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General introduction

Primary cutaneous lymphomas represent a heterogeneous group of non-Hodgkin lymphomas (NHL) presenting in the skin without evidence of extracutaneous disease at diagnosis. After the gastro-intestinal tract lymphomas, primary cutaneous lymphomas are the second most common group of extra-nodal NHL with an estimated annual incidence of 1:100.000 individuals.[1] Primary cutaneous lymphomas often have a completely different clinical behaviour and prognosis when compared to morphologically similar lymphomas arising in lymph nodes, and therefore require different types of treatment.[2] For this reason they have been included as separate entities in recent classifications systems for non-Hodgkin lymphomas, such as the World Health Organization - European Organization for Research and Treatment of Cancer (WHO-EORTC) classification for cutaneous lymphomas and the WHO classification of lymphoid neoplasms 2008.[2;3] Within these classifications two main groups of primary cutaneous lymphomas can be distinguished: primary cutaneous T-cell lymphomas (CTCL) accounting for 75% of the cases in the Western world, and primary cutaneous B-cell lymphomas (CBCL) that account for the remaining 25%.[2]

Mycosis fungoides (MF) and Sézary syndrome (SS) are the most well-known types of CTCL. MF has generally an indolent disease course with over the years or decades slow progression from patches and plaques to eventually tumors and in some cases extracutanous disease. SS is regarded a leukemic variant of CTCL with often a poor prognosis. The studies in this thesis focused on diagnostic and prognostic parameters in MF and SS. In this introductory chapter the clinical features, histology, molecular aspects, differential diagnosis and prognostic features of these two types of CTCL are presented.

MYCOSIS FUNGOIDES

CLINICAL FEATURES

MF is the most common type of CTCL, accounting for almost 50% of all cutaneous lymphomas.[2] MF usually affects older adults with a median age around 60 years, but may occur in children and adolescents as well.[4-7] Men are affected more often than women, with a male-to-female ratio of 1.6–2:1.[4-8] MF is clinically characterized by the slow evolution of patches and plaques to eventually tumors.[2] Extracutaneous dissemination occurs in a minority of patients. Preferred localizations of skin lesions are the buttocks and other non-sun-exposed areas. Patients with tumor stage MF usually show a combination of patches, plaques and (ulcerating) tumors. The staging of mycosis fungoides is based on the tumor-node-metastasis-blood (TNMB) staging system, which classifies both type and extent of skin lesions, the presence and degree of lymph node, visceral and blood involvement (**Tables 1 and 2**).[9] This staging system is important, since it determines management and treatment and has prognostic significance.

Table 1. TNMB classification of mycosis fungoides and Sézary syndrome.[9]

Classification	Description
T (skin)	
T_1	Limited patch/ plaque (< 10% of total skin surface)
T_2	Generalized patch/ plaque (≥ 10% of total skin surface)
T_3	One or more tumors (≥ 1 cm diameter)
T_4	Erythroderma (≥ 80% of total skin surface)
N (lymph node)	
N_0	No clinically enlarged lymph nodes
N_1	Clinically enlarged lymph nodes, histologically uninvolved
N_2	Clinically enlarged lymph nodes, histologically involved (nodal architecture uneffaced)
N_3	Clinically enlarged lymph nodes, histologically involved (nodal architecture (partially) effaced)
M (viscera)	
M_0	No visceral involvement
M_1	Visceral involvement
B (blood)	
B_0	No circulating atypical (Sézary) cells (or < 5% of lymphocytes)
B_1	Low blood tumor burden (≥ 5% of lymphocytes are atypical (Sézary) cells, but does not meet criteria B_2)
B_2	High blood tumor burden (≥ 1000/μL Sézary cells with positive clone)

Table 2. Clinical staging system for mycosis fungoides and Sézary syndrome.[9]

