIGINAL ARTICLE

WILEY

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

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Abstract

Sézary syndrome (SS) is an aggressive form of cutaneous T-cell lymphoma (CTCL) characterized by the presence of circulating malignant CD4+ T cells (Sézary cells) with many complex changes in the genome, transcriptome and epigenome. Epigenetic dysregulation seems to have an important role in the development and progression of SS as it was shown that SS cells are characterized by widespread changes in DNA methylation. In this study, we show that the transmembrane protein coding gene *TMEM244* is ectopically expressed in all SS patients and SS-derived cell lines and, to a lower extent, in mycosis fungoides and in a fraction of T-cell lymphomas, but not in B-cell malignancies and mononuclear cells of healthy individuals. We show that in patient samples and in the T-cell lines *TMEM244* expression is negatively correlated with the methylation level of its promoter. Furthermore, we demonstrate that *TMEM244* expression can be activated in vitro by the CRISPR-dCas9–induced specific demethylation of *TMEM244* promoter region. Since both, *TMEM244* expression and its promoter demethylation, are not detected in normal lymphoid cells, they can be potentially used as markers in Sézary syndrome and some other T-cell lymphomas.

KEYWORDS
CRISPR-dCas9, DNA methylation, Sézary syndrome, TET1, TMEM244

1 | INTRODUCTION

Sézary syndrome (SS) is an aggressive, leukaemic cutaneous T-cell lymphoma (CTCL) variant characterized clinically by severe erythroderma, pruritus and lymphadenopathy,[1] and the presence of atypical, malignant Sézary cells in blood, lymph nodes and skin with CD3/CD4 expression and heterogeneous naïve/memory maturation phenotype.[2] SS accounts for 4% of CTCL with the incidence of 0.4/1000 000, yet the prognosis for patients is poor (5-year survival of 30%).[1,3] SS is characterized by many complex changes in the genome and epigenome[4-7] that influence the transcriptome thereby leading to malignant transformation. Epigenetic dysregulation seems to have an important role in the development and progression of SS, as it was shown that genes involved in methylation, like *DNMTs* and *TETs*, are often mutated in SS,[3] and SS cells are characterized by widespread changes in DNA

TABLE 1 *TMEM244* expression and promoter methylation in haematological patients, healthy individuals and T-cell lines

Patient ID	Sex age	Diagnosis	WBC G/L	Lymph G/L	CD4+	CD4/CD8	Sample	Relative *TMEM244* expression $2^{-\Delta CT}/2^{-\Delta\Delta CT}$	*TMEM244* promoter region methylation (%mean ± SD)
Sézary syndrome and Mycosis fungoides									
P1	80 F	SS	24.8	18.3 (74%)	95%	15.9	PB	**371E-6/198.84**	**33.47 ± 2.94**
P2	65 M	SS	9.5	1.5 (15%)	83%	7.2	PB	**1 150E-6/61.50**	**67.85 ± 6.54**
P3	54 M	SS	12.5	6.1 (49%)	74%	4.5	PB	**521E-6/27.86**	**59.86 ± 2.94**
SS1	85 M	SS	18.9	13.8 (73%)	92%	16.7	PB	**8 224E-6/439.79**	**4.28 ± 0.70**
SS2	72 M	SS	9.3	4.5 (48%)	90%	45	PB	**173E-6/9.25**	**33.37 ± 1.74**
MF1	76 M	MF	7.0	1.0 (14%)	52%	1.9	PB	**198E-6/10.59**	**59.01 ± 4.2**
MF2	68 M	MF	10.2	3.0 (29%)	60%	1.7	PB	**351E-6/10.59**	**69.99 ± 6.8**
				(13%)	55%	1.5	BM	57E-6/3.05	83.75 ± 7.4
							SB	9E-6/0.48	71.31 ± 13.7
MF3	39 F	MF	5.3	1.5 (29%)	54%	2.6	PB	48E-6/2.57	75.53 ± 5.78
							BM	50E-6/2.67	83.01 ± 1.63
Other T-cell lymphomas and acute T-cell lymphoblastic leukaemia									
							PB	34E-6/1.82	84.23 ± 3.7
TCL1	47 M	EATL					BM	**234E-6/12.51**	73.36 ± 13.9
TCL2	72 F	PTCL					PB	18E-6/0.96	91.59 ± 4.9
TAL1	25 M	ETP-ALL					PB	**165E-6/8.82**	80.29 ± 23.5
TAL2	50 F	T-ALL					PB	91E-6/4.87	76.36 ± 4.4
TAL3	52 F	T-ALL					LN	89E-6/4.76	84.79 ± 17.2
							PB	34E-6/1.82	84.23 ± 3.7
B-cell leukaemia									
CLL1	58 F	CLL					PB	2E-6/0.11	92.54 ± 4.7
CLL2	51 M	CLL					PB	4E-6../.0.0.21	95.96 ± 1.5
CLL3	69 M	CLL					PB	8E-6/0.43	83.16 ± 19.9
CLL4	73 M	CLL					PB	1E-6../.0.0.05	92.49 ± 1.8
CLL5	73 M	CLL					PB	**433E-6../.0.23.16**	**53.73 ± 22.5**
CLL6	61 M	CLL					PB	2E-6/0.11	94.09 ± 2.3
CLL7	71 M	CLL					PB	24E-6/1.28	78.74 ± 24.3
CLL8	59 M	CLL					PB	30E-6/1.60	80.40 ± 23.3
HCL1	79 F	HCL					PB	15E-6/0.80	93.81 ± 1.6
HCL2	43 M	HCL					BM	59E-6/3.16	80.36 ± 7.1
BAL	22 F	B-ALL					BM	8E-6/0.43	91.28 ± 5.2
Healthy individuals									
BM1	63 F	HI					BM	16E-6/0.86	91.44 ± 4.0
C1	49 M	HI					PB	23E-6/.0.1.23	87.61 ± 5.7
C2	42 M	HI					PB	21E-6../.0.1.12	88.83 ± 6.6
C3	50 F	HI					PB	1E-6/0.05	84.80 ± 7.84
C4	41 M	HI					PB	15E-6/0.80	85.72 ± 8.30
C5	42 M	HI					PB	36E-6--/1.93	86.44 ± 6.16
T-cell lines									
SeAx		SS					CC	**2 536E-6/135.61**	**1.9 ± 1.03**
HH		CTCL					CC	**363E-6/19.41**	**3.2 ± 0.47**
Hut78		CTCL					CC	50E-6/2.67	**56 ± 25.81**
HDLM2		T-cell HL					CC	**1 482E-6/79.25**	**1.8 ± 0.52**
Jurkat		T-ALL					CC	0E-6/0.00	85.5 ± 18.69

Note: Meaningful *TMEM244* expression (>100E-6) and meaningful promoter hypomethylation (<70%) are given in bold.

Abbreviations: ALL, acute lymphoblastic leukaemia; B-ALL, B-cell acute lymphoblastic leukaemia; BM, bone marrow; CC, cell culture; CLL, chronic lymphocytic leukaemia; CTCL, cutaneous T-cell lymphoma; EATL, enteropathy-associated T-cell lymphoma; HCL, Hairy cell leukaemia; HI, healthy individual; HL, Hodgkin lymphoma; LN, lymph node; MF, mycosis fungoides; PB, peripheral blood; SB, skin biopsy; SS, Sézary syndrome; T-ALL, T-cell acute lymphoblastic leukaemia.

methylation,[8] including hypermethylation of tumour suppressor genes.[9] In cancer, disruption in methylation pattern, especially global hypomethylation of the genome, leads to chromosomal instability and consequently to altered transcription and impaired signalling pathways.[10]

In our previous study, we identified ectopic expression of transmembrane protein gene (*TMEM244*), with unknown biological function, in SS patients but not in healthy individuals.[11] The purpose of this study was to unravel the mechanism responsible for *TMEM244* activation. We found a negative correlation between *TMEM244* expression and promoter methylation in patient samples and in T-cell lines suggesting methylation to be a mechanism responsible for regulation of *TMEM244* expression. This concept was proved in vitro using CRISPR-dCas9 epigenome editing system, by activating *TMEM244* expression in Jurkat cells upon specific demethylation of selected CpGs in *TMEM244* promoter region.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Five Sézary syndrome blood samples were included in the study: P1 (F-female, age: 80), P2 (M-male, age: 65), P3 (M, age: 54) from the previous study,[11] and two new SS patients: SS1 (M, age: 85) and SS2 (M, age: 72). All SS samples were received from the Department of Dermatology, University of Medical Sciences, Poznan, Poland. Three of them were mononuclear cells (PBMCs) purified by density gradient centrifugation in Histopaque-1077 (Sigma-Aldrich, Germany) (P1-P3), while two others (SS1 and SS2) were sorted $CD4^+$ T cells, separated with Human CD4 + T Cell Enrichment Kit (StemCell Technologies).

Three mycosis fungoides samples (MF1-MF3) were collected from the Department of Hematology and Marrow Transplantation, University of Medical Sciences, Poznan, Poland: MF1 (blood), MF2 (blood, bone marrow, skin biopsy) and MF3 (blood, bone marrow). CD4 + lymphocytes were separated from blood and bone marrow by density gradient centrifugation in Histopaque-1077 (Sigma-Aldrich) and Human CD4 + T Cell Enrichment Kit (StemCell Technologies). Lymphocytes from the skin biopsy (size: 0.5 cm^2) were isolated as described by Salimi et al[12]

Thirteen blood samples, 3 bone marrow samples from different haematological malignancies and 1 healthy bone marrow sample (BM1) were obtained from the Department of Hematology and Marrow Transplantation, University of Medical Sciences, Poznan, Poland (Table 1). Five blood samples from healthy individuals from the previous study were used as controls (C1-C5).[11] PBMCs were purified from those samples using Histopaque-1077 (Sigma-Aldrich).

The use of human material was approved by the Local Ethics Committee (Decision1095/17) and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all individual participants involved in the study.

Samples were used to extract DNA (Gentra Puregene Blood Kit, Qiagen) and RNA (TRI Reagent, SIGMA) according to the manufacturer's protocol. The quantity of RNA and DNA was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific™, Waltham, CA), and the quality was determined by 1% agarose gel electrophoresis with ethidium bromide staining. cDNA was synthetized from 0.5 μg of RNA using QuantiTect Reverse Transcription Kit with random hexamer primers (Qiagen, Germany).

2.2 | Cell lines

Five established cell lines were included in the study: 3 CTCL cell lines (Hut-78—Sézary syndrome, ATCC TIB-161; HH—aggressive cutaneous T-cell leukaemia/lymphoma, ATCC CRL-2105; and SeAx—Sézary syndrome, kindly provided by Dr Markus Möbs[13]), T-cell acute lymphoblastic leukaemia (T-ALL) cell line Jurkat (SIGMA 88042803), and T-cell Hodgkin lymphoma cell line HDLM2 (DSMZ ACC17). They were cultured in a HEPES-buffered RPMI1640 medium with L-glutamine (Thermo Fisher Scientific™), 10%-20% foetal bovine serum (Sigma) and 1% penicillin/streptomycin (Life Technologies), according to manufacturer's instructions. Medium for SeAx was supplemented with Il-2 (200 U/mL) (Sigma-Aldrich) and medium for Jurkat with 1% sodium pyruvate (1 mmol\L) and 0.25% glucose (0.5 g/L) (Life Technologies).

2.3 | Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed using the CFX96 Real-Time System (Bio-Rad, Hercules, CA). *TMEM244* expression was analysed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) (Hs02340633_m1), with intron-spanning primers located in the second and third exons. Beta-2 microglobulin (*B2M)* (Hs00984230_m1), with intron-spanning primers located in the first and second exons, was used as a reference gene for sample normalization. For both genes, Applied Biosystems TaqMan MGB (minor groove binder) dual-labelled hydrolysis probes were used, incorporating a 5' fluorescent reporter dye and a 3' nonfluorescent quencher (NFQ). All samples were assayed in triplicates, and median value was used to calculate relative gene expression, according to the Livak method ($2^{-\Delta CT}$). The $2^{-\Delta\Delta CT}$ Livak equitation was additionally calculated for the patient samples, using as calibrator the mean value of healthy individuals. The $2^{-\Delta CT}$>100E-6, corresponding to the $2^{-\Delta\Delta CT}$>5.35, was considered to be significant expression of *TMEM244*.

2.4 | Plasmids and sgRNA cloning

Two lentiviral CRISPR-dCa9 vectors: pLV[Exp]-Bsd-EF1A > dCas9*:active TET1 (Tet Methylcytosine Dioxygenase 1) (ID: VB190118-1114rnk) and pLV[Exp]-Bsd-EF1A > dCas9*:inactive domain TET1 (VB190118-1116tye) were designed by VectorBuilder (Chicago, USA) (Supplementary). Both vectors have catalytically inactive Cas9 (dCas9) fused to either active or inactive (mutated) domain of TET1 demethylase.[14]

3rd generation lentiviral gRNA expression vector: pU6-sgRNA Ef1 alpha Puro-T2A-GFP was a gift from dr LA Gilbert.[15]

Four single guide RNAs (sg5: GAGAACTCCATCGTTTAATA; sg6: ACGCAGTAG-TGCAGGATGAT, sg7:AATTACTCATACAGCCAGAG, sg8:GATAGTGCGGCAAATAG-GCA) were designed using CRISPOR program (http://crispor.tefor.net/) to target the three CpG dinucleotides that were investigated for DNA methylation level. The sgRNA localization was selected in close proximity to the CpGs, to avoid binding of the large dCas9-TET1 protein complex directly to the CpGs (Figure 1). To exclude any off-target effects, two no-targeting sgRNAs were used: (NT3: ACGGAGGCTAAGCGTCGCAA and NT4: ATCGTTTCCGCTTAACGGCG). For functional experiments, Jurkat cell line, not expressing *TMEM244* and showing hypermethylated promoter region of the gene, was chosen. Sense (5' TTG---GTTTAAGAGC 3') and antisense (5' TTAGCTCTTAAAC---CAACAAG 3') oligonucleotides were annealed and cloned into a lentiviral pU6 vector backbone using BstXi and BlpI restriction sites and respective enzymes (NEB). Annealed oligonucleotides were ligated into a vector, before the sgRNA scaffold, using T4 ligase (Promega). Plasmid was replicated in TOP10 electrocompetent E. coli (Thermo Fisher Scientific™) and purified using Qiagen Plasmid Plus Kit (Qiagen, Germany). Cloned sgRNA constructs were sequenced to confirm the correctness of the inserted sequence.

2.5 | Lentiviral production

HEK293T (DSMZ ACC 635) cells were cultured in DMEM (Lonza) supplemented with 10% FBS (Sigma) and 1% penicillin/streptomycin (Life Technologies). Lentiviral vectors were co-transfected with 3rd generation packaging plasmids: pMSCV-VSV-G, pRSV.REV, pMDLgPRRE into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). Medium was replaced 24 hours after transfection. 48 and 72 hours post-transfection, viral supernatant was collected, sterile filtered through 0.45 μm syringe filter and stored at −80°C.

2.6 | Jurkat transduction

Jurkat cells were first transduced with lentivirus particles containing dCas9-TET1 active/inactive fusion. Virus supernatant was added to cells together with polybrene (4 μg/mL). To establish pure population of cells expressing dCas9/TET1, protein selection with blasticidin was performed for 7-14 days (15 μg/mL).

Jurkat cells with stable dCas9/TET1 expression were transduced with lentivirus particles containing sgRNAs. Nine separate transductions were performed: each sgRNA separately (sg5, sg6, sg7, sg8), mixture of two sgRNAs (sg5,6), mixture of all sgRNAs (sg5,6,7,8), two NT (NT3, NT4) and mixture of two NT (NT3,4). Selection with puromycin was performed for 5-7 days (2 μg/mL). Cells were harvested for DNA, RNA and protein at three time points—passage 1-st, 3-rd and 5-th post–puromycin selection.

2.7 | Western Blot

Cells were treated with RIPA lysis buffer (Sigma). Protein concentration was determined using Bicinchoninic Acid Kit (Sigma). Protein samples were mixed with the Laemmli 4X sample buffer (Sigma), denatured and run on the Mini-PROTEAN Stain-free gel (Bio-Rad) with Mini-PROTEAN® Tetra electrophoresis system (Bio-Rad). Proteins were semi-dry transferred onto PVDF membrane (The Mini Trans-Blot® cell system, Bio-Rad), blocked and incubated with Cas9 (7A9-3A3) mouse primary monoclonal antibody (Cell Signaling Technology; Leiden, Netherlands). After incubation with secondary Ab-HRP (sc-2005, Santa Cruz Biotechnology), the signal was detected by chemiluminescence with Clarity Western ECL Substrate (Bio-Rad) using ChemiDoc™ Imaging Systems (Bio-Rad). Quantitative analysis was performed using ImageLab™ Software. The WB results were normalized using stain-free technique, by measuring total protein directly on the WB membrane. In this method, trihalo compounds, included in the gel, react with tryptophan residues and after activation by UV light produce fluorescent signal that can be quantified in order to measure the relative amount of sample total protein.

2.8 | DNA methylation analysis by bisulfite pyrosequencing

The pyrosequencing assay for the analysis of DNA methylation level in *TMEM244* promoter region was designed using the PyroMark Assay Design Software 2.0.1.15 (Qiagen,

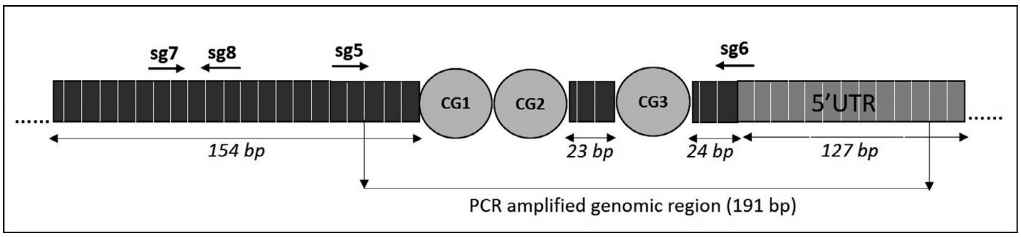

FIGURE 1 *TMEM244* promoter region. Genomic position of three CpG dinucleotides: chr6:130,182,479-130,182,514; GRCh37/hg19; four sgRNAs are indicated by arrows

Hilden, Germany). The assay included the (Forward) 5′-AGGATGTTTATTTTGGTATTTA-GTAGTT-3′, (Reverse) 5′-biotin-labelled- AAAATAATAAAAACCCCACTCCT-3′ and (Sequencing) 5′- TTTATTTTGGTATTTAGTAGTTT-3′ primers. The PCR amplified genomic region (chr6:130,182,353-130,182,543 GRCh37/hg19) (191 bp) was located upstream of *TMEM244* and overlapped partially the 5′UTR region of the gene (Figure 1). The amplified region overlapped the ENCODE regulatory region as well as several transcription factors binding sites.

DNA methylation level was calculated as the mean methylation measured at three CG dinucleotides at the genomic positions CpG_1 chr6:130,182,513; CpG_2 chr6:130,182,511; and CpG_3 chr6:130,182,486 (GRCh37/hg19) (Figure 1). For the PCR reactions, the PyroMark PCR kit (Qiagen) was used to prepare the following master mix: 12.5 μL PyroMark buffer; 0.5 μL F and R primer each (20 μmol\L); 2.5 μL Coral Load; 8 μL H_2O; and 1 μL of bisulfite-converted DNA (in total 25 μL). DNA bisulfite conversion was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Germany) according to the manufacturer's protocol. The reaction mix was cycled in the following conditions: 95°C for 15 minutes × 1; 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds × 45; and 72°C for 10 minutes × 1; 4°C ∞, and the PCR products were visualized on 1.5% agarose gel stained with SimplySafe (EURx) under UV light (BioDoc-it Imaging System, UVP, USA). The PyroMark Q24 purification station and sequencer were used to obtain single strand DNA and subsequent sequencing as described previously.[16] Each run included commercially available fully methylated and unmethylated controls (CpGenome™ Human Methylated & Non-Methylated DNA Standard Set, Sigma-Aldrich).

3 | RESULTS

3.1 | *TMEM44* is expressed in SS and CTCL cell lines with hypomethylation of the promoter region

Our previous study showed that *TMEM244* is expressed in SS patients (P1-P3 mean ± SD = 681 ± 413E-6).[11] In this study, *TMEM244* expression was quantified in different T- and B-cell lymphoma and leukaemia patients, in mononuclear cells of healthy individuals and in four T-cell lymphoma (TCL) and one TALL cell lines (Table 1). Only trace *TMEM244* expression was detected in healthy individuals (C1-C5 mean ± SD=19E-6 ± 13E-6; BM1 16E-6) (Table 1), while in majority of T-cell leukaemia/lymphoma cases and CTCL T-cell lines the expression of *TMEM244* was present.

TMEM244 was detected in both SS samples, yet the level was significantly higher for the SS1 patient (SS1 vs SS2 = 8,224E-6 vs 173E-6). In MF, that belong to the same group of CTCL lymphomas as SS, the expression of *TMEM244* was detected in blood samples of two patients (mean ± SD MF1-2PB = 275E-6 ± 108E-6), but much lower trace expression was measured in either bone marrow (mean ± SD MF2-3BM = 52E-6 ± 4E-6) or skin biopsy (MF2SB = 8E-6). Besides CTCLs, one of two non-cutaneous peripheral T-cell lymphoma (PTCL) patients and one of three T-ALL patients showed a meaningful *TMEM244* expression (TCL2: 234E-6 and TAL2: 165E-6). The expression was also detected in CTCL cell lines: SeAx (2,536E-6), HH (363E-6) and, at a very low level, in Hut78 (50E-6). HDML2 cell line, derived from a T-cell Hodgkin lymphoma, had a relatively high expression of *TMEM244* (1,482E-6), while no expression was detected in Jurkat cells.

All but one B-cell leukaemia samples (10/11) showed only a trace *TMEM244* expression (mean ± SD=15.3E-6 ± 18.3E-6), suggesting that this gene may be exclusive for T-cell malignancies. In one CLL sample, otherwise not different, a meaningful expression was detected (CLL5: 433E-6).

In order to determine whether *TMEM244* expression is regulated by DNA methylation, three CpG dinucleotides in the *TMEM244* promoter region were analysed by bisulfite pyrosequencing. The results showed that in cells without or with trace *TMEM244* expression the CpG sites were highly methylated, including healthy individuals (C1-C5 mean ± SD=86.68% ± 1.58; BM1 = 91.44%), Jurkat cell line (85.5%) and most B-cell leukaemias (mean ± SD = 88.3% ± 6.8). On the contrary, in cells expressing *TMEM244* at a significant level the promoter was hypomethylated. In homogeneous cell lines with high *TMEM244* expression, like SeAx, HDLM2 and HH, the promoter was completely demethylated (1.9%, 1.8%, 3.2% respectively). Similar effect was observed for SS1. This sample consisted of a homogenous population of malignant CD4 + lymphocytes with the highest expression of *TMEM244* detected in our study and complete demethylation of its promoter region (4.3%). In SS2 and Hut78, lower *TMEM244* expression was observed, and methylation analysis showed that it is a result of partial demethylation of *TMEM244* promoter (33.4% and 56% respectively). Three samples from a previous study (P1-P3) consisted of a heterogeneous population of mononuclear cells. Therefore, despite 33.5%-67.9% methylation, most likely derived from the admixture of non-malignant cells, the samples showed high *TMEM244* expression. In 2/3 MF patients, partial demethylation of *TMEM244* promoter in the blood samples (59% and 70%) was accompanied by a moderate *TMEM244* expression (198E-6 and 351E-6). In one PTCL and one T-ALL samples with a weak *TMEM244* expression, the mean methylation level was only slightly decreased compared with controls (73% and 80% respectively).

Among the 39 samples collected from patients, healthy donors and cell lines, a meaningful *TMEM244* expression was observed in 13 samples: five SS, two peripheral blood of MF, one CLL, one T-ALL, one T-cell lymphoma, and three T-cell lines. In those samples, the mean promoter methylation level was 44.11%±30.4 and the mean *TMEM244* expression was 1,246E-6 ± 2,206E-6. In the samples with trace or no *TMEM244* expression (27E-6 ± 26E-6), the mean methylation level was markedly higher (85% ± 8.5). Based on the obtained results, a cut-off value for promoter hypomethylation was set at 70% and for *TMEM244* expression at 100E-6. Using these cut-offs, 11/12 samples with promoter hypomethylation expressed *TMEM244* and 25/27 samples with methylated promoter did not express *TMEM244* ($P < 0.000001$ in Fisher exact test; Table 1). Pearson correlation coefficient test showed a strong negative correlation between

TMEM244 expression and the square of its promoter methylation (Figure 2; $R = -0.7813$; $P < .00001$).

3.2 | In vitro demethylation of *TMEM244* promoter activates *TMEM244* expression

In order to prove the mechanism of *TMEM244* transcriptional activation by promoter demethylation, the CRISPR-dCas9-TET1 system was used for directed demethylation. In the first step, Jurkat cell line was transduced with the dCas9-Tet1 expressing vector. After selection, the expression of this fusion was confirmed on protein level using Western blot and anti-dCas9 antibody (Figure 3A). Secondly, cells were transduced with vectors expressing sgRNAs. sgRNAs were used separately, or in combination of 2 or 4, as described in the Section 2. The GFP marker was used to confirm sgRNAs expression. Cells were harvested for analysis at three different time points that were stated as passage 1, 3, and 5 post–antibiotic selection. At each time point, the protein level was evaluated and the quantitative analysis using stain-free technology showed that the expression of dCas9-TET1 fusion protein was stable over time (Figure 3B). At each time point, the expression of *TMEM244* mRNA was checked, as well as methylation level of three CpG dinucleotides in the promoter region. Overall, analysis of *TMEM244* expression and promoter methylation level of all samples, in all time points, showed that the specific sgRNAs with the dCas9-TET1 complex decreased DNA methylation level in the studied region and activated the expression of *TMEM244*. In contrast, inactive TET1 did not affect methylation of chosen CpG dinucleotides (Supplementary).

Four sgRNAs, targeting different sites in the promoter region, were used. Transduction with sg5 and sg6 resulted in a significant reduction of methylation level by 30% and 25% respectively. Acting together the decrease was only by 10%. No effect was detected for two other sgRNAs, sg7 and sg8, and also for two NTs. Decreased DNA methylation was accompanied by activated *TMEM244* expression in Jurkat cells. The expression was low; however in wild-type Jurkat (JWT), *TMEM244* expression was completely absent.

To check the correlation between *TMEM244* expression and promoter methylation, we performed the Pearson correlation coefficient test (Figure 4) for all dCas9-TET1 samples. The analysis showed that the level of *TMEM244* expression is negatively correlated with the square of the methylation level in the promoter region ($R = -0.4766$), and this correlation is highly significant ($P < 0.0002$) (Figure 4).

4 | DISCUSSION

In this study, we showed that methylation is a key regulatory mechanism of *TMEM244* expression. Samples with *TMEM244* expression, among them mostly SS and a few other T-cell leukaemia/lymphoma cases, had promoter region hypomethylated, while in all samples not expressing the gene, the promoter was methylated. The negative correlation between *TMEM244* expression and promoter methylation was confirmed, and the mechanism was verified using CRISPR-dCas9-TET1 system for directed demethylation of the specific sites in the promoter region. This approach has not been used in CTCL studies so far. The only methylation modification was performed using 5-aza-2'deoxycitidine, a pan-demethylating compound. Upon 5-aza treatment, a down-regulated expression of two tumour suppressors, *THBS4* and *PTPRG* were restored,[9] as well as a potential epigenetic diagnostic marker CMTM2[8] and miR200c involved in activation of Notch pathways in CTCL.[17] The advantage of using CRISPR-dCas9-TET1 approach is its specificity to the region of interest, without affecting global methylation patterns. Therefore, the observed expression activation can be directly correlated with the *TMEM244* promoter methylation changes introduced by TET1 protein. In our study, we managed to 'switch on' the expression in Jurkat cell line with no basal *TMEM244* expression by demethylation using TET1 fused to dCas9 and guided by sgRNAs. sgRNAs design is the crucial step for CRISPR-Cas9 technology. In our case, the best results were obtained for sgRNAs that were localized closely to CpGs sites, no further than 100 bp. Two sgRNAs situated > 100 bp form CpGs did not have the expected effect.

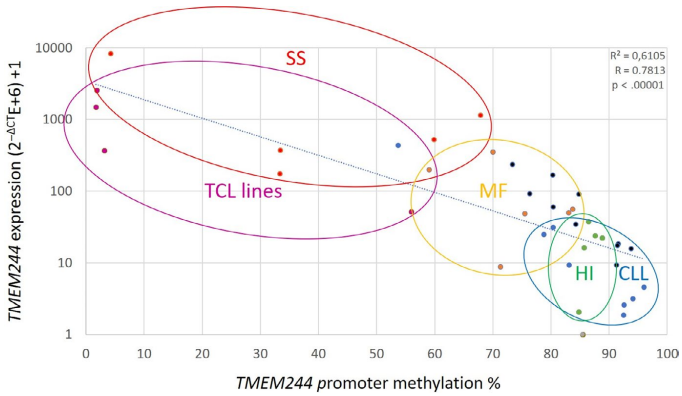

FIGURE 2 Correlation between promoter DNA methylation and *TMEM244* expression in lymphoid malignancies and in T-cell lines (SS: Sézary syndrome—red, MF: Mycosis fungoides—orange, CLL: chronic lymphoblastic leukaemia—blue, HI: healthy individuals—green, T-cell lymphoma cell lines—violet)

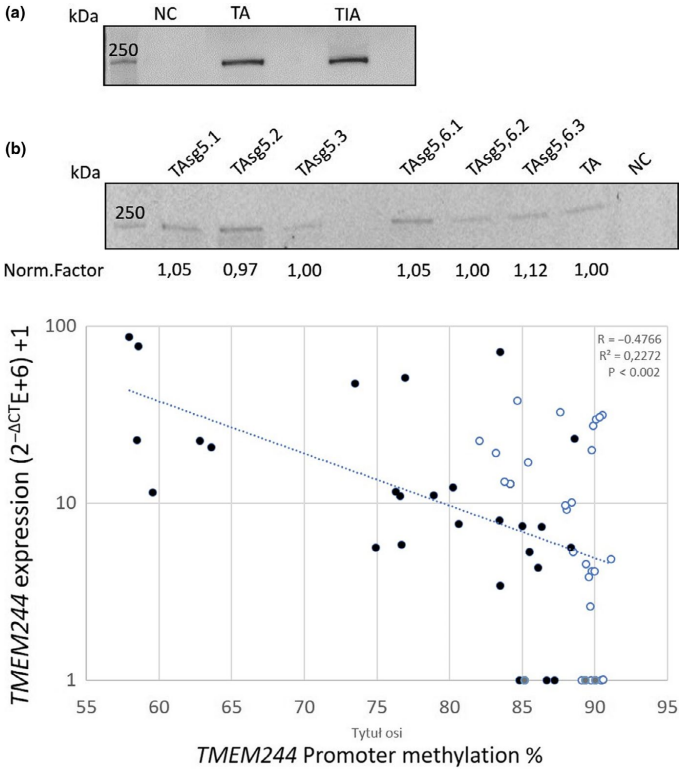

FIGURE 3 Western blot analysis of dCas9-TET1 expression. (a) Confirmation of dCas9-TET1 fusion protein expression in transduced Jurkat cells, (b) Quantitative analysis of dCas9-TET1 expression over time. NC: negative control (JWT); TA: TET1 active domain; TIA: TET1 inactive domain.; TAsg: TET1 active domain with single guided RNA; .1, .2, .3: time points

FIGURE 4 Correlation between methylation and *TMEM244* expression. Black dots: TET1 active; empty dots: TET1 inactive; grey dots: no sg

Contrary to the reports showing enhanced effect of combining two or more sgRNAs, in our hands two sgRNAs were less efficient than individual sgRNAs.

Genome-wide methylation analysis in CTCLs revealed that more CpG sites were hypomethylated than hypermethylated.[8,18] Hypomethylation leads to chromosomal instability and is often observed in cancer genomes. However, there are only two reports that actually describe a hypomethylation of specific genes in CTCLs. Wong et al described hypomethylation-mediated overexpression of *PLS3*, *GATA3* and *TWIST3*.[18] *GATA3* overexpression in CTCL was confirmed by Kamijo et al[19] The study showed that hypomethylation-mediated *GATA6* overexpression promotes tumour progression via overexpression of *CD137L* that together with *CD137* activates pathways leading to cell proliferation, tumour survival, growth and migration.

More studies were published on hypermethylated genes in CTCL, as they are often tumour suppressor genes involved in DNA repair, cell cycle, proliferation and apoptotic pathways. Hypermethylation in the promoter region, followed by decreased expression level, was detected for several tumour suppressors, including *CDKN2B* (p15), *CDKN2A* (p16) and *MGMT*,[20] *BCL7A*, *PTPRG* and *TP73* (p73)[9] and *RUNX3*/p46.[21] Promoter methylation not always resulted in gene silencing, and overexpression of *IL-15* in *CTCL* was actually associated with hypermethylation of the promoter, preventing binding of ZEB1 transcription repressor.[22]

Little is known about the *TMEM244* gene itself. It belongs to a family of transmembrane proteins (TMEMs) that are components of various membranes (cell membranes, mitochondrial, ER, lysosomal, Golgi membranes), present in different cells and fulfil important physiological functions. Many TMEMs are differentially expressed in different cancers.[23] So far, the role of *TMEM244* is unknown and no studies have been conducted in order to unravel its function. Although many RNAseq analysis has been performed for CTCLs samples,[4,24,25] only our team paid attention to that gene, probably due to its relatively low expression.

Our current results show that the expression of *TMEM244* gene is associated with T-cell lymphomas, especially with Sézary syndrome, and is a result of specific hypomethylation of its promoter. Since the expression of *TMEM244* and the hypomethylation of its promoter are specific to T-cell lymphoma, with the highest expression in SS, they could be used as a diagnostic marker in this type of CTCL.

ACKNOWLEDGEMENTS

This project was funded by the National Science Centre, decision No 2017/27/B/NZ5/01540 (KI, KR, MŻ, KN and GKP).

Supplementary Table1. Methylation analysis of 3 CpG dinucleotides localized in *TMEM244* promoter in patients, healthy donors and T-cell cell lines.

	CpG1 (%)	CpG2 (%)	CpG3 (%)	Mean (%)	SD
P1	35.59	34.7	30.11	33.47	2.94
P2	72.9	70.19	60.47	67.85	6.54
P3	62.12	60.92	56.54	59.86	2.94
SS1	4.35	3.55	4.95	4.28	0.70
SS2	34.52	34.21	31.37	33.37	1.74
MF1	63.14	59.18	54.72	59.01	4.21
MF2	76.11	71.17	62.68	69.99	6.79
MF2	89.02	85.58	76.65	83.75	6.38
MF2	83.41	74.06	56.45	71.31	13.69
MF3	84.73	82.83	81.48	83.01	1.63
MF3	80.43	77.01	69.16	75.53	5.78
TCL1	88.44	82.59	81.65	84.23	3.68
TCL2	89.35	66.84	63.89	73.36	13.93
TAL1	96.11	92.32	86.33	91.59	4.93
TAL2	95.35	92.28	53.24	80.29	23.48
TAL3 BL	81.02	75.69	72.38	76.36	4.36
TAL3 LN	95.55	93.9	64.93	84.79	17.22
CLL1	96.47	93.81	87.33	92.54	4.70
CLL2	97.62	95.67	94.6	95.96	1.53
CLL3	95.94	93.26	60.27	83.16	19.87
CLL4	94.49	92.04	90.93	92.49	1.82
CLL5	67.77	65.66	27.75	53.73	22.52
CLL6	96.53	93.78	91.96	94.09	2.30
CLL7	93.77	91.76	50.7	78.74	24.31
CLL8	95.37	92.24	53.58	80.40	23.28
HCL1	95.58	93.2	92.66	93.81	1.55
HCL2	86.04	82.61	72.43	80.36	7.08
BAL	95.95	92.19	85.69	91.28	5.19
BM1	94.97	92.32	87.02	91.44	4.05
C1	92.24	89.36	81.23	87.61	5.71
C2	94.34	90.69	81.45	88.83	6.64
C3	90.85	87.6	75.94	84.80	7.84
C4	92.45	88.25	76.45	85.72	8.30
C5	91.59	88.1	79.62	86.44	6.16
SeAx	1.5	1.13	3.07	1.90	1.03
HDLM2	0.65	1.1	3.42	1.72	1.49
Hut78	81.62	56.29	30.01	55.97	25.81
HH	3.45	2.63	3.42	3.17	0.47
Jurkat	97.01	95.51	63.91	85.48	18.69

Supplementary Table 2. Methylation of 3 CpG dinucleotides in *TMEM244* promoter in Jurkat cells with expression of dCas9-Tet1 active and dCas9-Tet1 inactive fusion protein, transduced with sgRNAs. measured at 3 time points.

Sample ID	CpG1	CpG2	CpG3	mean
Jurkat	95.76	94.26	77.98	89.33
Jurkat Tet active	96.39	90.23	68.89	85.17
Jurkat Tet inactive	97.27	94.85	78.01	90.04
Jurkat Tet active sg5 P1	75.25	65.44	37.96	59.55
Jurkat Tet active sg5 P3	74.12	68.24	46.01	62.79
Jurkat Tet active sg5 P5	68.62	60.44	44.76	57.94
Jurkat Tet inactive sg5 P1	97.42	92.3	62.85	84.19
Jurkat Tet inactive sg5 P3	96.68	93.22	59.71	83.20
Jurkat Tet inactive sg5 P5	96.91	91.45	65.62	84.66
Jurkat Tet active sg6 P1	73.99	69.5	47.25	63.58
Jurkat Tet active sg6 P3	70.8	63.36	41.3	58.49
Jurkat Tet active sg6 P5	70.19	61.59	43.99	58.59
Jurkat Tet inactive sg6 P1	96.53	92.28	62.46	83.76
Jurkat Tet inactive sg6 P3	97.3	93.79	65.12	85.40
Jurkat Tet inactive sg6 P5	95.77	90.83	59.64	82.08
Jurkat Tet active sg7 P1	95.81	90.97	63.65	83.48
Jurkat Tet active sg7 P3	99.57	90.89	59.86	83.44
Jurkat Tet active sg7 P5	96.03	91.2	63.27	83.50
Jurkat Tet inactive sg7 P1	97.66	95.48	76.23	89.79
Jurkat Tet inactive sg7 P3	97.56	94.24	77.02	89.61
Jurkat Tet inactive sg7 P5	97.35	93.77	80.38	90.50
Jurkat Tet active sg8 P1	99.05	97.73	40	78.93
Jurkat Tet active sg8 P3	93.2	87.13	61.51	80.61
Jurkat Tet active sg8 P5	92.56	83.63	64.52	80.24
Jurkat Tet inactive sg8 P1	97.98	95.08	80.3	91.12
Jurkat Tet inactive sg8 P3	97.42	93.71	80.69	90.61
Jurkat Tet inactive sg8 P5	97.64	92.65	79.06	89.78
Jurkat Tet active nt3 P1	96.63	92.98	69.44	86.35
Jurkat Tet active nt3 P3	97.28	90.78	67.04	85.03
Jurkat Tet active nt3 P5	97.54	89.45	67.49	84.83
Jurkat Tet inactive nt3 P1	98.05	94.29	77.6	89.98
Jurkat Tet inactive nt3 P3	97.23	94.12	76.84	89.40
Jurkat Tet inactive nt3 P5	97.42	92.45	79.71	89.86
Jurkat Tet active nt4 P1	96.38	93.24	75.45	88.36

Jurkat Tet active nt4 P3	96.49	91.43	70.34	86.09
Jurkat Tet active nt4 P5	97.65	91.56	72.46	87.22
Jurkat Tet inactive nt4 P1	97.9	94.9	78.66	90.49
Jurkat Tet inactive nt4 P3	97.29	94.04	77.77	89.70
Jurkat Tet inactive nt4 P5	96.67	92.97	79.53	89.72
Jurkat Tet active sg5.6.7.8 P1	86.38	82.53	61.15	76.69
Jurkat Tet active sg5.6.7.8 P3	83.02	79.31	62.38	74.90
Jurkat Tet active sg5.6.7.8 P5	84.69	79.99	66.18	76.95
Jurkat Tet inactive sg5.6.7.8 P1	95.71	93.12	75.34	88.06
Jurkat Tet inactive sg5.6.7.8 P3	97.07	92.88	75.54	88.50
Jurkat Tet inactive sg5.6.7.8 P5	96.56	92.11	74.21	87.63
Jurkat Tet active sg5.6 P1	82.18	79.75	67.85	76.59
Jurkat Tet active sg5.6. P3	81.7	77.27	69.94	76.30
Jurkat Tet active sg5.6. P5	79.83	73.95	66.69	73.49
Jurkat Tet inactive sg5.6 P1	97.36	93.98	73.95	88.43
Jurkat Tet inactive sg5.6 P3	97.61	92.56	73.74	87.97
Jurkat Tet inactive sg5.6 P5	97.06	93.94	79.24	90.08
Jurkat Tet active nt3.4 P1	96.89	89.32	70.23	85.48
Jurkat Tet active nt3.4 P3	96.82	92.5	70.67	86.66
Jurkat Tet active nt3.4 P5	96.07	90.76	78.97	88.60
Jurkat Tet inactive nt3.4 P1	97.22	94.4	75.67	89.10
Jurkat Tet inactive nt3.4 P3	97.96	94.05	79.32	90.44
Jurkat Tet inactive nt3.4 P5	97.87	93.13	79.96	90.32
Methylated control 1	97.86	97.15	95.39	96.80
Unmethylated control 1	15.44	9.57	4.19	9.73
Methylated control 2	98.14	96.36	95.09	96.53
Unmethylated control 2	13.06	7.69	2.96	7.90
Methylated control 3	97.5	95.6	98.26	97.12
Unmethylated control 3	16.09	9.2	3.77	9.69

Supplementary Table3. *TMEM244* expression (2^-ΔCT) in Jurkat cells with expression of dCas9-Tet1 active and dCas9-Tet1 inactive fusion protein, transduced with sgRNAs. measured at 3 time point.