IA	T_1	N_0	M_0	B_{0-1}
IB	T_2	N_0	M_0	B_{0-1}
IIA	T_{1-2}	N_{1-2}	M_0	B_{0-1}
IIB	T_3	N_{0-2}	M_0	B_{0-1}
III	T_4	N_{0-2}	M_0	B_{0-1}
IVA_1	T_{1-4}	N_{0-2}	M_0	B_2
IVA_2	T_{1-4}	N_3	M_0	B_{0-2}
IVB	T_{1-4}	N_{0-3}	M_1	B_{0-2}

HISTOLOGY AND PHENOTYPE

The histology of patch and plaque MF is characterized by a band-like infiltrate in the papillary dermis consisting of atypical lymphocytes with small- to medium-sized, indented (cerebriform) nuclei and histiocytes.[2;10] In these early stages the malignant cells are preferentially localized in the epidermis (epidermotropism). Intraepidermal collections of atypical cells (Pautrier microabscesses) are highly characteristic, but observed in only a minority of cases.[11] In tumor stage MF, the dermal infiltrate becomes more diffuse containing variable numbers of small, medium-sized, to large cerebriform cells and blast

cells with prominent nuclei, and epidermotropism may be lost. The atypical cells in MF have a CD3+, CD4+, CD45RO+ and CD8− memory T-cell phenotype, but in rare cases a CD4−, CD8+ or a CD4−, CD8− T-cell immunophenotype is found.[12-15] Loss of pan-T cell antigens such as CD2, CD3, CD5 and CD7 is a common aberration in MF.[10]

GENETIC FEATURES

Several studies on tumor stage MF using array-based comparative genomic hybridization reported the same recurrent genetic aberrations including gains of chromosome 7q21-22 (55−60%), 8q24 (32%) and 17q21 (37−41%) and loss of 9p21 (30−42%) and 13q14 (20−36%).[16-18] Loss of 9p21 harboring *CDKN2A*, *CDKN2B* and *MTAP* tumor suppressor genes, has been associated with a shorter survival in patients with tumor stage MF.[16-19]

Several studies reported constitutive activation of the NF-κB pathway in MF, which may be explained in part by down-regulation of *NFKBIZ*, an inhibitor of this pathway.[20;21] Gene expression studies in early stage MF revealed overexpression of *TOX*, which may turn out to be a useful diagnostic marker.[22]

PROGNOSIS AND PROGNOSTIC FEATURES

The prognosis of MF patients is closely correlated with clinical stage, and in particular the type and extent of skin lesions and the presence of extracutaneous disease.[4-6] While the survival in MF stage IA is comparable with age-, race- and sex-matched control population, the prognosis deteriorates with progression of disease.[4;23;24] The 10-year disease-specific survival (DSS) is 95−97 % for stage IA, 77−83% for stage IB, 42% for stage IIB, but only 20% for patients with stage IV.[5;8] Patients usually die of systemic involvement or infections. Apart from clinical stage, advanced age, male sex, folliculotropic MF and large cell transformation have been associated with adverse prognosis in MF.[5;6;8;25-33]

In the current classification patients with only skin tumors are categorized in one group (stage IIB), but clinical observations show considerable variation in number of tumors and time interval between each tumor occasion in these patients with MF stage IIB disease. Previous studies that investigated the relation between tumor formation and survival focussed on tumor distribution (solitary, localized, regional or generalized) only.[8;26;34] Talpur et al found that patients who have generalized skin tumors at diagnosis of MF have a reduced survival compared to those who present with only a solitary tumor.[26] Benner et al described similar results for the number of tumors in patients with transformed MF.[34] However, these studies did not quantify the exact number of tumors, nor investigated the number of tumors that developed during follow-up.