	1st	2nd	3rd
J WT	0.0E-6	0	0
TET AC	0.0E-6	0	0
TET AC sg5	10.5	21.5	86.1
TET AC sg6	19.7	21.6	76.1
TET AC sg7	2.4	7.0	70.4
TET AC sg8	10.0	6.6	11.2
TET AC sg5.6.7.8	4.8	4.6	50.2
TET AC sg5.6	9.9	10.6	46.2
TET AC NT3	6.3	6.4	0.0
TET AC NT4	4.6	3.3	0.0
TET AC NT3/4	4.3	0.0	22.1
TET IN	0.0	0.0	0
TET IN sg5	11.8	18.1	36.9
TET IN sg6	12.2	16.0	21.5
TET IN sg7	3.1	2.8	30.3
TET IN sg8	3.8	0.0	18.9
TET IN sg5.6.7.8	8.2	4.3	31.6
TET IN sg5.6	9.1	8.7	28.7
TET IN NT3	3.1	3.5	26.2
TET IN NT4	0.0	1.6	0.0
TET IN NT3/4	0.0	0.0	29.5

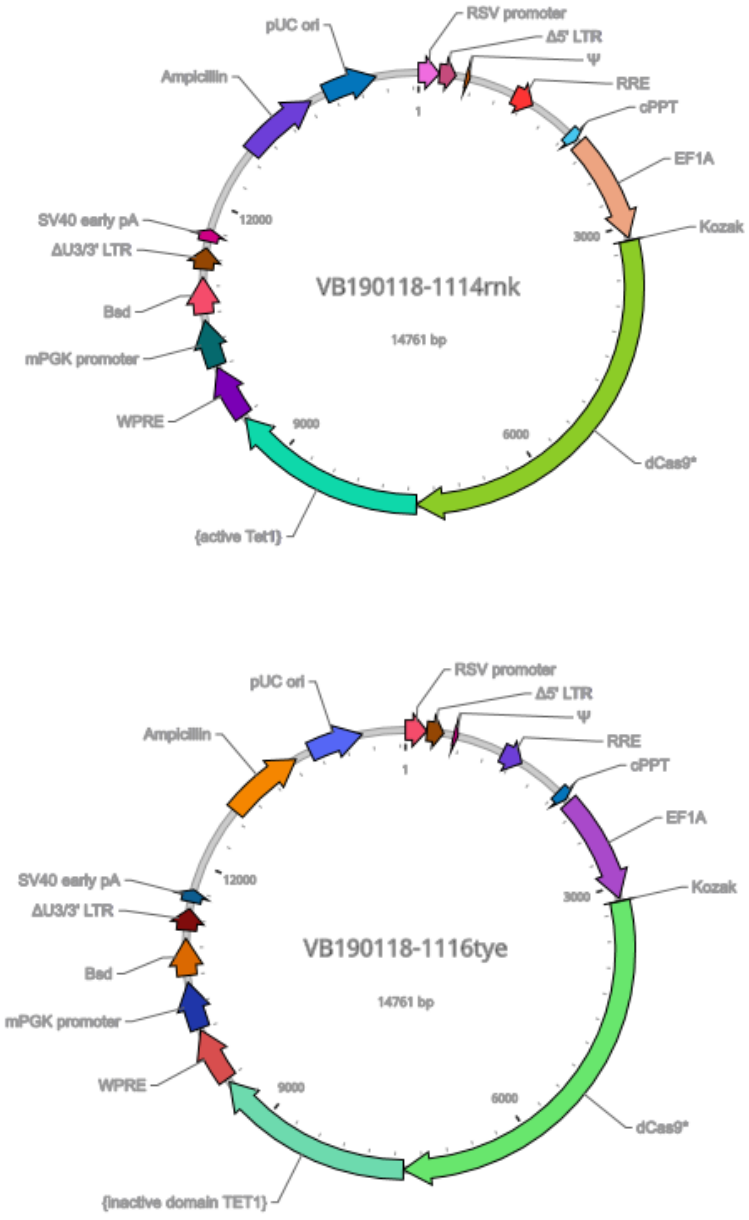

Figure 1. Map of two lentiviral CRISPR-dCa9 vectors: pLV[Exp]-Bsd-EF1A>dCas9*:active TET1 (ID: VB190118-1114rnk) and pLV[Exp]-Bsd-EF1A>dCas9*:inactive domain TET1 (VB190118-1116tye).

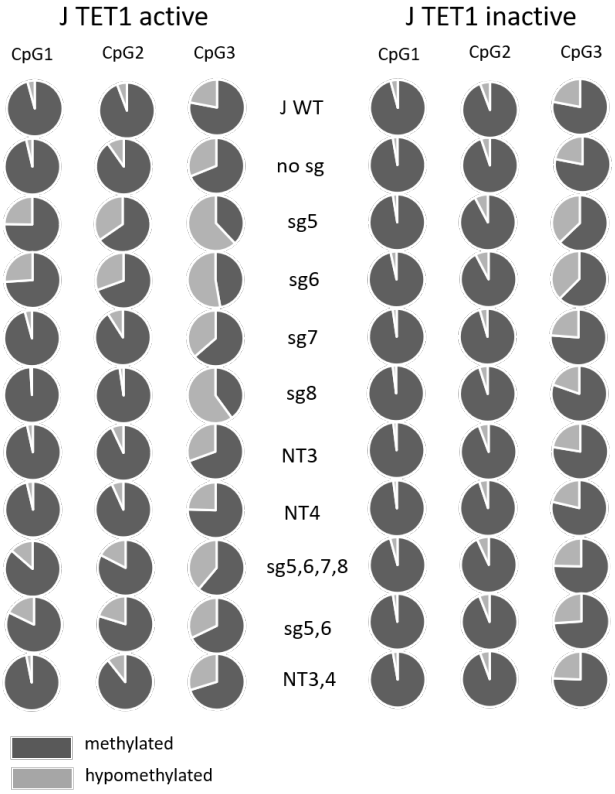

Figure 2. Comparison of methylation of three CpG dinucleotides between samples transduced with active and inactive TET1 domain and different sgRNAs. in the 1st time point.

2nd ARTICLE ENTITLED:

***TMEM244* is a long non-coding RNA necessary for CTCL cell growth**

Karolina Rassek, Katarzyna Iżykowska, Magdalena Żurawek, Monika Pieniawska, Karina Nowicka, Xing Zhao, Grzegorz Krzysztof Przybylski

MDPI International Journal of Molecular Sciences, 2023, 24(4), 3531.

Background: *TMEM244* gene was originally reported by prof. Przybylski group to be ectopically expressed in SS, however, the function of this gene in cells has been unknown (Izykowska et al., 2017). *TMEM244* gene was annotated to the TMEM family yet, the expression of the TMEM244 protein has not been experimentally confirmed. In the previous paper we demonstrated a mechanism of *TMEM244* activation (Izykowska et al., 2020). Because *TMEM244* overexpression was demonstrated to be a characteristic feature of SS patients, and associated with poor overall survival in T-cell lymphoma patients, we have hypothesized that it can be also crucial in the pathogenesis of CTCL (Chen et al., 2022, Izykowska et al., 2020). To our knowledge, this is the first study focusing on establishing *TMEM244* function.

Results: To identify a protein encoded by the *TMEM244* gene, first we examined its expression using qRT-PCR in different CTCL (SeAx, HH) and non-CTCL cell lines (HDLM2, D341med, and COLO684). For that purpose, we used cell lines with predicted high levels of *TMEM244* transcript as well as established Jurkat cell lines with induced *TMEM244* overexpression. Upon confirmation of *TMEM244* expression on the mRNA level, we used the Western Blot method to examine the expression of TMEM244 protein. Because our lentiviral construct used for establishing TMEM244 expression in cell lines included a FLAG tag sequence, for protein detection we used anti-flag and anti-TMEM244 antibodies. Based on the Human Protein Atlas database, for Western Blot experiments, we have added brain lysate with a high expression of *TMEM244* mRNA. Because TMEM244 protein was not found, we performed *in silico* analysis using the CPAT tool, which showed a very low coding probability of *TMEM244* (Wang et al., 2013). In the next step, we decided to establish the subcellular localization of the *TMEM244* transcript. Based on the bioinformatic localization predictor, we have foreseen the *TMEM244* transcript to be localized mainly in the cytoplasm and partially in the nucleus. By using subcellular fractionation followed by qRT-PCR and FISH analysis, we confirmed this prediction in SeAx and HDLM2 cell lines revealing higher TMEM244 transcript expression in

the cytoplasm (SeAx=67%; HDLM2=55%) than in the nucleus (SeAx=29%; HDLM2=39%). Because *TMEM244* overexpression was found exclusively in CTCL patients, we have decided to check whether *TMEM244* influences the growth of CTCL cell lines. To address that issue, we have transduced two CTCL cell lines with shRNAs targeting its transcript and performed a GFP competition assay. We have found a significant decrease in the growth of the GFP+ cell population for all constructs in both CTCL cell lines compared to scrambled and non-targeting constructs. This inhibitory effect may be at least partially mediated by apoptosis, which in our flow cytometry analysis showed a slight difference in the number of apoptotic cells in both cell lines. As the final step, we performed RACE PCR to search for alternative transcripts of *TMEM244*. The experiment revealed two novel transcripts with no protein-coding potential.

Conclusions: In this study, we have demonstrated that despite cellular localization, *TMEM244* does not encode a protein, but rather belongs to the long non-coding RNA group. Moreover, our results showed that *TMEM244* expression is necessary for the growth of CTCL cells. We have also demonstrated two new transcript variants with no significant coding potential and unknown function.

KR contribution to this article

- cell lines culturing
- viral transductions
- FISH assays
- RNA isolations
- reverse transcriptions and qRT-PCRs
- Western Blot experiments
- expression data analyses
- most GFP competition assays
- most apoptosis assays
- figures preparation
- writing the manuscript draft and preparing the final manuscript version.

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Article

TMEM244 Is a Long Non-Coding RNA Necessary for CTCL Cell Growth

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Abstract: Transmembrane protein 244 (TMEM244) was annotated to be a member of the TMEM family, which are is a component of cell membranes and is involved in many cellular processes. To date, the expression of the TMEM244 protein has not been experimentally confirmed, and its function has not been clarified. Recently, the expression of the *TMEM244* gene was acknowledged to be a diagnostic marker for Sézary syndrome, a rare cutaneous T-cell lymphoma (CTCL). In this study, we aimed to determine the role of the *TMEM244* gene in CTCL cells. Two CTCL cell lines were transfected with shRNAs targeting the *TMEM244* transcript. The phenotypic effect of *TMEM244* knockdown was validated using green fluorescent protein (GFP) growth competition assays and AnnexinV/7AAD staining. Western blot analysis was performed to identify the TMEM244 protein. Our results indicate that *TMEM244* is not a protein-coding gene but a long non-coding RNA (lncRNA) that is necessary for the growth of CTCL cells.

Keywords: *TMEM244*; Sézary syndrome; CTCL; lncRNA

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1. Introduction

The transmembrane protein (TMEM) family comprises proteins that are embedded in the cell membrane and span both intracellular and extracellular environments. TMEMs are components of various cell membranes, such as mitochondrial membranes, Golgi membranes, lysosomes, and the endoplasmic reticulum [1]. They are involved in many cellular processes, such as the transport of ions and molecules across impermeable membranes, membrane trafficking, and signaling transduction pathways [2]. Studies showed that TMEMs' expression can be down- or upregulated in several cancers [3,4] and is associated with tumor progression, disease stage, and patient survival [5,6]. Because membrane proteins are involved in essential cellular pathways, they are often targets of pharmaceutical agents [7]. Yet, for most TMEMs, the mechanism of their involvement in carcinogenesis is still unknown.

The *TMEM244* gene is located in chromosome 6q22.33 and comprises five exons. Under physiological conditions, *TMEM244* is expressed at a low level in the brain and the pituitary glands. To date, the existence of the TMEM244 protein has not been experimentally demonstrated, but the gene is supposed to encode a protein of 128 amino acids and a molecular mass of 14,657 Da. Izykowska et al., were the first to pay attention to the *TMEM244* gene, as it was identified among four other genes (*EHD1*, *MTMR2*, *RNF123*, and *TOX*) to be involved in the rearrangements affecting gene expression in Sézary syndrome (SS) patients compared to controls [8]. Further studies showed that *TMEM244* is expressed in T-cell lymphomas as a result of specific hypomethylation of its promoter, and this expression is associated with poor overall survival in T-cell lymphoma patients [9,10]. A significantly higher expression of *TMEM244* was identified in Sézary syndrome

patients, not only compared to healthy individuals but also to SS clinical mimickers, such as mycosis fungoides and erythrodermic manifestations of non-malignant diseases, therefore indicating its diagnostic potential. Moreover, higher expressions of *TMEM244* in CD4+ and CD8+ subsets of memory cells (CD45RO+) were identified, which is in line with the immunophenotype of Sézary cells [11]. The purpose of this study was to establish the function of the *TMEM244* gene, which has not been investigated yet.

2. Results

2.1. TMEM244 Has a Low Protein-Coding Potential and Cannot Be Detected at the Protein Level

For the detection of the TMEM244 protein, two CTCL cell lines were examined: SeAx and HH, as well as other non-CTCL cell lines, HDLM2, D341med, and COLO684, with predicted high levels of the TMEM244 transcript.

A high level of *TMEM244* expression in the Jurkat cell line was induced using two lentiviral systems with different promoters (human cytomegalovirus; CMV or 3-phosphoglycerate kinase; PGK). FLAG tags were introduced either at the N- or C-terminus of the *TMEM244* gene. *TMEM244* expression on the mRNA level, as determined using RT-qPCR, was the highest in Jurkat-CMV (mean = $390 \times 10^3 \pm 102 \times 10^3$) and Jurkat-PGK (mean = $190 \times 10^3 \pm 69 \times 10^3$). In cell lines with endogenous *TMEM244* expression, the highest level was detected in D341med (mean = $180 \times 10^3 \pm 67 \times 10^3$), followed by COLO684 (mean = $59 \times 10^3 \pm 16 \times 10^3$), SeAx (mean = $2.5E \times 10^3 \pm 0.82 \times 10^3$), HDLM2 (mean = $2.4 \times 10^3 \pm 0.88 \times 10^3$), and HH (mean = $0.7 \times 10^3 \pm 0.2 \times 10^3$) (Figure 1). Upon confirmation of the expression of *TMEM244* in selected cell lines, Western blot analysis using a custom-made anti-TMEM244 antibody and an anti-FLAG antibody, with a wild-type cell line used as a control, was performed.

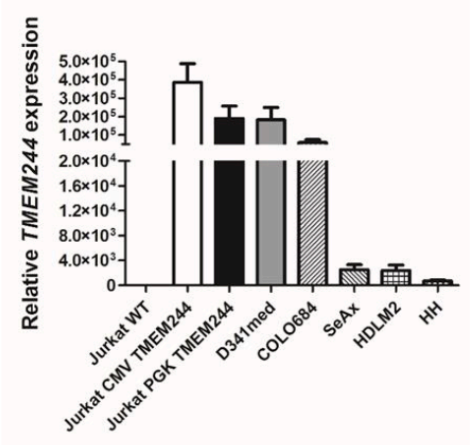


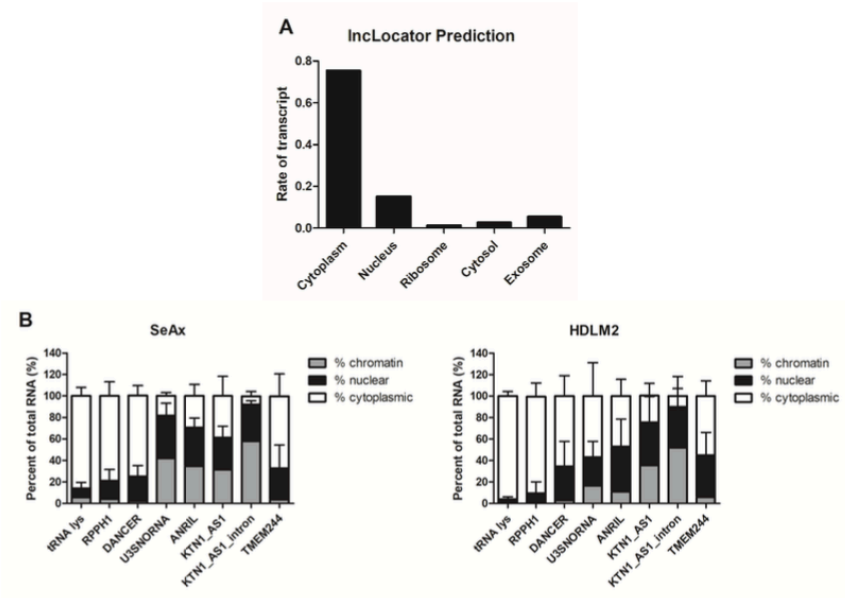
Figure 1. Relative *TMEM244* expression analyzed using RT-qPCR in cell lines with endogenous expression and in cell lines stably transduced with *TMEM244* ORF vectors. The expression was normalized to beta-2-microglobulin. Data are represented as mean ± SD ($n = 3$).

Moreover, commercially available brain lysate was analyzed for TMEM244 protein expression, as according to the Human Protein Atlas database *TMEM244* is expressed on the mRNA level in the brain (https://www.proteinatlas.org/ENSG00000203756-TMEM244/tissue; accessed on 30 January 2020). All Western blot analyses showed signals from positive controls, but they failed to detect the TMEM244 protein (Figure S1). The TMEM244 protein was neither detected with the anti-FLAG antibody in cell lines with

induced *TMEM244* overexpression (Figure S1A) nor with the specific anti-TMEM244 antibody in the same cell lines, and it was not detected in cell lines or brain tissue with high endogenous *TMEM244* expression (Figure S1B,C). Furthermore, *in silico* analysis showed a very low coding probability of *TMEM244* [0.097], with a cutoff <0.364 indicating a noncoding sequence [12]. Taken together, these results indicate that *TMEM244* is not expressed at the protein level.

2.2. TMEM244 Transcript Is Primarily Localized in the Cytoplasm

Using the long non-coding RNA subcellular localization predictor (lncLocator; http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/; accessed on 2 September 2021), the *TMEM244* transcript was predicted to be present, mainly in the cytoplasm and partially in the nucleus (Figure 2A). To confirm this prediction, subcellular fractionation was performed in SeAx and HDLM2 cell lines and FISH RNA analysis in SeAx and HDLM2 cells. In both cell lines, the level of the *TMEM244* transcript in the cytoplasm (SeAx = 67%; HDLM2 = 55%) was higher than that observed in the nucleus (SeAx = 29%; HDLM2 = 39%) (Figure 2B). FISH results further confirmed that in SeAx and HDLM2 cells, the prominent distribution of *TMEM244* was in the cytoplasm (Figure 2C).



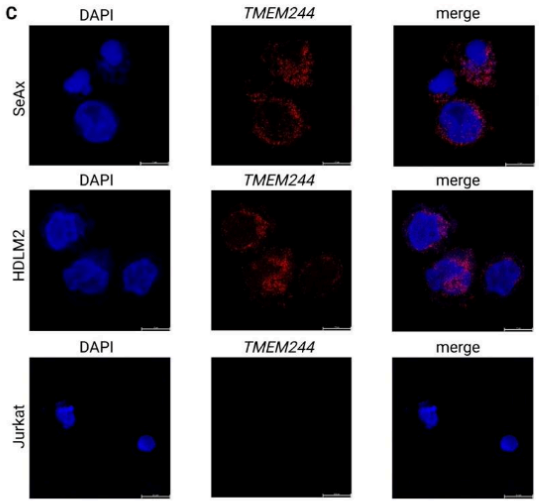


Figure 2. Localization of *TMEM244* transcript. (**A**) lncLocator prediction; (**B**) subcellular fractionation and RT-qPCR analysis of *TMEM244* expression in SeAx and HDLM2 cells; tRNA lys, RPPH1, and DANCER were used as cytoplasmic controls; U3SNORNA and ANRIL-nuclear controls; KTN1_AS1_ and KTN1_AS1_intron-chromatin controls. The mean values ± SD of 3 independent experiments are shown (**C**) FISH analysis of *TMEM244* in SeAx, HDLM2 and Jurkat cells (negative control); *TMEM244* FISH signal in red, DAPI counterstain in blue.

2.3. Inhibition of TMEM244 Results in Decreased Cell Growth in CTCL Cell Lines

To establish the function of *TMEM244* in cancer cells, the effect of *TMEM244* knockdown was analyzed in SeAx and HH cell lines with endogenous *TMEM244* expression. The effectiveness of the shRNAs targeting the *TMEM244* transcript was confirmed for both cell lines (Figure S2). *TMEM244* silencing resulted in a strong negative effect on cell growth. On day 22 after transduction, the ratio of GFP-positive cells decreased by more than 50% in the cell lines treated with *TMEM244*-specific shRNAs compared to non-targeting and scrambled controls (Figure 3).

The knockdown of *TMEM244* decreased in the GFP+ cell population for all constructs in both CTCL cell lines. In HH, all shRNAs showed a strong effect, with a reduction of 57%, 55%, and 68% for shRNA 1, shRNA 2, and shRNA 3, respectively, compared to SCR and NT. The effects on the growth of SeAx cells were stronger for shRNA 2 and shRNA 3, with a reduction of 78% and 74% compared to SCR, and only a mild effect was observed for shRNA 1 (36%).

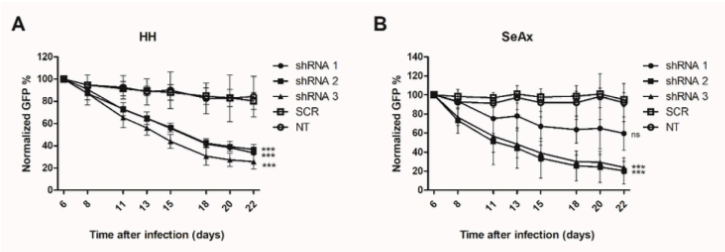


Figure 3. Green fluorescent protein (GFP) growth competition assay with shRNAs targeting *TMEM244* in (**A**) SeAx and (**B**) HH. The effect of *TMEM244* knockdown on cell growth was assessed

by following the percentage of GFP+ cells for 22 days post-transduction, with the GFP percentage normalized to day six ($n = 3$); *** $p < 0.001$, ns—non-significant, based on mixed model analysis; NT—non-targeting, SCR—scrambled.

To further investigate the mechanism of growth inhibition upon *TMEM244* knockdown on cancer cells, Annexin V/7AAD staining was conducted (Figure 4).

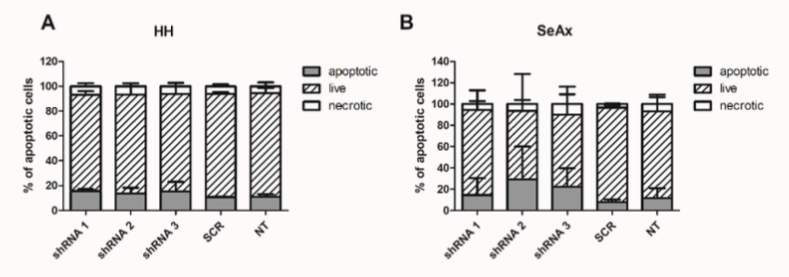


Figure 4. Cell viability upon *TMEM244* knockdown in (**A**) HH; (**B**) SeAx cells. The percentages of apoptotic, live, and necrotic cells were determined using flow cytometry with Annexin V/7AAD staining. The mean values ± SD of 3 independent experiments are shown. NT—non-targeting, SCR—scrambled.

Only a slight, statistically non-significant difference in the number of apoptotic cells in the HH cell line was detected. In SeAx, although also non-significant, the effect was stronger, with an increase of 7% and 3% using shRNA 1; 21%, and 18% using shRNA 2; and 14% and 11% using shRNA 3, compared to SCR and NT, respectively.

2.4. Identification of Novel Alternative Transcripts of TMEM244

This study led to the identification of two novel alternative *TMEM244* transcripts. Besides the two known transcripts—variant one with 5 exons (RefSeq NM_001010876; ENST00000368143.6) and variant two with an extra 5′ exon (ENST00000438392.2), available in the Genome Browser—two additional variants were identified: variant three without exon 4 and variant four without exons 2 and 3 (Figures 5A and S4). The expression profile of each splice variant in cell lines with *TMEM244* expression (SeAx, HH, Hut78, HDML2, and D341med) was performed using RT-qPCR with variant-specific primers. In most cell lines, the expression level was as follows; variant1 > variant2 > variant3 > variant4, except for D341med, where the expression of variant three was higher than that of variant two (Figure 5B).

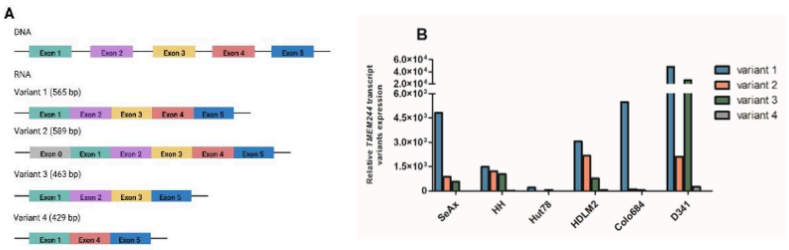


Figure 5. *TMEM244* transcript variants identified with RACE method. (**A**) Transcript variant length scheme; (**B**) relative expression of *TMEM244* transcript variants analyzed by RT-qPCR.

To assess the coding potential of each transcript variant, the *in silico* analysis was performed using CPAT. The analysis confirmed that neither of the detected variants had

the potential to encode a protein. The coding potential was 0.0976, 0.0003, 0.0359, and 0.0043 for variants one, two, three, and four, respectively.

3. Discussion

*TMEM*s are a very heterogeneous group of more than 300 genes, which have been included based on the *in silico* analysis of their DNA sequence. To be annotated as a *TMEM* gene, the predicted protein structure must contain at least one putative transmembrane segment that spans completely or partially through biological membranes (Guo et al. [13]). Some *TMEMs* have been experimentally shown to encode a protein and, upon functional characterization, have been renamed and reclassified (Marx et al. [14]) . Still, for many of them, including *TMEM244*, neither the protein nor the function have been experimentally demonstrated.

Our study demonstrated that despite its annotation, based only on the in silico analysis of the predicted protein structure, the *TMEM244* gene does not seem to encode a protein but, rather, belongs to the long-non-coding RNA (lncRNAs) family. LncRNAs are defined as ≥200 nucleotides long RNAs that are spliced and polyadenylated like mRNAs; however, they lack protein-coding activity. *In silico* analysis revealed minor coding potential of the 545 nucleotides long *TMEM244* transcript. *TMEM244* has an open reading frame (ORF) but, as shown for other lncRNAs, such as LINC00116 or LINC00948, possessing an ORF does not determine protein production [15,16]. *TMEM244*, like lncRNAs, is poorly conserved and its expression level is lower compared to protein-coding genes [17]. In addition, the *TMEM244* mRNA level is highest in the brain and pituitary glands, which is typical for lncRNAs.

While mRNAs are very specifically located on the ribosomes in the cytoplasm, lncRNAs may occupy diverse sites, including chromatin, subnuclear domains, nucleoplasm, and cytoplasm [18]. Furthermore, in tumors, the cellular localization of lncRNAs is related to their functions. LncRNAs located in the nuclear compartment usually control transcription and post-transcriptional processing. Since *TMEM244* is mainly located in the cytoplasm, this suggests its involvement in the regulation of translation, mRNA turnover, protein stability, sponging of cytosolic factors, and the modulation of signaling pathways [15,19].

Recently, emerging evidence showed that lncRNAs could promote cell proliferation and, therefore, be engaged in carcinogenesis. For instance, the lncRNA HOXD cluster antisense RNA 1 (*HOXD-AS1*) was upregulated and promoted cell proliferation in cervical cancer, while lncRNA *EPIC1* promoted proliferation and inhibited apoptosis of gallbladder cancer cells [16,20]. LncRNAs can also affect apoptosis by acting as a competitive endogenous RNA (ceRNA) for miRNA and binding to the sequence at the 5′ end of the miRNA, therefore reducing target mRNA expression and ultimately affecting cell apoptosis. Moreover, lncRNAs can act directly or indirectly on death receptors [16]. Previous studies have demonstrated that in some cases, a non-protein-coding locus can give rise to functionally distinct transcript isoforms [21–24]. Recently, it was shown that the switch in the lncRNA HOTAIR start site after the induction of differentiation promotes the inclusion of HOTAIR exon 3, containing a protein-binding domain, which likely changes its function [25]. We showed that *TMEM244* is necessary for the growth of cells where its expression is at a relatively high level, such as in CTCL cell lines. However, the mechanism behind this observation is still unknown.

It is, however, worth mentioning that the experiments were focused on the sense strand of *TMEM244* based on the GenBank (NCBI) transcription annotation (Gene ID: 253582), and it is not known if the anti-sense strand of *TMEM244* is expressed or whether it plays any role in cell proliferation.

4. Materials and Methods

4.1. Cell Lines

Seven established cell lines were included in the study. Four were lymphoid cell lines: HH—established from an aggressive cutaneous T-cell leukemia/lymphoma patient (ATCC CRL-2105), SeAx—the Sézary syndrome cell line, kindly provided by Markus Möbs [26], Jurkat—a T-cell acute lymphoblastic leukemia (T-ALL) cell line (88042803; Merck KGaA, Darmstadt, Germany), and HDLM2—a T-cell Hodgkin lymphoma cell line (DSMZ ACC17). In addition, three non-lymphoid cell lines were used: D341 med—a medulloblastoma cell line (ATCC HTB-187), COLO 684—human uterus adenocarcinoma (ECACC 87061203), and HEK293T (DSMZ ACC 635). CTCL and COLO 684 cell lines were cultured in a HEPES-buffered RPMI1640 medium with L-glutamine (Thermo Fisher Scientific™, Waltham, MA, USA), 10–20% fetal bovine serum (FBS) (Merck KgaA, Darmstadt, Germany), and 1% penicillin/streptomycin (Thermo Fisher Scientific™, Waltham, MA, USA), according to the manufacturer's instructions. The medium for SeAx was supplemented with Il-2 (200 U/mL) (Merck KgaA, Darmstadt, Germany) and the medium for Jurkat with 1% sodium pyruvate (1 mmol/L) and 0.25% glucose (0.5 g/L) (Thermo Fisher Scientific™, Waltham, MA, USA). The D341 med cell line was cultured in Eagle's Minimum Essential Medium (ATCC 30-2003™), supplemented with 20% FBS (Merck KgaA, Darmstadt, Germany), according to the manufacturer's protocol. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland) with 10% FBS (Merck KgaA, Darmstadt, Germany) and 1% penicillin/streptomycin (Thermo Fisher Scientific™, Waltham, MA, USA).

4.2. Fluorescence In Situ Hybridization (FISH) Assay

Fluorescence-labeled probes for *TMEM244* and *GAPDH* RNA were designed and synthesized, and FISH experiments were performed according to the manufacturer's protocol, using the Stellaris™ FISH technology kit (Biosearch Technologies, Hoddesdon, UK). Twenty Quasar® 570-labeled probes for the *TMEM244* transcript were designed using Stellaris® Probe Designer version 4.2 (LGC Biosearch Technologies, Berlin, Germany). The nuclei were stained with DAPI, and Human GAPDH with the Quasar® 570 Dye Stellaris® FISH Probe was used as a cytoplasmic marker (Figure S3). Images were acquired using a Leica DMI8 laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Cells were imaged with an HC PL APO CS2 100×/1.40 oil objective lens and processed using Leica Application Suite X software (Leica Microsystems, Wetzlar, Germany). All samples were imaged under the same optical conditions.

4.3. Generation of Cells with Knockout or Stable Expression of TMEM244

In brief, lentiviral vectors were co-transfected with 3rd-generation packaging plasmids—pMSCV-VSV-G, pRSV.REV, and pMDL-gPRRE—into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific™, Waltham, MA, USA). At 24 h post-transfection, the medium was replaced. At 48 and 72 h post-transfection, viral supernatant was collected, sterile filtered through a 0.45 µm syringe filter, and stored at −80 °C. For *TMEM244* knockdown miRZIP, KLHL6 plasmid was used. Three short hairpins RNAs were designed using the Broad Institute program (https://portals.broadinstitute.org/gpp/public/seq/search; accessed on 13 December 2021) to knock down TMEM244 (shRNA 1 on exon 3, sense: GATCCATCCCTCATGGCTCAACATAATTCAAGAGATTATGTTGAGCCATGAGGGATTTTTTG, antisense: AATTCAAAAAATCCC TCATGGCTCAACATAATCTCTTGAATTATGTTGAGCCATGAGGGATG; shRNA 2 on exon 4, sense: GATCCGAAGAATGGGTTTGGGATTATTTCAAGAGAATAATCCC AAACCCATTCTTCTTTTTG, antisense: AATTCAAAAAGAAGAATGGGTTTGGGATTATTCTCTTGAAATAATCCCAAACCCATTCTTCG; shRNA 3 on exon 5, sense: GATCCGTGGGCTGCTTTAGGTATATCTTCAAGAGAGATATACCTAAAGCAGCCC

ACTTTTTG, antisense: AATTCAAAAAGTGGGCTGCTTTAGGTATATCTCTCTTGAAGATATACCTAAAGCAGCCCACG) (Figure S5). Control NT2 and SCR vectors were a kind gift from Prof. Anke van den Berg and Dr. Joost Kluiver [27]. Jurkat cells were transduced with lentiviral vectors: pLV-CMV-Tmem244 (flag, 6xHis), pLV-hPGK-Tmem244 (flag, 6xHis), and pLV_flag_CMV_Tmem244. Virus supernatant was added to cells together with polybrene (4 μg/mL). Seax, HH, and D341med cells were transduced with lentiviral vector miRZIP KLHL6. To validate the *TMEM244* overexpression level, cells were infected, aiming at an infection percentage of >70%. To establish a pure population of cells, selection with puromycin was performed for 5–7 days (2 μg/mL). The efficiency of the transduction was measured by flow cytometry using the green fluorescent protein (GPF) signal. Cells were harvested for RNA and protein.

4.4. Western Blot

Whole cell lysates were prepared from 5–10 × 10^6 cells. Cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer (Merck KgaA, Darmstadt, Germany) containing 1X protease inhibitor cocktail (Bioshop Canada Inc., Burlington, ON, Canada) for 30 min on ice. Samples were centrifuged at 14,000× *g* for 30 min to remove DNA or debris. As an additional control, MG132 proteosome inhibitor (Merck KgaA, Darmstadt, Germany) was added to the cell cultures to prevent possible TMEM244 degradation. After 6 h incubation with 1 μM MG132 or 4 h incubation with 20 μM of MG132, cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer (Merck KgaA, Darmstadt, Germany) containing 1X protease inhibitor cocktail (Bioshop Canada Inc., Burlington, ON, Canada) for 30 min on ice. Samples were then sonicated (3 cycles, ON 20 s, OFF 30 s). Total protein concentrations of the cell extracts were measured using the Pierce BCA Protein Quantitation kit (Thermo Fisher Scientific™, Waltham, MA, USA), and the samples were stored at −80 °C until assayed. Prior to loading on gel, samples were heated at 95 °C for 5 min in a heating block. A synthetic peptide-fragment of the putative TMEM244 protein used for mice immunization was used as a positive control. Not centrifuged and nonheated proteins were used as controls for the sample preparation procedure.

Human brain whole tissue lysate was commercially available (Novus Biologicals LLC a Bio-Techne Brand, Centennial, CO, USA). Western blotting was performed as previously described [9]. Primary antibodies (anti-FLAG (F1804, 1:1000, Merck KGaA, Darmstadt, Germany), anti-TMEM244 (custom-made; 1:000, Proteogenix, Schiltigheim, France)) were used, as well as HRP-labeled secondary antibodies (sc-2005, 1:10,000, Santa Cruz Biotechnology, Dallas, TX, USA). The signal was detected by chemiluminescence with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) using ChemiDoc™ Imaging Systems (Bio-Rad, Hercules, CA, USA). Quantitative analysis was performed using ImageLabTM Software. The WB results were normalized using a stain-free technique, by measuring total protein directly on the WB membrane.

4.5. GFP Competition Assay

SeAx and HH cells were infected with miRZIP lentivirus, aiming at the infection percentage of 50%. The percentages of GFP-positive cells were measured using the flow cytometry (CytoFLEX S Flow Cytometer, Beckman Coulter, Indianapolis, IN, USA) on day 4 post-transduction and monitored tri-weekly for three weeks. Data were analyzed using Kaluza Analysis Software (Beckman Coulter, Indianapolis, IN USA). To determine the effect on cell growth, the percentage of GFP-positive cells on day 6 was set to 100%, and the fold difference relative to this starting point was calculated for each time point. To determine significant differences in the GFP assays, we used mixed model analysis as described previously [27].

4.6. Apoptosis Assay

The percentages of apoptotic cells were determined in SeAx and HH cells harvested on day 8 after transduction with the lentiviral miRZIP vectors aiming at an infection percentage of >95%. Briefly, cells were washed twice with cold phosphate-buffered saline and resuspended at a concentration of 1×10^6 cells/mL in 1X Binding Buffer. Cells were stained with Annexin V APC and 7AAD according to the manufacturer's protocol (BD Biosciences) and analyzed via flow cytometry (CytoFLEX S Flow Cytometer, Beckman Coulter, Indianapolis, IN, USA).

4.7. RACE-PCR

Both 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed using the SMARTer® RACE 5′/3′ kit (Takara Bio Inc., San Jose, CA, USA), according to the manufacturer's instructions. Briefly, 1 µg of total RNA isolated from the SeAx cell line was converted into the RACE-Ready first-strand cDNA. For the preparation of 5′-RACE-Ready cDNA/3′-RACE-Ready cDNA, the 5′-CDS Primer A/3′-CDS Primer A, respectively, were mixed with RNA. Two rounds of PCR amplification were performed, the first one with a specific primer 10XUPM (universal primer mix) and a gene-specific primer: TMEM244r primer for the 3′ RACE and TMEM244f primer for the 5′RACE. To perform nested PCR, Universal Primer Short (UPM short) was added, as well as TMEM244r2 inner primer for the 3′RACE and TMEM244f2 inner primer for the 5′RACE. PCR products were analyzed using 1% agarose gel. Prior to qRT-PCR, the PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Subsequently, colony PCR was performed using RedTaq Polymerase (Merck KGaA, Darmstadt, Germany) and M13f,M13r primers. Different size bands were sequenced to identify the possible isoforms. Isoforms detected using RACE were confirmed using RT-PCR and primers designed to identify isoforms. The GAPDH gene was used as a positive control. All primer sequences are listed in Table S1.

4.8. RNA Extraction and Real-Time Quantitative PCR (RT-qPCR)

RNA was extracted using TRI Reagent (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. The quantity of RNA was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA), and the quality was determined by 0.8% agarose gel electrophoresis with ethidium bromide staining. cDNA was synthesized from 0.3 µg or 0.5 µg of RNA using SuperScript™ IV Reverse Transcriptase with random hexamer primers (Invitrogen™, Waltham, MA, USA). *TMEM244* expression was analyzed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) (Hs02340633_m1) with intron-spanning primers located in the second and third exons. Beta-2 microglobulin (B2M) (Hs00984230_m1), with intron-spanning primers located in the first and second exons, was used as a reference gene for sample normalization. Relative gene expression was calculated using the median ct value method ($2^{-\Delta CT}$). Expression levels of different isoforms were measured using 5× HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne, Tartu, Estonia) and primers specific to each isoform. The results were normalized to the *GAPDH* reference gene. Standard curves for each isoform were prepared as follows: PCR products for each *TMEM244* isoform and *GAPDH* gene were cloned into the pGEM®-T Easy Vector and transformed into bacteria. Vectors were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific™, Waltham, MA, USA), sequenced, and digested with the BstXI enzyme (NEB, Ipswich, MA, USA). Serial dilutions were prepared to obtain the concentration from 10^9 to 10^1 copy numbers. RT-qPCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

4.9. Isolation of Nuclear and Cytoplasmic RNA

Cytoplasmic, nuclear, and chromatin RNA were isolated using an adaptation of the CD4+ T-cell nuclei extraction by Danko et al. [28,29]. RNA from the isolated fractions was reverse transcribed and used for qRT- PCR as described above. All samples were tested for *TMEM244* as well as tRNA lys, RPPH1, DANCER (cytoplasmic controls), U3SNORNA, ANRIL (nuclear controlss), and KTN1_AS1, KTN1_AS1_intron (chromatin controls). The sum of the cytoplasmic, nuclear, and chromatin expression levels of each transcript was set to 100%, and the percentage of each transcript localized to each compartment was determined. All controls showed the expected localization in each experiment, confirming successful fractionation. The primers used for RT-qPCR are listed in Table S1.

5. Conclusions

Our study is the first to experimentally verify the presence of TMEM244 protein. Different *TMEM244* transcript variants were identified; however, none of them had significant coding potential and they were all expressed at a lower level compared to the main transcript variant. Although *TMEM244* transcripts are localized in the cytoplasm, it appears that they do not encode a protein but are, rather, lncRNAs. Obtained results demonstrate that *TMEM244* mRNA is necessary for cellular growth of CTCL cells; therefore, it might be considered a new therapeutic target for the treatment of CTCL. Further study is needed to elucidate the in vivo effect and the downstream signaling pathway through which *TMEM244* functions in CTCL cells, as well as the function of its novel transcript variants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24043531/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials

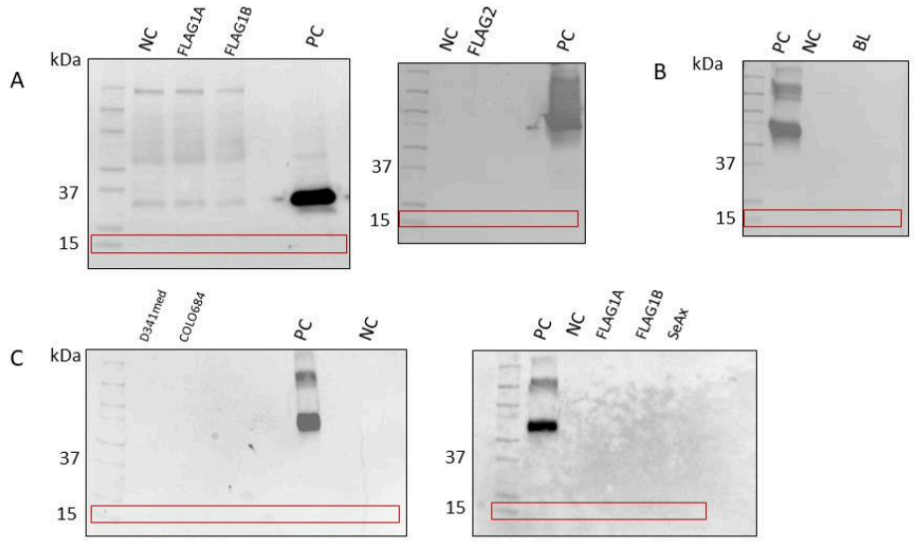

Figure S1. Western blot analysis of TMEM244 expression. Predicted protein size: TMEM244- 15kDa, TMEM244-flag- 16kDa. (a). Western blot analysis using anti-flag antibody. NC- negative control (Jurkat WT), PC- positive control (protein with flag- tag), FLAG1A- Jurkat pLV-CMV-Tmem244_flag, FLAG1B- Jurkat pLV-PGK_Tmem244_flag, FLAG2 – Jurkat pLV_flag_CMV_Tmem244. (b) Western blot analysis using anti-TMEM244 custom made antibody in brain lysate. PC- positive control (peptide used for anti-TMEM244 ab production), NC- negative control (Jurkat WT), B L- brain lysate. (c) Western blot analysis using anti-TMEM244 custom made antibody in cell lines. PC- positive control (peptide used for anti-TMEM244 ab production), FLAG1A- Jurkat pLV-CMV-Tmem244_flag, FLAG1B- Jurkat pLV-PGK_Tmem244_flag, NC- negative control (Jurkat WT).

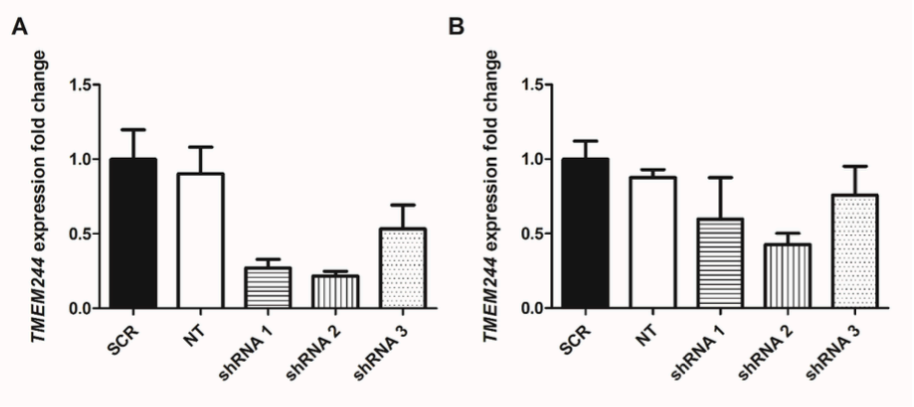

Figure S2. Confirmation of *TMEM244* expression knockdown by qRT-PCR in cell lines. (a) in SeAx cell line; (b) in HH cell line; SCR- scrambled, NT- non-targeting.

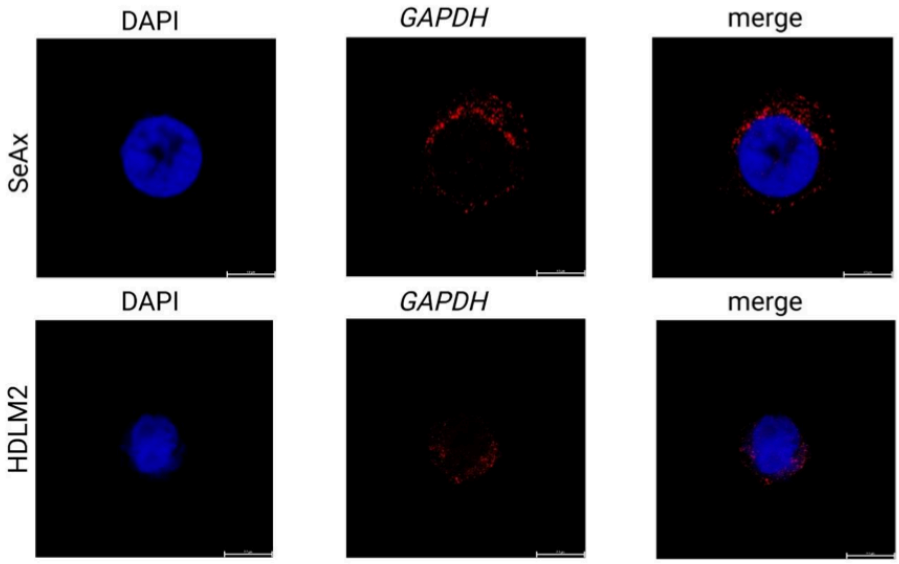

Figure S3. FISH analysis of GAPDH in SeAx and HDLM2 cells; GAPDH FISH signal in red, DAPI counterstain in blue.

A transcript_variant3 [organism=Homo sapiens] transmembrane protein 244 (TMEM244)

ACTACTGCGTCTCACACTTAGTCTCCAGGAGTAATTGAAAAGCTCACGGTGACAATTGTGTCTTCTTCCA
I GATGTCATCGCTATAAGGAGTGGGGCTTTCATCACCTCCTTGACGTAGGATGTGTACATGGCTCTCCAGG
TCAGAGTTGCTCCAAGCAAGGTTGTTTTGCAGAAGTTTCTTCTATGTGTCATTCTTTTCTACACTGTGTA
II CTATGTGTCCCTGAGCATGGGCTGCGTGATGTTTGAGGTGCATGAGTTGAATGTCCTGGCTCCATTTGAT
III TTCAAAACAAATCCCTCATGGCTCAACATAAACTATAAAGTTATGTTGGAATTCCCCTTGACATCACATT
V GGTGGGCTGCTTTAGGTATATCAAAATTGCTTGTTTAGATTCTCTAATGCACAGAAATAATGTTAAATAG
AATAACTGTGGAAATATATTTTATTTTCTCATAGATTTTACAA

B transcript_variant4 [organism=Homo sapiens] transmembrane protein 244 (TMEM244)

ACTACTGCGTCTCACACTTAGTCTCCAGGAGTAATTGAAAAGCTCACGGTGACAATTGTGTCTTCTTCCA
I GATGTCATCGCTATAAGGAGTGGGGCTTTCATCACCTCCTTGACGTAGGATGTGTACATGGCTCTCCAGG
TCAGAGTTGCTCCAAGCAAGTTCTTTTAGTTTCAACAGAGGTCACCTACTTTGTTTGTGGATTGTTTTTT
IV GTTCCAGTTGTGGAAGAATGGGTTTGGGATTATGCTATTTCAGTCACTATTCTTCATGTTGCCATCACTT
CAACTGTTATGTTGGAATTCCCCTTGACATCACATTGGTGGGCTGCTTTAGGTATATCAAAATTTGCTTG
V TTTAGATTCTCTAATGCACAGAAATAATGTTAAATAGAATAACTGTGGAAATATATTTTATTTTCTCATA
GATTTTACAA

Figure S4. Sequences of identified *TMEM244* transcript variant: (a) transcript variant 3; (b) transcript variant 4. Green underline- exon 1; pink underline- exon 2; yellow underline- exon 3; red underline- exon 4; blue underline- exon 5.

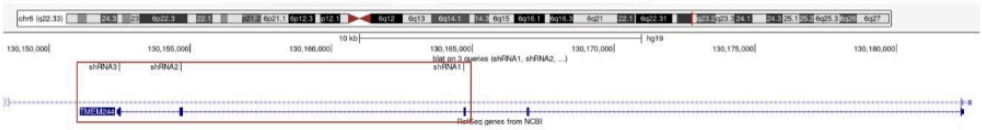

Figure S5. Schematic ilustration of the binding site for the shRNAs in the TMEM244 mRNA.

Table S1. The list of primers used in this study.

Primer name	Sequence (5′-3′)
Primers for RACE analysis	
TMEM244r	AGCAATTTTGATATACCTAAAGCAGCCCAC
TMEM244f	CACGGTGACAATTGTGTCTTCTTCCAG
TMEM244r2	CTAAAGCAGCCCACCAATGTGATGTC
TMEM244f2	GCTATAAGGAGTGGGGCTTTCATCAC
Ex1f	GACAATTGTGTCTTCTTCCAG
Ex5r	CTATGAGAAAATAAAATATATTTCCACAG
Ex3,5f	AAAGTTATGTTGGAATTCCCC
Ex1,4f	GTTGCTCCAAGCAAGTTCTTTTAG
Ex0f	CACGCTTCAACATGAATGCAG
Ex4f	CAGTTGTGGAAGAATGGGTTTG
GAPDHf	GACAGTCAGCCGCATCTTCT
GAPDHr	GCGCCCAATACGACCAAATC
M13 f	GTAAAACGACGGCCAG
M13 r	CAGGAAACAGCTATGAC
Primers for gene expression analysis by quantitative Real Time PCR	
tRNAlys_f	CGGCTAGCTCAGTCGGTAGA
tRNAlys_r	CCAACGTGGGGCTCGAAC
RPPHf	AGCTTGGAACAGACTCACGG
RPPHr	AATGGGCGGAGGAGAGTAGT
DANCERf	CGTCTCTTACGTCTGCGGAA
DANCERr	TGGCTTGTGCCTGTAGTTGT
U3SNORNAf	AACCCCGAGGAAGAGAGGTA
U3SNORNAr	CACTCCCCAATACGGAGAGA
ANRILf	AAGCCGCTCCGCTCCTCTTCT
ANRILr	GCCGTGTCCAGATGTCGCGT
KTN1_AS1f	GCAAAGACACAAGGCTCACA
KTN1_AS1r	ATGGTATTGGGGCACGTACA
KTN1_AS1_intronf	TTGGCTGCTATTTACTACCCTCC
KTN1_AS1_intronr	GCTGGGTGTGTTGCTAATCC
TMEM244f	GCTATAAGGAGTGGGGCTTTCATCAC
TMEM244r	CTAAAGCAGCCCACCAATGTGATGC

Table S2. The list of Stellaris® RNA FISH probes for *TMEM244* transcript used in this study.

Probe	Probe (5'-> 3')
1	GAAGACACAATTGTCACCGTG
2	CTTATAGCGATGACATCTGGA
3	CGTCAAGGAGGTGATGAAAGC
4	CTGGAGAGCCATGTACACATC
5	AACAACCTTGCTTGGAGCAAC
6	CACATAGAAGAAACTTCTGCA
7	GGACACATAGTACACAGTGTA
8	ATGCACCTCAAACATCACGCA
9	ATGGAGCCAGGACATTCAACT
10	GAGGGATTTGTTTTGAAATCA
11	CTTTATAGTTTATGTTGAGCC
12	GGTGACCTCTGTTGAAACTAA
13	TTCTTCCACAACTGGAACAAA
14	AAATAGCATAATCCCAAACCC
15	GGCAACATGAAGAATAGTGAC
16	CCAACATAACAGTTGAAGTGA
17	CCAATGTGATGTCAAGGGGAA
18	TTTGATATACCTAAAGCAGCC
19	TTTCTGTGCATTAGAGAATCT
20	ATTTCCACAGTTATTCTATTT

3rd ARTICLE ENTITLED:

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

Karolina Rassek, Katarzyna Iżykowska, Magdalena Żurawek, Karina Nowicka, Monika Joks, Karolina Olek-Hrab, Berenika Olszewska, Małgorzata Sokołowska- Wojdyło, Wojciech Biernat, Roman Janusz Nowicki, Grzegorz Krzysztof Przybylski

Journal of Investigative Dermatology, 2023, 143, 344-347.

Background: SS is known to be a disease, with a high degree of inter-and intra-patient heterogeneity as well as phenotypic changes over time, therefore making it very difficult to diagnose (Najidh et al., 2021). The disease is characterized by the presence of circulating malignant cells with a post-thymic T, helper, "central memory" (CD3+, CD5+, CD28+, TCRαβ+, CD4+CD8−, CD45RO+, CCR7+, CD27+) phenotype (Novelli et al., 2015b). However, besides the common expression of CD4 and CD45RO antigens, the immunophenotype of Sézary cells is diverse among patients (Novelli et al., 2015a). Additionally, SS can mimic many common benign chronic dermatoses such as psoriasis, pityriasis rubra pilaris, dermatitis, as well as mycosis fungoides (Vakiti et al., 2022). The currently proposed ISCL criteria for SS diagnosis combine clinical, histopathological, immunophenotypic, and molecular tests and include absolute Sézary cells count ≥1000/μL, a CD4/CD8 ratio ≥ 10, aberrant expression of pan-T-cell antigens (i.e., loss of CD7 and/or CD26 expression in at least 40% or 30% of cells, respectively), demonstration of T-cell clonality (TCR) by molecular methods, or cytogenetic demonstration of an abnormal clone (Hristov et al., 2019). Additionally, WHO classification requires the presence of erythroderma, generalized lymphadenopathy, and Sézary cells in the skin, peripheral blood, and lymph nodes (Hristov et al., 2021). From the clinical point of view, these complex criteria make SS diagnosis difficult and challenging. Skin histopathological changes often resemble those seen in MF, with less visible epidermotropism, although skin biopsy changes can be paradoxically subtle and nonspecific (Scheffer et al., 1986). Moreover, the traditional PCR of TCR used to identify the presence of a T-cell clone in clinical samples has a high false-negative rate in early-stage SS (Kirsch et al., 2015). Lack of correct diagnostic tools often leads to delays in diagnosis and inappropriate treatment that adversely affects the outcome of this aggressive disease. Therefore,

there is a need for an efficient diagnostic tool, that will not only allow fast diagnosis but also sensitive disease monitoring and accurate assessment of treatment response. Therefore, the aim of this study was to unravel whether *TMEM244* may serve as an easy diagnostic tool for SS and to identify the specific population of peripheral blood mononuclear cells with the highest basic expression of *TMEM244*.

Results: Using qRT-PCR we have indicated that median *TMEM244* expression is significantly higher in SS samples (1 500E-06; n=13) compared with healthy individuals (HI) (29.4E-06; n=30) as well as with mycosis fungoides/erythroderma (MF/E) samples (23.7E-06; n=9) in PBMC. Moreover, we have shown that median *TMEM244* expression is significantly higher within sorted CD4+ T-cell population from SS patients (2 360E-06; n=6) compared with CD4+ T-cells from HI (27.5E-06; n=14) and MF/E (70E-06; n=14). By using PE-conjugated antibody staining and positive selection on magnetic beads followed by qRT-PCR analysis, we have demonstrated that in normal subsets of lymphocytes, *TMEM244* is preferentially expressed, although on a low level, in both CD4+ helper (median= 392E-06) and CD8+ cytotoxic (median= 557E-06) subsets of memory cells (CD4RO+). In parallel, we have performed multiplex RT-PCR analysis to amplify rearrangements in TRB genes expression and found the clonal expansion of T cells, expressing the TRBV10-1—J2-7—C2 gene only in one SS patient which has shown the highest ratios of atypical Sézary cells (45.7%) and CD4+/CD8+ cells (25.5).

Conclusions*:* The presented results proved that expression of *TMEM244* is significantly higher in SS patients compared to HI or diseases with similar clinical presentation: MF and erythroderma of non-malignant origin. Moreover, *TMEM244* expression is higher in both CD4+ and CD8+ subsets of memory cells (CD4RO+) in blood from healthy individuals, which is in line with the immunophenotype of Sézary cells. Taken together, obtained results proved that *TMEM244* expression in the blood can be used to distinguish Sézary syndrome from MF and non-malignant erythroderma therefore significantly improving the disease diagnosis.

KR contribution to this article

- patient samples preparation: density gradient centrifugation of peripheral blood mononuclear cells and $CD4^+$ T cells sorting
- RNA isolations
- reverse transcriptions and qRT-PCRs

- expression data analyses
- preparation of figures and tables
- writing the manuscript draft and preparing the final manuscript version.

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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; doi: 10.1016/j.jid.2022.08.046

TO THE EDITOR

Sézary syndrome (SS) is a rare, aggressive leukemic variant of cutaneous T-cell lymphoma (CTCL). The disease is characterized by severe erythroderma, generalized lymphadenopathy, and the presence of circulating Sézary cells that are a clonal proliferation of CD4+/CD45RO+ malignant T lymphocytes primarily involving the skin (Huet et al., 2006; Willemze et al., 2005). SS is a leukemic variant of CTCL and is closely related to mycosis fungoides (MF), a more indolent and slowly progressing type (Booken et al., 2008). MF is the most common type of CTCL (50–70%), whereas patients with SS represent approximately 5% of CTCL cases. The incidence rate of CTCL is increasing and is currently 0.77 of 100,000 (Bradford et al., 2009; Criscione and Weinstock, 2007). The International Society of Cutaneous Lymphomas–proposed criteria for SS diagnosis integrate clinical, immunophenotyping, histopathologic, and molecular studies (Hristov et al., 2019). However, owing to great molecular heterogeneity between individual patients and the fact that SS can mimic many common benign chronic dermatoses such as psoriasis, pityriasis rubra pilaris, dermatitis, etc. as well as MF, the correct diagnosis is often very challenging (Vakiti et al., 2022). Moreover, the lack of correct diagnostic tools often leads to delays in diagnosis and inappropriate treatment that adversely affect the outcome of this aggressive disease. Despite advances in therapy, the prognosis still remains poor, with a 5-year overall survival of 30% (Najidh et al., 2021).

In our previous study, we identified ectopic expression of *TMEM244*, with unknown biological function, in patients with SS but not in healthy individuals (HIs) (Iżykowska et al., 2017). In this study, our purpose was to unravel whether *TMEM244* may serve as a diagnostic tool for SS identification. RT-qPCR was used to investigate the expression of *TMEM244* in PBMCs and CD4+ T cells isolated from the blood of patients with SS, MF, and erythroderma and also in HIs. In addition, we examined *TMEM244* expression in subpopulations of blood cells of six HIs.

The study is a combination of retrospective data (SS1–SS14 and MF1–MF3) (Iżykowska et al., 2020, 2017) and an analysis of new samples (SS15 and SS16, MF4–MF6, erythroderma E1–E8) collectively, including 16, 6,

Abbreviations: CTCL, cutaneous T-cell lymphoma; HI, healthy individual; MF, mycosis fungoides; SS, Sézary syndrome

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Table 1. Clinical Data and *TMEM244* Expression of Patients

Patient ID	Age/ Sex	Diagnosis	WB C, g/l	Lymph, g/l	Atypical Sézary Cells, %	CD4/ CD8	Therapy before Sample Collection	Sample	*TMEM244* Expression $2^{-\Delta CT}$
SS16	64/M	SS IVA1 + high count monoclonal B-cell lymphocytosis	19.13	11.75 (50.8%)	45.7	25.5	None	PBMC	782E-06
								CD4+	1750E-06
SS17	78/M	SS IVA1 + high count monoclonal B-cell lymphocytosis	15.03	6.45 (23.3%)	16.4	16.4	None	PBMC	380E-06
								CD4+	2150E-06
								SB	143E-06
MF4	75/F	Mycosis fungoides IIB	7.63	0.91 (9.6%)	1.6	3.9	None	PBMC	8E-06
								CD4+	28E-06
MF5	52/F	Mycosis fungoides IB	4.62	0.72 (8.7%)	0.5	4.7	IFN 90 µg	PBMC	29E-06
								CD4+	123E-06
MF6	57/F	Mycosis fungoides IB	5.18	1.05 (11.9%)	1.2	2.6	None	PBMC	70E-06
								CD4+	195E-06
E1	88/M	E of unknown etiology + monoclonal B-cell lymphocytosis	9.97	1.96 (15%)	1.8	7.4	None	CD4+	188E-06
								SB	10E-06
E2	66/F	E of unknown etiology	5.07	0.65 (11.5%)	3.3	2.5	50 mg/day acytretin + 20 mg/day prednizon	CD4+	25E-06
								SB	43E-06
E3	57/M	Drug-induced E	NA	4.1%	0.0	5.4	None	PBMC	42E-06
								CD4+	55E-06
								SB	31E-06
E4	73/M	E, pityriais rubra pilaris susp/MF susp	NA	NA	NA	NA	None	PBMC	7E-06
								CD4+	40E-06
								SB	60E-06
E5	67/F	E and atopic dermatitis	7.43	1.66 (18.8%)	0.4	2.8	None	PBMC	18E-06
								CD4+	101E-06
								SB	14E-06
E6	68/F	Drug-induced E	NA	NA	NA	NA	None	PBMC	11E-06
								CD4+	85E-06
E7	41/M	E and atopic dermatitis	NA	NA	NA	NA	None	PBMC	64E-06
								CD4+	22E-06
								SB	18E-06
E8	70/M	E of unknown etiology	9.32	1.88 (15.9%)	1.5	2.8	None	PBMC	24E-06
								CD4+	53E-06
								SB	4E-06

Abbreviations: E, erythroderma; F, female; ID, identification; Lymph, lymphocyte; M, male; MF, mycosis fungoides; NA, not available; SB, skin biopsy; SS, Sézary syndrome; susp, suspected; WBC, white blood count.

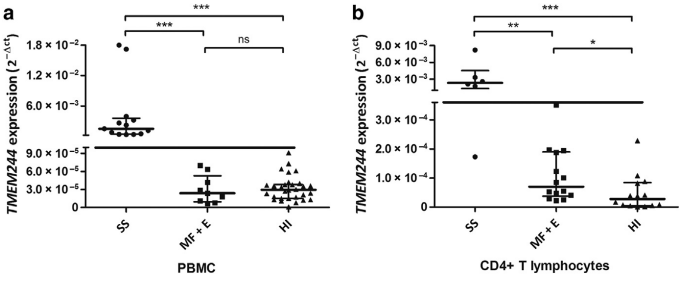

Figure 1. Relative *TMEM244* expression in patients' samples. Relative *TMEM244* expression analyzed by RT-qPCR in HI and in patients with MF + E and SS. (**a**) in PBMC samples of SS patients (n = 13), MF/E patients (n = 9), HI (n = 30); (**b**) in CD4+ T cells of SS patients (n = 6), MF/E patients (n = 14), HI (n = 14). Data are expressed as median. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. E, erythroderma; HI, healthy individual; MF, mycosis fungoides; SS, Sézary syndrome.

and 8 patients with SS, MF, and erythroderma, respectively, and 44 HIs (Table 1). The patients were diagnosed according to the International Society of Cutaneous Lymphomas/European Organization for Research and Treatment of Cancer classification for primary CTCL and the European Organization for Research and Treatment of Cancer/World Health Organization (Willemze et al., 2019, 2005). The study was approved by the Bioethics Committee at the Karol Marcinkowski Medical University of Poznań (Poznań, Poland) (decision 1095/17) and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all individual participants involved in the study. PBMCs were purified by density gradient centrifugation. CD4+ lymphocytes were separated by immunomagnetic-negative selection with EasySep™ Human CD4+ T Cell Enrichment kit. Selected subpopulations of PBMCs from six HIs were separated by phycoerythrin-conjugated antibody staining and positive selection on magnetic beads. *TMEM244* expression was analyzed by RT-qPCR using TaqMan Gene Expression Assays, with *B2M* and *GAPDH* used as reference genes. All samples were assayed in triplicates; the median value was used to calculate relative gene expression ($2^{-\Delta CT}$). Data distribution was verified by the Shapiro–Wilk normality test. Statistical differences between non-normally distributed medians were evaluated with the Mann–Whitney test.

Results from the PBMC samples showed that median *TMEM244* expression was significantly higher in patients with SS (1,500E-06, n = 13) than in HIs (29.4E-06, n = 30; $P < 0.00001$) as well as in those with MF/erythroderma (23.7E-06, n = 9; $P = 0.0001$) (Figure 1a). Similarly, median *TMEM244* expression was significantly higher in separated CD4+ T-cell population from SS (2,360E-06, n = 6) than in CD4+ T cells from HIs (27.5E-06, n = 14; $P = 0.00084$) and those from MF/erythroderma (70E-06, n = 14; $P = 0.002$) (Figure 1b). The cut-off value considered positive for SS diagnosis was set at 100E-06 for PBMCs and 360E-06 for sorted CD4+ cells.

The expression of *TMEM244* was analyzed in the following separated subpopulations of blood cells from six HIs: CD4+, CD8+, CD45RO+, CD45RA+, CD31+, CD56+, CD14+, and CD19+ (Supplementary Figure S1 and Supplementary Table S1). *TMEM244* expression was higher in T cells—both CD4+ (median = 392E-06) and CD8+ (median = 557E-06) subpopulations than in CD19+ B cells (median = 23E-06), CD56+ NK cells (median = 106E-06), and CD14+ monocytes (median = 514E-06). Moreover, the analysis of CD45RO+ memory T cells (median = 189E-06), CD45RA+ naïve T cells (median = 51E-06), and the youngest subset of CD31+ naïve T cells (median = 75E-06) showed that the expression of *TMEM244* was associated with the postactivation state of T cells (Supplementary Figure S2).

In two SS (SS16 and SS17), three MF (MF4–6), and eight erythroderma (E1–8) samples, clonality analyses were performed using multiplex RT-PCR of the expressed *TCRβ* gene. Amplification products of the separated CD4+ fractions of SS16 and SS17, MF4 and MF5, and E1–4 samples were further analyzed by nucleotide sequencing. Clonal expansion of T cells, expressing the *TRBV10-1—J2-7—C2* gene, was detected only in SS16, which has shown the highest ratios of atypical Sézary cells (45.7%) and CD4+/CD8+ cells (25.5).

Complex criteria and huge heterogeneity among patients make SS diagnosis difficult and challenging. Cutaneous histopathological lesions often resemble those in MF, with less epidermotropism, although skin biopsy findings can be paradoxically subtle and nonspecific (Hristov et al., 2019). Moreover, analysis of *TCR* by PCR used to identify the presence of a T-cell clone in clinical samples has a high false-negative rate in early-stage SS (Kirsch et al., 2015). There has been a search for a simple but unequivocal diagnostic marker that would allow the identification of this lymphoma. Recently, a new study revealed 86 different driver genes mutated in CTCL, with 19 genes not previously associated with this disease and two not previously associated with any malignancy (Park et al., 2021). However, many driver genes are shared between MF and SS. Our study showed that the expression of *TMEM244* is significantly higher in patients with SS than only in HIs but also with clinical mimickers: MF and erythrodermic manifestations of nonmalignant diseases, which could be especially helpful in the diagnostic context. Because SS has a worse prognosis than MF, this is in line with a recent study, showing that high expression of *TMEM244* is associated with poor survival of patients with CTCL (Chen et al., 2022). Our study also revealed higher expression of *TMEM244* in CD4+ and CD8+ subsets of memory cells (CD45RO+), which is in line with the immunophenotype of Sézary cells. Therefore, measuring *TMEM244* expression using RT-qPCR could be used as an easy and cheap blood diagnostic marker to distinguish SS from diseases with similar clinical presentation.

Data availability statement

RT-qPCR and flow cytometry data will be provided on request from the corresponding author.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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Conceptualization: GKP; Funding Acquisition: GKP, KI; Investigation: KR, KI, MŻ, KN; Methodology: KR, KI, MŻ, KN; Project Administration: GKP; Resources: MJ, KOH, BO, MSW, WB, RJN; Supervision: GKP, KI: Validation: KR, KI; Visualization: KR, MŻ; Writing - Original Draft Preparation: KR, KI; Writing - Review and Editing: KI, GKP, MŻ

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SUPPLEMENTARY MATERIAL
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Counting Monkeypox Lesions in Patient Photographs: Limits of Agreement of Manual Counts and Artificial Intelligence

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TO THE EDITOR

The extent of cutaneous involvement is a key aspect of diagnosing and monitoring monkeypox disease, which is considered the most important orthopoxvirus in humans (Sklenovska et al., 2018). The spread of monkeypox cases in Europe and North America in May 2022 raised global public health concerns (Muyembe-Tamfum, 2022), leading to the World Health Organization declaring a public health emergency on July 23, 2022 (World Health Organization, 2022).

Abbreviation: AI, artificial intelligence

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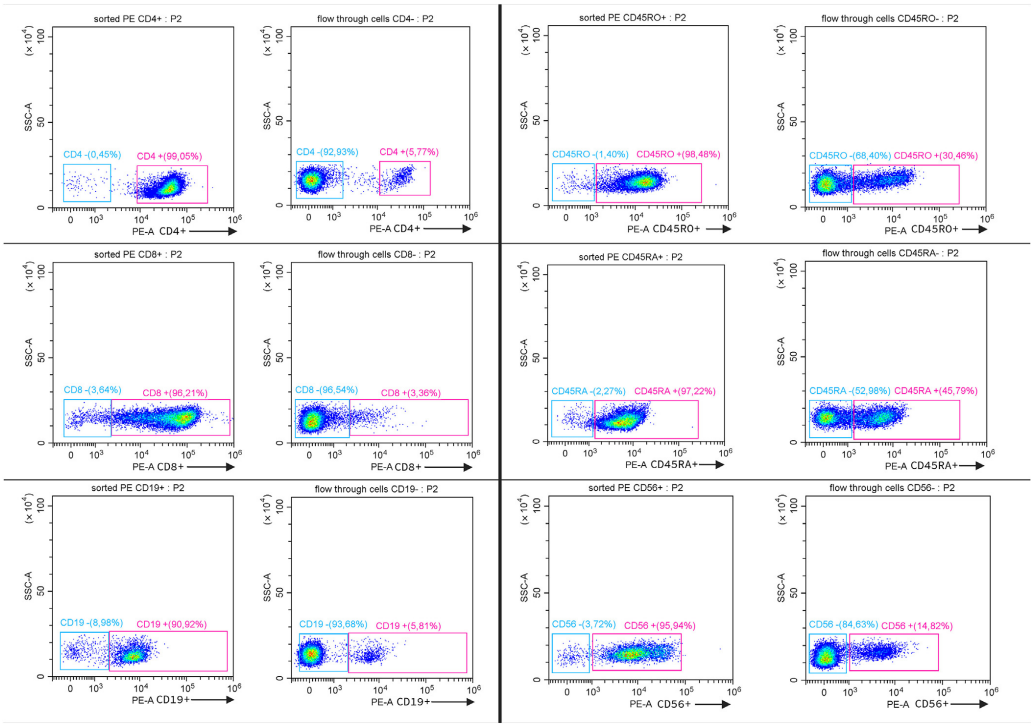
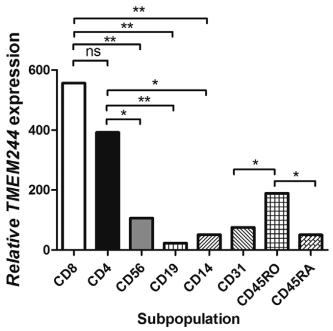


Supplementary Figure S1. Flow cytometry analysis of mononuclear cells in the blood from healthy donors. Representative flow cytometry results show an enriched population of PE-positive cells and flow-through to assess the purity of the enriched population in the blood. SSC-A, side scatter area; PE, phycoerythrin.



Supplementary Figure S2. Relative *TMEM244* expression in subpopulations of blood cells from healthy individuals. Relative *TMEM244* expression was analyzed by RT-qPCR in HI blood subpopulations (n = 6). Data are expressed as median. $*P < 0.05$ and $**P < 0.01$. ns, not significant.

Supplementary Table S1. Relative *TMEM244* Expression in Subpopulations of Mononuclear Cells in the Blood of HIs

Patient ID	Age/Sex	Diagnosis	Sample	Cell Subpopulation	Relative *TMEM244* Expression $2^{-\Delta CT}$
HI_1	NA	HI	Buffy coat	CD8	3.5E-04
				CD4	2.5E-04
				CD56	3.9E-05
				CD19	2.1E-05
				CD14	1.5E-05
				CD45RO	3.8E-04
				CD45RA	1.7E-05
				CD31	2.2E-05
HI_2	NA	HI	Buffy coat	CD8	1.3E-03
				CD4	1.0E-03
				CD56	1.6E-04
				CD19	2.3E-05
				CD14	2.3E-04
				CD45RO	1.3E-03
				CD45RA	1.1E-04
				CD31	1.3E-04
HI_3	27/M	HI	Buffy coat	CD8	5.3E-04
				CD4	5.3E-04
				CD56	4.9E-05
				CD19	-
				CD14	1.2E-04
				CD45RO	1.1E-04
				CD45RA	6.8E-05
				CD31	6.1E-05
HI_4	23/M	HI	Buffy coat	CD8	1.0E-03
				CD4	2.3E-04
				CD56	1.8E-04
				CD19	2.6E-05
				CD14	2.1E-05
				CD45RO	9.4E-05
				CD45RA	3.8E-05
				CD31	3.3E-05
HI_5	36/M	HI	Buffy coat	CD8	5.9E-04
				CD4	5.7E-04
				CD56	2.5E-04
				CD19	4.5E-05
				CD14	5.1E-05
				CD45RO	1.8E-04
				CD45RA	1.7E-05
				CD31	9.0E-05
HI_6	47/M	HI	Buffy coat	CD8	3.6E-04
				CD4	1.9E-04
				CD56	2.7E-05
				CD19	1.8E-05
				CD14	-
				CD45RO	2.0E-04
				CD45RA	6.3E-05
				CD31	1.2E-04

Abbreviations: HI, healthy individual; ID, identification; M, male; NA, not available.

4th ARTICLE ENTITLED:

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

Karolina Rassek, Katarzyna Iżykowska

MDPI Cancers, 2020, 12(8), 2129.

Background: The knowledge of CTCLs molecular genetics has been continuously growing over the past years. Advances in single-cell gene expression profiling in patient samples opened new possibilities for analyzing cancer cell heterogeneity. Several studies were performed to analyze gene expression profiling in skin or blood samples from CTCL patients compared to skin samples from healthy donors, patients with atopic dermatitis, or benign inflammatory dermatoses. Even though the knowledge about CTCLs genotype and immunophenotype largely increased, there are still many differences between the analyzed samples, due to large inter- and intra- tumor heterogeneity within patients. The review summarizes the current knowledge on the genomic heterogeneity of CTCL gained through the application of novel RNA sequencing approaches, and highlights the common findings.

Results: In this review, we compared available studies where molecular features of malignant cells through high-throughput sequencing techniques were analyzed. Based on our systematic review, a huge heterogeneity between CTCL patients was revealed with the use of the RNA-sequencing approach, while single-cell RNA- sequencing enabled to find high intratumor heterogeneity and divided the malignant population into distinct clusters within a single CTCL patient. Interestingly, multiple protein-coding genes, including the *TOX* gene as well as lncRNAs differentially expressed in CTCL cells were revealed. The involvement of the HTLV-1 virus in CTCLs pathogenesis was excluded. In addition to this comparative analysis, we have also evaluated affected pathways, included: T-cell receptor signaling, IL-2-mediated signaling, and cell cycle progression, crucial for CTCLs pathogenesis..

Conclusions: We have described several genomic features deregulated in CTCL patients identified in multiple studies. We concluded that high throughput analysis is essential to advance our understanding of the disease and improve disease treatment.

KR contribution to this article

- selection of the reviewed literature
- preparation of figures and tables
- writing the manuscript draft and preparing the final manuscript version.

cancers

MDPI

Review

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

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Abstract: Cutaneous T-cell lymphomas (CTCLs) represent a large, heterogeneous group of non-Hodgkin lymphomas that primarily affect the skin. Among multiple CTCL variants, the most prevalent types are mycosis fungoides (MF) and Sézary syndrome (SS). In the past decade, the molecular genetics of CTCL have been the target of intense study, increasing the knowledge of CTCL genomic alterations, discovering novel biomarkers, and potential targets for patient-specific therapy. However, the detailed pathogenesis of CTCL development still needs to be discovered. This review aims to summarize the novel insights into molecular heterogeneity of malignant cells using high-throughput technologies, such as RNA sequencing and single-cell RNA sequencing, which might be useful to identify tumour-specific molecular signatures and, therefore, offer guidance for therapy, diagnosis, and prognosis of CTCL.

Keywords: Sézary syndrome; mycosis fungoides; cutaneous T-cell lymphomas; single-cell heterogeneity; RNA-sequencing

1. Introduction

Cutaneous T-cell lymphomas (CTCLs) are a large, heterogeneous group of lymphoproliferative hyperplasias derived from mature skin-homing T lymphocytes, with a different stage of malignancy. Sézary Syndrome (SS) and Mycosis fungoides (MF) constitute about 65–80% of all CTCLs cases [1]. MF is the most prevalent form of CTCL, constituting approximately 50% of all lymphomas arising primarily in the skin, with an incidence rate of six to seven cases per million persons. Early-stage MF has an excellent prognosis, and 90% of patients do not progress to the tumour stage [2,3]. The incidence of SS, an aggressive and leukemic variant of CTCL, is 0.1–0.3 cases per million persons, accounting for only 2.5% of all CTCLs [4]. MF affects African Americans more often than Caucasians, while the incidence rate of SS is higher in Caucasians than in African Americans. However, the 2:1 male-to-female ratio is the same in both lymphomas [3,4]. Both SS and MF were shown to be closely related; the clinical features of the late-stage MF might resemble those of SS. Moreover, both diseases are characterized by similar genetic profiles and by great diversity in gene expression, mutations, and chromosomal aberrations [5]. Despite much study, the driver mutations of CTCLs are still unknown [2]. Moreover, due to the lack of specific markers and resemblance to different dermatologic conditions, such as chronic actinic dermatitis, psoriasis, idiopathic erythroderma or chronic eczematous dermatitis, the diagnosis of CTCLs is very challenging, with limited treatment options available [6–8]. Recent studies on genotyping and immunophenotyping of CTCLs indicate that, although arising as an expansion of mature helper memory T-cells, the population of cells derived from each patient shows some level of heterogeneity by forming complex aberrant clonal hierarchies and subclones [9–12]. Determining intratumoral heterogeneity is challenging, but is crucial for understanding tumour pathogenesis and evolution, and might have important implications for the diagnosis and treatment [13]. In this review,

we highlight recent insights into the genomic heterogeneity of CTCLs through the application of novel RNA sequencing approaches.

2. Clinical and Immunological Features of CTCLs

Classic Alibert–Bazin MF is characterized by the progressive appearance of plaques, patches, and, ultimately, in the case of some patients, tumours [3]. Malignant lymphocytes of MF resemble those of the α/β memory T-helper phenotype (T-cell receptor [TCR]ß$^+$, TCRγ^-, cluster of differentiation CD3$^+$, CD4$^+$, CD5$^+$, CD8$^-$, CD45RO$^+$, T-cell intracellular antigen [TIA]-1$^-$). In most conventional cases of MF, cells do not express cytotoxic markers, such as TIA-1, granzyme B, and perforin. However, sometimes, these cells can exhibit a T-cytotoxic phenotype (TCRß$^+$, TCRγ^-, CD3$^+$, CD4$^-$, CD5$^+$, CD8$^+$, TIA-1$^+$ or TCRß$^-$, TCRγ^+, CD3$^+$, CD4$^-$, CD5$^+$, CD8$^+$/$^-$, TIA-1$^+$). In advanced stages of MF, CD4$^+$/CD8$^+$ or CD4$^-$/CD8$^-$ phenotypes can be observed [3].

SS is typically characterized by erythroderma, lymphadenopathy, and severe pruritus. Neoplastic T lymphocytes (Sézary cells) present in skin, lymph nodes, and peripheral blood express the CD3$^+$CD4$^+$CD8$^-$ phenotype. Expression of CD3, CD4, CD45RO, and CCR4 indicates a mature memory T-cell phenotype, and expression of CCR7, L-selectin, and CD27, a central memory T-cells phenotype of malignant cells. Sézary cells also express T-regulatory profile (CD25 and FOX-P3) phenotypes, which result in suppression of the immune response [4].

Both MF and SS lymphocytes can express a T-helper type 2 phenotype, characterized by inreased IL-4, IL-5, IL-10 and IL-13 production [14]. In early MF Th1 phenotype could be detected, but it switches to Th2 as this phenotype creates more beneficial microenvironmet for tumor growth. The role of Th17 and Th22 cells in the pathogenesis of CTCL was also investigated and it was shown that IL-22 is higly expressed in lesional skin of CTCL, in contrast to low expression of Il-17.

3. High-Throughput RNA Sequencing Techniques

High-throughput technologies, such as RNA sequencing (RNA-seq), have become irreplaceable tools for transcriptional analysis of differential gene expression. By sequencing a huge number of cells from one sample, it is now possible to investigate aspects of RNA biology, such as its structure, interactions, and pathways of translation or transcription [15]. Because of unbiased analysis of the entire transcriptome, RNA sequencing enables us to identify previously undescribed transcripts, such as lncRNAs, gene isoforms, or pathways of gene expression regulated by enhancer RNAs. Another advantage of the RNA-seq method is the ability to identify non-human transcripts, for example, those of viral origin, that can confirm or exclude a potential infectious aetiology of human diseases [16,17].

Single-cell RNA sequencing, a recent development of RNA-seq, is a revolutionary tool with several distinct advantages over bulk RNA-seq, such as investigation of expression patterns of individual cells. By using scRNA-seq, it is now possible to track cell lineages during differentiation or examine rare cell populations, which could not be detected using bulk RNA-seq [18,19]. Many scRNA-seq protocols and approaches have been introduced during method development. However, all of them follow the same basic steps. Common principles required for the generation of scRNA-seq libraries include the isolation of cells from each other, cell lysis, reverse-transcription into the first-strand cDNA, and cDNA amplification [20]. Although experimental methods are increasingly developing, there are still some important drawbacks of scRNA-seq that should be considered. Because of the low amount of material, there is a low mRNA capture efficiency and a high dropout rate. Therefore, an efficient cell lysis strategy is needed. Additionally, compared to bulk RNA-seq, scRNA-seq produces more variable and nosier data, which pose challenges for the computational analysis of the results. Although some tools have been designed and commercial companies (e.g., 10× Genomics and Illumina) have provided software to handle raw data files, this area requires further improvement (Table 1), (Figure 1) [19,21].

Table 1. Comparison of RNA sequencing methods.

Features	Single-Cell RNA Sequencing	RNA Sequencing
Transcriptome analysis	Unique transcriptome expression of each of many diverse cell types	Average transcriptome expression of many cells
Heterogeneity of the population	Cell to cell variability	Cells from tissues considered to be homogeneous
Populations of cells	Identifying rare populations	-
Conditions of cells	Cells from one condition are generally captured and sequenced	Compares differentially expressed genes under multiple conditions
Statistical power	Increased (capturing thousands of cells in one condition)	-
Cells uniqueness	Revealing latent changes, new cell types, cells subpopulations	-
Generated data	Noisier, more variable data	Less background noise, less variable
Capture efficiency	Low	High

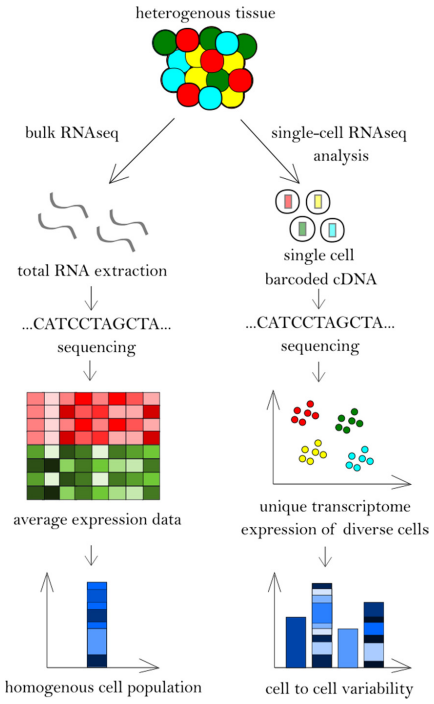

Figure 1. Bulk RNA sequencing and single-cell RNA sequencing workflow.

4. RNA Seq Analysis of CTCL Patients

Litvinov et al. were the first to use TruSeq targeted RNA gene expression analysis to study formalin-fixed and paraffin-embedded (FFPE) samples from a cohort of CTCL patients and benign inflammatory dermatoses [22]. The comparison of gene expression using clustering analysis revealed a

significant cross-classification between CTCL and benign samples, and highlighted a significant degree of heterogeneity with respect to gene expression changes within different CTCL samples and even within the same patient, where samples taken at different times did not cluster together. The analysis of over 280 highly studied biomarkers and candidate genes for CTCL pathogenesis confirmed several important gene expression changes that, in combination with other techniques, have diagnostic and prognostic potential. The authors especially underlined the upregulation of the TOX, FYB, LEF1, CCR4, ITK, EED, POU2AF, IL-26, STAT5, BLK, and GTSF1 genes and downregulation of the PSORS1C2 gene in CTCL patients compared to controls (benign inflammatory dermatoses). The overexpression of the thymocyte selection-associated high-mobility group box (TOX) gene was especially highlighted, as TOX was previously reported to be upregulated in MF and SS and correlated with increased risk of disease progression and poor prognosis [23,24]. In this study, the statistical analysis of gene expression between early and advanced stages of CTCLs revealed upregulation of the TOX, FYB, and GTSF1 genes and downregulation of the LTB4 gene in advanced stages of the disease, consistent with previous studies that identified those genes as molecular markers of progression [25]. Moreover, it was shown that the TOX, FYB, and CCR4 genes are upregulated in stage I patients that were at risk of cancer progression. The study also revealed overexpression of STAT5 in CTCL samples, which was previously shown as a driver of expression of oncogenic BIC/miR-155 in cancer and promoter of the proliferation of malignant T-cells [26]. Moreover, upregulation of various inflammation mediating genes, such as CD70, STAT signalling genes, LTA, NFKB1, NFKB2, IL-15, and other inflammatory cytokines was observed in CTCL samples compared to controls. The analysis of several selected genes with respect to the clinical stage of the disease enabled the authors to identify upregulation of genes connected with poor prognosis or inflammation. The CD30, GNLY, CD70, and GTSF1 genes were expressed at later stages of the disease, while in early stages, BCL7A (a favourable prognosis gene) was expressed. The bioinformatics follow up of the Litvinov et al. study was conducted by another group of Lefrancois et al. TruSeq gene expression patterns in older (≤2008) vs. more recent (≥2009) FFPE samples were analyzed in order to examine if previous clustering and gene expression patterns can be confirmed when analyzed based on the year of biopsy [27]. Both analyses showed nearly identical trends and findings. In addition, Lefrancois et al. validated known upregulated in CTCL targets such as STAT signaling genes, and inflammatory interleukins and identified novel differentially expressed genes that were not statistical significant in Litvinov et al. study, including upregulated: BCL11A, SELL, IRF1, MAD1, CASP1, BIRC5 and MAX and downregulated MDM4, SERPINB3 and TBS4 genes.

To investigate the mutational landscape of SS genomes and possible fusion transcripts, Prasad et al. analyzed SS samples using whole-exome sequencing and RNA-seq [28]. Fusion transcripts that are expressed on the RNA level as a result of genomic rearrangements are often involved in malignant transformation as they might result in disruption of tumour suppressor genes or activation of oncogenes. In ten SS patients analyzed, 86 potential fusion transcripts were detected. Among them, TYK2-UPF1, COL25A1-NFKB2, FASN-SGMS1, SGMS1-ZEB1, SPATA21-RASA2, PITRM1-HK1, and BCR-NDUFAF6 were validated and discussed to have a potential role in pathogenesis due to the involvement of a fusion partner in signaling pathways, T-cell differentiation, transcriptional regulation, or proliferation.

Fusion transcripts were also identified in the SS patients studied by Iżykowska et al. [29] and Wang et al. [30]. Iżykowska et al., using whole genome sequencing and RNA-seq technology, analyzed 9 SS patients and SS derived cell line, SeAx. Many copy number variations and rearrangements were detected, fifteen rearrangements resulted in the expression of new fusion transcripts [29], with only one (TFG-GPR128) reported before [31]. Five of the detected transcripts resulted in ectopic expression of fragments of genes not present in normal T- cells (BAIAP2, CPN2, GPR128, CAPN12 and FIGLA) and nine of the transcripts were in frame (EHD1-CAPN12, TMEM66-BAIAP2, MBD4-PTPRC, PTPRC-CPN2, MYB-MBNL1, TFGGPR128, MAP4K3-FIGLA, DCP1A-CCL27, MBNL1-KIAA2018). Wang et al. investigated CD4+ T-cells from peripheral blood of 37 advanced stage SS patients [30]. Among 41 in-frame fusions, 29 were validated, and one of them, CD28-CTLA4, was previously detected in CTCLs. CTLA4–CD28 gene fusion was detected in several types of lymphoma [32] and thus could

provide a target for potential immunotherapy. A case study was even conducted where a SS patient was treated with a CTLA4 inhibitor [33]. Recently, it was proposed that, in cancer immunotherapy, the CD28 agonists could be used together with anti-PD1 antibodies to increase the effectiveness of therapies targeting PD1, which are also tested in terms of MF/SS [34,35]. Moreover, using RNA-seq, Wang et al. [30] was able to distinguish 345 upregulated transcripts. Several CD molecules and chemokines required for T-cell development and function, as well as interleukins and interleukin receptors, were upregulated (IL32, IL2RG, CD3G, CD27, CCR4, and CCR8). High expression of IL2RG, which encodes IL2 receptor common gamma chain, was detected in all examined patients. The most upregulated chemokine in all but one patient was IL32, a proinflammatory cytokine important in T-cell communication, tumorigenesis, and autoimmune diseases. Previous studies have suggested that IL32 might be involved in an autocrine signalling loop stimulating the growth of Sézary cells and that high expression of IL32 in MF patients is correlated with disease activity [36].

5. Identification of *Long Non-Coding* RNAs in CTCL

The development of high-throughput sequencing technologies has enabled the detection and classification of cancer-associated non-coding RNA. Long non-coding RNAs (lncRNAs) are classified as more than 200 nt long transcripts, which lack protein-coding potential. It has been shown that lncRNAs are involved in many cellular processes, such as chromosome structure modulation, transcription, splicing, and post-translational modifications [37]. In recent years, lncRNAs dysregulation has been linked to the pathogenesis of some disorders, such as cardiovascular diseases, metabolic disorders, and cancer [38–40]. Moreover, it has been suggested that lncRNAs can serve as potential diagnostic and prognostic markers [41,42] or targets of drug treatment in some cancers [43]. Therefore, the reliable identification of lncRNAs might be critical for understanding the molecular pathogenesis of CTCLs. Because the RNA-seq technique is more sensitive to detecting less-abundant transcripts and identifying novel splicing isoforms, it is a technique of choice to study gene expression signatures specific to tissues or cell types [44].

To obtain a pure population and minimize the detection of less relevant differences in mRNA expression, Lee et al. compared Sézary cells (SCs) to patient-matched polyclonal CD4+ T-cells from three individuals [45]. In this study, the role of lncRNAs in SS and MF was investigated for the first time. The authors identified 21 annotated SC-associated lncRNAs differentially expressed in SS cells, and the presence of them in the majority of 24 examined MF tumours was confirmed. Among them, there were 13 previously unreported Sézary cell-associated transcripts (SeCATs) with differential expression, 12 with highly conserved regions that are predicted to be noncoding and have potential functional importance. Furthermore, Lee et al. performed the analysis of protein-coding genes and found that 525 were commonly upregulated and 519 were downregulated. Within 1044 genes, they detected upregulation of: TNFSF11 (RANKL), PTHLH, EPHA4, ZNF331, DDX41, KCNN4, ITGB1, CNIH4, and CD52 and downregulation of APBA2, STAT4, NEDD4L, MXI1, TGFBR2, BCL2L11, SATB1, SP140, and RPS2, as reported in previous studies [46–48]. Moreover, the study revealed an increased expression of several genes that encode transmembrane (TMEM) proteins in all three SS patients: ACVR2A, ADAM8, ANK1, APP, CD4, CD59, EMP3, EPHA4, GPR68, KCNN4, PDCD1, PSEN1, SIGIRR, and TNFRSF1B. The authors noted that presenilin-1 is a part of the y-secretase complex, important in the oncogenic pathway in T-cell acute lymphoblastic leukaemia [49], that KCNN4 seems to be responsible for T-cell activation and proliferation [50], and that PCDCD1 is considered to be important in T-cell function and contributes to the prevention of autoimmune diseases [51]. Given their accessibility to therapeutic antibodies, TMEM proteins seem to be attractive targets for future studies.

6. Single-Cell RNA Seq in CTCL Studies

6.1. The Population of Malignant Sézary Cells can be Divided into Distinct Subpopulations

Surface antigens signatures are important for establishing cells in different conditions or unravelling molecular changes in cells, and therefore achieving a better understanding of the disease [52]. Furthermore, the identification of populations of cells exhibiting different gene signatures is crucial for effective clinical treatment. Researchers in the following studies used scRNA-seq to analyze blood samples and skin biopsies of CTCLs patients to identify potential tumour-specific molecular signatures.

Buus et al. investigated peripheral blood mononuclear cells from seven patients diagnosed with SS [10]. Using the T-distributed Stochastic Neighbor Embedding (t-SNE) visualization algorithm of single-cell expression of multiple markers at the same time, they were able to divide the population of malignant cells into subpopulations exhibiting distinct combinations of surface markers. The analysis of those populations showed that not all patients had similar heterogeneity of all examined markers and all markers showed differential expression within at least one patient. Only cutaneous lymphocyte-associated antigen (CLA) had bimodal expression in the malignant populations in all patients. Heterogeneous surface phenotypes were correlated to distinct mRNA transcript profiles within the malignant population studied in six SS patients. T-cell relevant genes were analyzed and, similarly to the expression of surface markers, mRNA transcripts also divided malignant cells into subpopulations. Among 110 T-cell related genes examined, only five (S100A4, S100A10, IL7R, CCR7, and CXCR4) were highly expressed by most of the malignant cells, and two of them (cancer-related genes S100A4 and S100A10) were ubiquitously expressed in all populations. A good correlation between the mRNA and protein expression at the single-cell level for the investigated genes (SELL, IL7R, CCR7, and CD4) encoding proteins was observed.

A study published by Borcherding et al. was based on single-cell RNA and T-cell receptor sequencing and comparison of pooled SS cells to normal CD4+ controls [12]. In an analyzed SS patient, 12 clusters were distinguished based on mRNA expression, and each cluster was defined by expression of five to seven top genes. Clusters were most closely associated with normal versus malignant cells, as six of them were constituted of normal CD4+ T-cells and five of malignant SS cells. The gene expression analysis of known marker genes showed that the majority of cells of both normal and malignant origin correlated with CD4+ central memory T-cells. The malignant population was clonal and exhibited an increase in expression of CD70 and a decrease in CD26 expression, also identified in multiple inflammatory diseases [53]. Moreover, malignant cells had increased CD5 expression and maintained a CD7 expression, which is usually lost in the case of CTCLs patients [54]. Normal and malignant cells were further analyzed in terms of differential gene expression, and several marker genes were identified; most of these had been previously described, but two were newly identified: SAMSN1 and TSPAN2.

Gaydosik et al. investigated skin samples from five CTCL patients and four healthy controls [11]. Cells were grouped according to expression profiles and, unlike in healthy samples where there was an overlap between cells, no overlap was detected between cells from both tumour and healthy samples or between tumour samples themselves. Based on the comparison of the transcriptomes of each lymphocyte subset from tumours and from four skin control samples, at least one cluster unique for each sample was identified. Those clusters were identified based on the expression of unique genes that were selected by differential expression analysis. Moreover, those unique clusters expressed TOX, a marker of malignant lymphocytes, and a significant although heterogeneous over-expression of genes associated with tumour cell proliferation, tumorigenesis, and resistance to apoptosis. In addition, gene expression analysis allowed the authors to identify highly proliferating lymphocytes and those clusters had 17-gene expression signature common to all tumours: ACTG1, ANP32B, ATP5C1, DUT, HMGN1, HN1, NPM1, NUSAP1, PCNA, PPA1, PPIA, PSMB2, RAN, RANBP1, SET, SMC4, and STMN1. Three of those genes, PCNA, ATP5C1, and NUSAP1, were confirmed to be co-expressed with TOX in

tumour samples, but not in normal skin and atopic dermatitis, and as a result, they have a potential to be a diagnostic marker in CTCL.

6.2. Heterogeneity of Malignant Population

The main challenge in cancer diagnosis and effective treatment is tumour heterogeneity. Single-cell RNA sequencing has enabled the gathering of molecular profiles for thousands of individual cells, thus enabling the quantitative characterization of cell heterogeneity. Identifying the origins of cellular heterogeneity and understanding how individual cells process information and respond to signals has now become a central challenge of medicine.

SS malignant cells are heterogeneous in terms of surface protein expression and mRNA profile. Buus et al., using single-cell methods, showed that SS cells have a heterogeneous expression of different T-cell markers that divide the population of malignant cells into subpopulations difficult to classified based on conventional T-cell classification [10]. Previously, it has been proposed that SS cells originate from central memory T-cells because of the expression of CD45RO, CD62L, and CD197. However, using flow cytometry, Buus et al. revealed that many malignant cells from SS patients expressed CD45RA, which is a marker of naïve T lymphocytes or stem-cell memory lymphocytes. Moreover, mRNA sequencing of 110 T-cell related genes revealed heterogeneous expression patterns between malignant T-cells. Surprisingly, even genes that have been considered to be classical biomarkers of CTCLs (ILR7, CCR7, and CXCR4) were heterogeneously expressed within some patients.

Borcherding et al. investigated the heterogeneity of the SS cells at the single-cell level and separated the malignant population into five clusters having distinct transcriptional states [12]. Differences in transcription factors expression between malignant clusters were analyzed, and a clonal evolution was predicted to start from FOXP3 positive cells to both GATA3A+ and IKZF2+. Moreover, immune phenotypes were investigated, and cells were analyzed for markers of skin-homing T-cells, central memory cells, and Tregs. Low expression of FUT7 and consistent expression of skin-homing markers (CCR4, SELPLG, and ITGB1) and central memory markers (CD28, CCr7, and SELL) have been noticed. Low expression of CD25, a Treg marker, was noticed in all clusters, but in one cluster there was a distinct population having a Treg cell-like phenotype. Functional heterogeneity between malignant cells was also confirmed based on analysis of different T-cell related gene sets and pathways.

Gaydosik et al., based on analysis of transcriptional profiles of T lymphocytes, identified large inter- and intratumour heterogeneity in advanced CTCLs skin samples [11]. A cluster analysis showed that each patient has a unique cluster characterized by expression of certain genes: RDH10, CXCL13, SCG2 (CTCL-2), FGR, IGFBP2/P6, NEFM (CTCL-5), ANO1, TNP1, CES4A, ZDHHC14 (CTCL-6), LGALS7, SERPINB3/B4, SPR2A (CTCL-8), NTRK2, and TMPRSS3 (CTCL-12). Furthermore, the expression signature of tumour-specific clusters showed activation of specific tumour-associated pathways involved in tumour cell survival, proliferation, and metastasis. Heterogeneity was also detected in the microenvironment of tumour cells, especially in the population of tumour-infiltrating CD8+ lymphocytes (TILs), which are responsible for killing cancer cells, but they are often incapable of mounting an efficient anti-tumour action. The molecular signature of those cells was based on analysis of effector molecules, checkpoint receptor inhibitors, and Treg markers, and the analysis revealed heterogeneity in both effector and exhaustion programs across patients.

7. Dysregulated Signalling Pathways Revealed in CTCL Patients by RNA Sequencing

High-throughput technologies have also become an important tool for identifying deregulations of specific signalling pathways that might be associated with disease progression and are crucial for understanding and diagnosis of haematological malignancies. So far, the alteration of several pathways has been shown to be involved in the pathogenesis of CTCL, including JAK/STAT signalling, the NFκB signalling pathway, T-cell signalling pathways, TCR associated enzymes, Th2 differentiation, epigenetic regulation, cell survival, and cell cycle checkpoint [30,55–58]. Consistent with previous reports, the following studies demonstrated a variety of affected pathways in the case of CTCLs.

Several pathways, connected with T-cell receptor signalling, IL-2 mediated signalling, and cell cycle progression, were detected in more than one study (Figure 2).

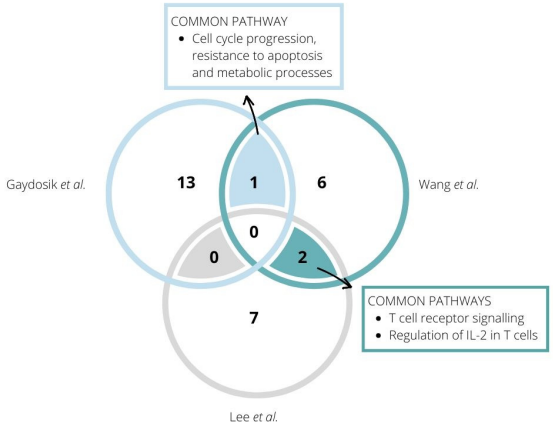

Figure 2. Unique and shared deregulated signaling pathways in Cutaneous T-cell lymphoma (CTCL) cells identified by high-throughput sequencing in the studies by Gaydosik et al., Wang et al. and Lee et al.

Lee et al.—using genes differentially expressed in three patients—demonstrated the deregulation of several signal transduction pathways in SS cells, including PI3K/Akt, TGFB, NF-kB, and T-cell receptor signalling [45]. Wang et al. performed a gene enrichment analysis that revealed alterations in pathways connected with cell cycle control, regulation of the immune system, TCR signalling, chemokine signalling, and MYC transcriptional activation [30].

Gaydosik et al. identified activation of many tumour-associated signalling pathways that are unique to each tumour: activation of eIF2, eIF4 mTOR signalling, NK-cell signalling, and virus entry via endocytic pathways [11]. Moreover, the expression of genes involved in tumour cell survival, proliferation, and metastasis (some common to glioma and non-small cell lung cancer) was identified. In addition, the inactivation of granzyme M—promoting tumour cell transformation, migration and drug resistance and related to skin inflammation and skin barrier dysfunction—was detected, as well as pathways associated with epithelial-mesenchymal-transition. Furthermore, the study reported the activation of pathways common to all proliferating T lymphocytes in each tumour, such as cell cycle progression, resistance to apoptosis, and metabolic processes.

Borcherding et al. found significant differences between malignant clusters in T-cell-related gene sets [12]. This study reported that one cluster, along with the previously noted increase in Tcm and skin-homing gene markers, was significantly enriched for gene signatures of type II interferon signalling, terminal differentiation, and cytolytic activity. Another alteration in gene set enrichment included high levels of hypoxia in another cluster. Contradictory to other clusters, in one cluster, the enrichment of anti-inflammatory and Treg markers was observed.

8. Role of Infectious Agents in Disease Onset and Progression

Many studies have investigated potential infectious involvement in triggering or promoting CTCLs. Unfortunately, the results of many of these studies are inconsistent. While a number of studies failed to show any association with pathogenic organisms, studies concluding a definite infectious role in CTCL aetiology have also been reported [59]. Several researchers examined an association with Chlamydophila pneumonia [60,61] and Borrelia burgdorferi [62,63]. However, most of the studies of possible bacterial pathogenesis have focused on investigating the role of Staphylococcus aureus [64,65].

Recently, a potential link between CTCL activity and antibiotic treatment of Staphylococcus aureus has been suggested [66]. Aggressive antibiotic treatment was associated with decreased proliferation of malignant T-cells and inhibition of the disease activity in lesional skin colonized by this pathogen, therefore providing a justification for treatment of Staphylococcus aureus in CTCL patients with severe disease. Attempts have also been made to evaluate the involvement of viral pathogens, such as human T-lymphotropic virus-1 (HTLV-1) [67–74], HTLV-2 [69,75], human immunodeficiency virus (HIV) [76–78], Epstein–Barr virus (EBV) [79–82], human herpesvirus (HHV) 6, 7, and 8 [83–86], polyomaviruses [87,88], and hepatitis C virus (HCV) [89]. Study of the HTLV-1 virus became especially controversial, as some of the researchers were convinced that it is a crucial factor in the pathogenesis of CTCL [67–69], while others indicated that MF and SS are not associated with HTLV-1 infection [70–74]. The study by Netchiporouk et al. focused on highlighting the differences between classic MF/SS an HTLV-1 driven disease [90]. It was shown that although both of the diseases on the molecular level show similar gene expression patterns, there are many differences between them. Classic MF/SS cells are mostly aneuploid, characterized by multiple chromosomal changes and large number of alterations, including mutation of TP53 and strong expression of GTSF1 negative prognostic marker. HTLV-1+ leukemia cells are mostly diploid, with only a minimal number of chromosomal aberrations and structural alterations. HTLV-1+ cells are also characterized by wild type TP53 and weak expression of GTSF1. Moreover, it was shown that while classic MF/SS cells are sensitive to HDAC inhibitor treatment, HTLV-1+ cells are relatively resistant to it. Therefore, authors indicate that HTLV-1 virus is likely not involved in the pathogenesis of CTCLs as it drives a different pathway of lymphomagensis. However, the hypothesis of a common exposure to some infectious agent supports several studies reporting CTCL occurrence in married couples [91], families [92], or even non-blood family members [93].

While searching for the activation of cancer-associated pathways in CTCLs, Gaydosik et al. detected the upregulation of several genes involved with virus entry via endocytic pathways [11]. However, Litvinov et al. were unable to detect HTLV-1 transcript in all but one patient from the endemic area [22]. Lee et al. also failed to reveal the presence of viral transcripts of HTLV-1 or other human viruses in Sézary cells [45]. However, it is possible that these transcripts escaped detection by bulk RNA-seq (but not in the more sensitive single-cell RNA seq method).

9. Clinical Significance of Novel Single-Cell RNA Sequencing Technologies

Single-cell techniques open new possibilities that can be used in the clinic, including dealing with drug resistance, designing individual and targeted treatments, and monitoring disease progression. Identification of patient-specific gene expression of malignant cells might be used in personalized therapy [11]. The analysis of unique malignant cells for each patient cluster creates a possibility for accurate therapy focused on specific pathways. On the other hand, signatures common to all tumours, such as TOX gene expression or the expression of certain genes identifying actively-proliferating lymphocytes, could be used for the diagnosis and monitoring of treatment. Importantly, scRNA-seq allows for the study of cells in the cancer microenvironment, such as TILs [11]. Understanding the heterogeneity of co-inhibitory receptor expression might be essential for efficient immunotherapy based on targeting the receptors and enhancing TILs antitumor response. The rapid and accurate diagnosis of the type and stage of the CTCLs is challenging. Borcherding et al. analyzed data from 152 CTCL patients at different stages of the disease using the same algorithm used for the differentiation of unique transcriptional states in malignant population during RNA-seq analysis [12]. Based on this analysis, genes with high expression predictive of early disease (FOXP3 and PTPN6) and late-stage disease (TGFB1, CD7, and SUZ12) were identified, which could be valuable for future diagnostic purposes. Another huge challenge is the resistance of cancer cells to drugs, which is observed beyond the treatment of CTCLs. Only 30% of CTCL patients respond to treatment with HDACi due to HDACi resistance. A recent study showed that there is a molecular explanation for it and it is associated with highly acetylated elements that may drive the high expression of genes promoting disease progression [94]. Using HDACi and single-cell technologies, Buus et al. identified a subpopulation

of SS cells that were resistant to treatment [10]. Those resistant cells were classified into the same subpopulations based on the surface markers. The consequences of cells escaping treatment are known to be serious, as they could lead to a relapse of even more aggressive diseases. This knowledge might permit the application of multiple treatments targeting different malignant populations.

10. Conclusions

High-throughput technologies provided an unprecedented view into the genomic heterogeneity of CTCLs, allowing a deeper understanding of the pathogenesis and molecular changes of CTCLs. While RNA-seq analysis showed a huge heterogeneity between patients, single-cell RNA-seq technology revealed high intratumor heterogeneity and divided malignant population within a single patient into distinct clusters. Moreover, RNA sequencing allowed the researchers to perform the transcriptome analysis not only in the fresh blood samples but also in FFPE samples, revealing many novel molecular changes, differentially expressed genes and fusion transcripts potentially involved in malignant transformation. The investigation of lncRNAs in CTCL patients uncovered previously unidentified Sézary-associated transcripts, while multiple protein coding and inflammation-mediated genes have been proposed as markers of CTCL progression or diagnosis. The overexpression of the TOX gene was highlighted, as it is considered to be correlated with an increased risk of disease progression and poor prognosis. In addition, multiple dysregulated signaling pathways were identified; however, only a few were common to more than one study. The obtained results also demonstrated a most unlikely involvement of HTLV-1 virus in CTCL pathogenesis.

As the treatment of CTCL patients remains challenging, it is hoped that personalized medicine will enable patient-specific pathways to be targeted during therapy. RNA sequencing technologies, especially those based on the single-cell analysis, can, therefore, help to advance our understanding of the disease and improve treatment.

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CONCLUSIONS

As a result of the performed analyses described in this dissertation, the following conclusions were made:

1. Increased expression of the *TMEM244* gene is associated with CTCL, especially with Sézary syndrome.
2. Expression of the *TMEM224* gene is a result of specific hypomethylation of its promoter.
3. The lack of coding potential, and not detecting expression at the protein level suggests that *TMEM244* transcripts belong to the family of long non-coding RNAs.
4. *TMEM244* is expressed at low levels in CD4+ and CD8+ subtypes of normal memory T cells (CD45RO+).
5. Silencing of *TMEM244* inhibits the growth of CTCL cell lines.
6. Quantitative analysis of *TMEM244* expression can be used as a new diagnostic marker to distinguish Sézary syndrome from diseases with a similar clinical presentation.

ABSTRACT

Cutaneous T- cell lymphomas (CTCLs) are a large, heterogenous group of T-cell skin malignancies. Among many CTCL subtypes, Sézary Syndrome (SS) and mycosis fungoides (MF) are the two most common clinical variants. Although characterized by great differences in gene expression, mutations, and chromosomal aberrations, the causes of CTCLs remain unknown. Many genetic alterations and dysregulation of signaling pathways have been reported in the CTCLs, however, the exact molecular mechanism of the pathogenesis is still to be unraveled. Due to the lack of diagnostic markers and the presence of multiple clinical presentations, the diagnosis of CTCL is difficult, resulting in an average of 6 years of confirmation from the disease onset. What's more, to date, there is no cure for CTCL, and the therapy mainly focuses on relieving symptoms and inhibiting disease progression. Because of that, there is a need for further intensive studies on CTCL, not only to understand tumor biology, but also to find a potential diagnostic marker and finally introduce new therapeutic strategies. We found that the *transmembrane protein coding 244 gene* (*TMEM244*) is ectopically expressed in all SS patients, SS-derived cell lines, and, to a lower extent, in MF and a fraction of T-cell lymphomas, but not in B-cell malignancies and peripheral blood mononuclear cells (PBMC) of healthy individuals. Therefore, the main aim of this dissertation was to identify the mechanisms of *TMEM244* expression and its function in CTCL biology that will allow to better understand this disease.

The first specifc aim of this dissertation was the identification of the mechanism responsible for *TMEM244* activation. Epigenetic dysregulations are known to play an important role in the development and progression of SS as it was shown that SS cells are characterized by widespread changes in DNA methylation. The presented results showed a negative correlation between *TMEM244* expression and promoter methylation in patient samples and T-cell lines. Moreover, by using the CRISPR-dCas9 epigenome editing system it was proved that demethylation of selected CpGs in the *TMEM244* promoter region activated *TMEM244* expression in examined cell lines, suggesting methylation to be a mechanism responsible for the regulation of its expression.

Considering elevated levels of *TMEM244* in CTCL patients and the fact that the *TMEM244* gene role has not been investigated yet, establishing the function and the coding potential of *TMEM244* was the second aim of this dissertation. By applying the GFP competition test, it was demonstrated that *TMEM244* is necessary for cellular growth in CTCL cells, therefore it might be considered a new potential therapeutic target for the treatment of

CTCL. Furthermore, by using RNA fractionation followed by qRT-PCR as well as RNA-FISH assay, cytoplasmic localization of *TMEM244* transcript was identified. Although *TMEM244* transcript is localized in the cytoplasm, by using the Western Blot method it was shown that it does not encode a protein but is rather a long non-coding RNA (lncRNA), whose specific function is still to be discovered.

The third aim of this dissertation was to examine *TMEM244* expression in a subpopulation of blood cells of healthy individuals and to address whether its expression may serve as an easy diagnostic tool for SS. By applying flow cytometry and qRT-PCR it was established that in physiological conditions generally higher expression of *TMEM244* was observed either in CD4+ or in CD8+ subsets of memory cells (CD4RO+) of peripheral blood mononuclear cells (PBMC), which is in line with the immunophenotype of Sézary cells. Additionally, as it was shown by applying qRT-PCR, *TMEM244* expression in either CD4+ T-cells or the whole population of PBMC of SS patients, can be used to distinguish this lymphoma from diseases with similar clinical presentations such as MF and non-malignant erythroderma and as a result significantly improve the diagnosis.

In summary, the results presented in this dissertation show that specific DNA demethylation of promoter is responsible for *TMEM244* expression. Moreover, *TMEM244* is necessary for the growth of CTCL cells and despite its annotation, does not code a protein but is rather a lncRNA, not previously described. Experimental studies allowed us to prove that analysis of *TMEM244* expression could be used as an easy and cheap blood diagnostic marker to distinguish SS from diseases with similar clinical presentation. Additionally, based on the available literature, the current state of knowledge on the heterogeneity of malignant cells in this lymphoma was summarized.

STRESZCZENIE

Chłoniaki skórne T-komórkowe (ang. cutenous T-cell lymphoma (CTCL)) to duża, heterogenna grupa nowotworów złośliwych charakteryzująca się nieprawidłowym nagromadzeniem złośliwych komórek T w skórze. Spośród wielu podtypów CTCL, dwoma najczęstszymi wariantami klinicznymi są Zespół Sézary'ego (ang. Sézary Syndrome (SS)) i ziarniniak grzybiasty (ang. mycosis fungoides (MF)). Pomimo wielu badań, które wykazały dużą różnorodność aberracji chromosomalnych i mutacji genowych, przyczyna SS pozostaje niezidentyfikowana. W CTCL odnotowano wiele zmian genetycznych i rozregulowanie szlaków sygnałowych, jednak dokładny mechanizm molekularny patogenezy jest nadal nieznany. Z powodu braku dostępnego markera diagnostycznego i różnorodnego obrazu klinicznego choroby, rozpoznanie CTCL jest trudne, co skutkuje średnio 6-letnim czasem diagnozy. Co więcej, do tej pory nie wynaleziono skutecznej terapii przeciwko CTCL, a leczenie koncentruje się głównie na łagodzeniu objawów i opóźnianiu postępów choroby. W związku z tym istnieje potrzeba dalszych intensywnych badań nie tylko w celu zrozumienia biologii tego chłoniaka, ale także znalezienia potencjalnego markera diagnostycznego, czy wprowadzenia nowych strategii terapeutycznych. Na podstawie własnych badań wykazano ektopową ekspresję genu kodującego białko transbłonowe 244 (ang. *transmembrane protein gene 244*, (*TMEM244*)) u wszystkich pacjentów z SS, liniach komórkowych pochodzących z SS oraz w mniejszym stopniu, w MF i w części chłoniaków z komórek T, ale nie w nowotworach z komórek B oraz komórkach jednojądrzastych krwi obwodowej (ang. peripheral blood mononuclear cells (PBMC)) zdrowych osób. W związku z tym, celem niniejszej rozprawy doktorskiej było scharakteryzowanie mechanizmów związanych z ekspresją i funkcją genu *TMEM244* w biologii CTCL w celu lepszego zrozumienie tej choroby.

Pierwszym zagadnieniem podjętym w ramach niniejszej rozprawy doktorskiej była identyfikacja mechanizmu odpowiedzialnego za aktywację genu *TMEM244*. Dane literaturowe wskazują, że deregulacja epigenetyczna odgrywa ważną rolę w rozwoju i progresji SS, w związku z tym, że komórki SS charakteryzują się licznymi zmianami w metylacji DNA. Przedstawione wyniki wykazały ujemną korelację między ekspresją genu *TMEM244,* a metylacją DNA jego regionu promotorowego w próbkach pozyskanych od pacjentów oraz w liniach komórkowych z limfocytów T. Ponadto za pomocą systemu edycji epigenomu CRISPR-dCas9 wykazano, że demetylacja wybranych wysp CpG w regionie promotorowym *TMEM244* aktywuje jego ekspresję w badanych liniach komórkowych, co sugeruje, że metylacja jest mechanizmem odpowiedzialnym za regulację jego ekspresji.

Biorąc pod uwagę indukcję ekspresji genu *TMEM244* u pacjentów z CTCL oraz fakt, że rola genu *TMEM244* nie została jak dotąd zbadana, celem drugiej części badań było zidentyfikowanie funkcji i potencjału kodujący genu *TMEM244*. Za pomocą testu kompetycji wzrostu komórek z białkiem zielonej fluorescencji (ang. green fluorescent protein (GFP)) wykazano, że ekspresja genu *TMEM244* jest niezbędna do wzrostu komórek w liniach z tej grupy chłoniaków, co pokazuje zasadność uznania go za nowy potencjalny cel terapeutyczny w leczeniu CTCL. Ponadto, stosując qRT-PCR poprzedzony frakcjonowaniem RNA, a także fluorescencyjną hybrydyzację in situ (ang. fluorescent in situ hybridization (FISH)), potwierdzono cytoplazmatyczną lokalizację transkryptu genu *TMEM244*. Pomimo, że transkrypt genu *TMEM244* zlokalizowany jest w cytoplazmie, przy pomocy metody Western Blot wykazano, że nie koduje on białka, ale jest raczej długim niekodującym RNA (ang. long non-coding RNA (lncRNA)), którego dokładna funkcja wciąż pozostaje do ustalenia.

W trzeciej części prowadzonych badań postanowiono sprawdzić ekspresję *TMEM244* w subpopulacjach krwi zdrowych osób oraz ustalić, czy ekspresja tego genu może służyć jako łatwe narzędzie diagnostyczne dla SS. Za pomocą cytometrii przepływowej i qRT-PCR w prawidłowych komórkach PBMC zaobserwowano wyższą ekspresję *TMEM244* zarówno w podgrupach CD4+, jak i CD8+ komórek pamięci (CD4RO+), co jest zgodne z immunofenotypem komórek Sezary'ego. Dodatkowo, jak wykazano stosując qRT-PCR, ekspresja *TMEM244* zarówno w limfocytach T CD4+, jak i w całej populacji PBMC pacjentów z SS, może być wykorzystana do odróżnienia SS od chorób o podobnym obrazie klinicznym, jak MF i erytrodermii nienowotworowej, a w rezultacie znacząco poprawić diagnostykę tej jednostki chorobowej.

Podsumowując, przeprowadzone badania wykazały, że metylacja DNA jest odpowiedzialna za regulację ekspresji genu *TMEM244*. Co więcej, udowodniono, że ekspresja genu *TMEM244* ma wpływ na wzrost komórek CTCL, a gen ten pomimo swojej adnotacji nie koduje białka, lecz jest prawdopodobnie długim niekodujących RNA. Badania eksperymentalne pozwoliły wykazać, że analiza ekspresji genu *TMEM244* może być wykorzystana jako łatwy i tani marker diagnostyczny z krwi służący do odróżnienia SS od chorób o podobnym obrazie klinicznym. Dodatkowo na podstawie dostępnej literatury usystematyzowano aktualny stan wiedzy na temat heterogenności komórek nowotworowych w tej grupie chłoniaków.

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ZHOU, X., POPESCU, N. C., KLEIN, G. & IMREH, S. 2007. The interferon-alpha responsive gene TMEM7 suppresses cell proliferation and is downregulated in human hepatocellular carcinoma. *Cancer Genet Cytogenet,* 177**,** 6-15.

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1st ARTICLE CO-AUTHOR STATEMENTS

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Poznan, 14.02.2023
Katarzyna Iżykowska
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I confirm that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

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.

My contribution to the cited publication was design of the study, isolation of lymphocytes T from the skin and blood, cell line culturing and transduction, vector and sgRNA design and cloning, RT-qPCR expression analysis, interpretation of the results, statistical analysis, table and figures preparation, writing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

(signature)

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Poznan, 13.02.2023
Magdalena Żurawek
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication which I am a co-author:

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

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.

My contribution to the cited publication was production of the viral vectors, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

Magdalena Żurawek
(signature)

Poznań, 05.02.2023
Karolina Olek-Hrab
Private Practice

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

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.

My contribution to the cited publication was providing patient samples along with clinical data, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,
K. Olek-Hrab
(signature)

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Poznań, 14.02.2023

Prof. Maciej Giefing, PhD
Head
Department of Cancer Genetics
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication which I am a co-author:

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

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.

My contribution to the cited publication included designing the DNA methylation analysis, evaluation of the results, contribution to the final version of manuscript, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

Z-CA DYREKTORA
ds. Naukowych
Instytutu Genetyki Człowieka PAN

With regards,
prof. dr hab. Maciej Giefing
.....
(signature)

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Poznań, 30.01.2023.
Prof. Grzegorz Przybylski, PhD, MD
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

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.**

My contribution to the cited publication was design of the study, overall oversee of the experiments, planning and preparing the final version of the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

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Poznan, 27.01.23
Karolina Rassek
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree, hereby I confirm that I have greatly contributed to the following publication which I am a co-author:

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

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.

My contribution to the cited publication was:

- patient samples preparation: density gradient centrifugation of peripheral blood mononuclear cells and $CD4^+$ T cells sorting
- cell lines culturing and viral transductions
- most DNA and RNA isolations
- reverse transcriptions and qRT-PCRs
- most Western Blot experiments
- expression data analyses
- reviewing and preparing the final manuscript version.

I would like to use the above publication in my PhD thesis entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,
Karolina Rassek

2nd ARTICLE CO-AUTHOR STATEMENTS

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Poznan, 14.02.2023
Katarzyna Iżykowska
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I confirm and state that Karolina Rassek greatly contributed to the following publication which I am co-author:

***TMEM244* is a long non-coding RNA necessary for CTCL cell growth**

Rassek K., Iżykowska K., Żurawek M., Pieniawska M., Nowicka K., Zhao X., Przybylski G.K.

My contribution to the cited publication was overall oversee of the experiments and data analysis, planning RACE and RNA-FISH experiments, performing RACE experiments, performing flow cytometry and analysis of the data, cell culturing, writing and correcting the manuscript, participating in the process related to the publishing procedure

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

(signature)

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Poznan, 13.02.2023
Magdalena Żurawek
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication which I am a co-author:

***TMEM244* is a long non-coding RNA necessary for CTCL cell growth**

Rassek K., Iżykowska K., **Żurawek M.**, Pieniawska M., Nowicka K., Zhao X., Przybylski G.K.

My contribution to the cited publication was cell culturing, flow cytometry analysis, writing, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,
Magdalena Żurawek
(signature)

Hanzeplein 1, 9713 GZ Groningen, 2023-2-13
Xing Zhao
University Medical Center Groningen

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication which I am a co-author:

***TMEM244* is a long non-coding RNA necessary for CTCL cell growth**

Rassek K., Iżykowska K., Żurawek M., Pieniawska M., Nowicka K., **Zhao X.**, Przybylski G.K.

My contribution to the cited publication was biostatistics analysis, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

Xing Zhao
(signature)

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Poznań, 30.01.2023.
Prof. Grzegorz Przybylski, PhD, MD
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

***TMEM244* is a long non-coding RNA necessary for CTCL cell growth**

Rassek K., Iżykowska K., Żurawek M., Pieniawska M., Nowicka K., Zhao X., **Przybylski G.K.**

My contribution to the cited publication was concept of the study, overall oversee of the experiments, reviewing and editing the final version of the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

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Poznan, 15.02.23
Karolina Rassek
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree, hereby I confirm that I have greatly contributed to the following publication which I am a co-author:

***TMEM244* is a long non-coding RNA necessary for CTCL cell growth**

Rassek K., Iżykowska K., Żurawek M., Pieniawska M., Nowicka K., Zhao X., Przybylski G.K.

My contribution to the cited publication was:

- cell lines culturing
- viral transductions
- FISH assays
- RNA isolations
- reverse transcriptions and qRT-PCRs
- Western Blot experiments
- expression data analyses
- GFP competition assays
- most apoptosis assays
- figures preparation
- writing the manuscript draft and preparing the final manuscript version.

I would like to use the above publication in my PhD thesis entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,
Karolina Rassek

3rd ARTICLE CO-AUTHOR STATEMENTS

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Poznan, 14.02.2023
Katarzyna Iżykowska
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I confirm that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

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.

My contribution to the cited publication was overall oversee of the experiments and data analysis, creating a database of patients, lymphocyte T separation from blood and skin biopsies, separation of peripheral blood mononuclear cell subpopulations, cell staining and flow cytometry performing and analysis, writing, reviewing and editing the final version of the manuscript, coordinating the process related to the publishing procedure as a corresponding author.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

(signature)

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Poznan, 13.02.2023
Magdalena Żurawek
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication which I am a co-author:

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

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.

My contribution to the cited publication was separation of lymphocytes T from the blood, reviewing and editing the final version of the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

Magdalena Żurawek
(signature)

Gdańsk, 30.01.2023

Berenika Olszewska, MD, PhD
Department of Dermatology, Venereology and Allergology,
Faculty of Medicine, Medical University of Gdansk,
Gdansk, Poland

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

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.

My contribution to the cited publication was selection of patients samples, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

.....
(signature)

Małgorzata Sokołowska-Wojdyło
Katedra i Klinika Dermatologii, Wenerologii i Alergologii Gumed
ul. Smoluchowskiego 17, Gdańsk
(adres na dniu Gumed ul. Skłodowskiej-Curie 3a, Gdańsk)

2023.04.06

Address, date
Name
Institution

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

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.

My contribution to the cited publication was selection and characterization of patient samples, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

(signature)

Prof. dr hab. n. med. Małgorzata Sokołowska-Wojdyło
specjalista dermatolog wenerolog
(mgr) biotechnolog
NR 4711498

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Poznan, 30.01.2023.

Prof. Grzegorz Krzysztof Przybylski, PhD, MD
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I state that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

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.**

My contribution to the cited publication was concept of the study, overall oversee of the experiments, reviewing and editing the final version of the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

G. Przybylski

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Poznan, 27.01.23
Karolina Rassek
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree, hereby I confirm that I have greatly contributed to the following publication which I am a co-author:

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

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.

My contribution to the cited publication was:

- patient samples preparation: density gradient centrifugation of peripheral blood mononuclear cells and $CD4^+$ T cells sorting
- RNA isolations
- reverse transcriptions and qRT-PCRs
- expression data analyses
- preparation of figures and tables
- writing the manuscript draft and preparing the final manuscript version.

I would like to use the above publication in my PhD thesis entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

Karolina Rassek

4th ARTICLE CO-AUTHOR STATEMENTS

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Poznan, 14.02.2023
Katarzyna Iżykowska
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree to Karolina Rassek, hereby I confirm that Karolina Rassek greatly contributed to the following publication of which I am a co-author:

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

Rassek K., Iżykowska K.

My contribution to the cited publication was co-design of the study, reviewing and editing the manuscript.

At the same time, I agree to use the above publication in the PhD thesis of Karolina Rassek entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

.....
(signature)

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Poznan, 27.01.2023
Karolina Rassek
Institute of Human Genetics PAS

STATEMENT FOR DOCTORAL THESIS

Regarding the procedure for award of PhD degree, hereby I confirm that I have greatly contributed to the following publication which I am a co-author:

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

Rassek K., Iżykowska K.

My contribution to the cited publication was:

- co-desing of the study
- selection of the reviewed literature
- preparation of figures and tables
- writing the manuscript draft and preparing the final manuscript version.

I would like to use the above publication in my PhD thesis entitled: *Identification of mechanisms related to the expression of TMEM244 gene and its role in cutaneous T-cell lymphomas*. This PhD thesis is prepared under the guidance of prof. Grzegorz Krzysztof Przybylski (supervisor) and Katarzyna Iżykowska (co-supervisor).

With regards,

Karolina Rasek