SÉZARY SYNDROME

DEFINITION AND CLINICAL FEATURES

Sézary syndrome (SS) is a rare and aggressive type of CTCL derived from CD4+ skin-homing memory T cells. SS is characterized historically by the triad of erythroderma, generalized lymphadenopathy and neoplastic T cells (Sézary cells) in skin, lymph nodes and peripheral blood.[35] Additional clinical features are ectropion, alopecia, onychodystrophy,

palmoplantar hyperkeratosis and severe pruritus. The diagnosis of SS is based on clinical presentation (erythroderma and lymphadenopathy) and demonstration of a T-cell clone in the peripheral blood (preferably the same clone in skin), in combination with one or more of the following criteria: an absolute Sézary cell count ≥ 1000 cells per mm^3; loss of T-cell markers CD2, CD3, CD4 and /or CD5; and /or an expanding population of CD4+ T cells leading to a CD4/CD8 ratio of more than 10.[2;3] However, rare cases of SS without erythroderma, but otherwise fulfilling the diagnostic criteria, have been described.[36]

HISTOLOGY AND PHENOTYPE

The histology of SS is variable. It may be similar to that of MF, but cases of SS more often show a monotonous band-like or perivascular infiltrate in the papillary dermis, that is mainly composed of lymphocytes with atypical or cerebriform nuclei. Epidermotropism may be present and Pautrier microabscesses may be found. However, in up to one third of SS cases histology may only show reactive changes.[37;38]

The malignant cells in SS consistently have a CD3+, CD4+ and CD8− T-cell phenotype. Flow cytometry studies of peripheral blood reported frequent loss of CD7 and CD26 and reported expression of killer cell immunoglobulin (KIR)-like receptors CD158a, CD158b and CD158k by Sézary cells.[39-51] Other studies described that Sézary cells have a "central memory" T-cell phenotype (CD27+, CD45RA−, CD45RO+).[42;45;52;53]

GENETIC FEATURES

Many studies have investigated the peripheral blood of SS patients for numerical and structural chromosomal alterations. Investigations on copy number alterations identified gain of *JUNB* (57%), *MYC* (75%) and loss of *MYC* antagonists *MNT* (55%) and *MXI1* (40%) as recurrent genetic lesions in the SS genome.[54-56] Mutations in *PLCG1*, *NRAS* and *P53* have been reported in SS, albeit at a low frequency.[57-60]

Other molecular studies describe altered gene expression of one or more genes in SS. Increased expression of *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *JUNB*, *TWIST1*, *EPHA4*, *MYC* and *TOX* and decreased expression of *STAT4* by Sézary cells have been reported and regarded as potential diagnostic markers for SS.[55;61-70] One study showed that a combination of *TWIST* and *PLS3* or *KIRD3DL2* expression could diagnose 98% of SS patients and found *TWIST* as the strongest diagnostic marker with positivity in 91% of SS patients.[71]

However, most of these molecular biomarkers were identified in small, single center studies with limited number of patients and controls and have not been confirmed in large independent studies.

EPIGENETIC FEATURES

Epigenetics is defined as heritable alterations in gene expression that are not caused by changes in primary DNA sequence and include aberrant DNA methylation, histone modification and non-coding RNAs (microRNAs).[72;73]

Epigenetic changes have been linked to the development and progression of cancer.[73] The importance of these changes in the molecular pathogenesis of SS is illustrated by the clinical efficacy of romidepsin, a histone deacetylase inhibitor, in 32% of SS patients.[74]

DNA hypermethylation of CpG islands in promoter regions of tumor suppressor genes leads to silencing of the gene, while global DNA hypomethylation is associated with chromosomal instability.[75;76] Previous studies that investigated DNA methylation in MF and SS have mainly focused on singles genes. In SS tumor suppressor genes *CDKN2A* and *FAS* were found to be frequently silenced by promoter hypermethylation.[77;78] One study describes genome-wide DNA methylation patterns in aggressive CTCL (transformed mycosis fungoides and primary cutaneous peripheral T-cell lymphoma, unspecified) and an indolent entity (CD30-positive large T-cell lymphoma, currently termed primary cutaneous anaplastic large cell lymphoma) and found widespread promoter hypermethylation associated with inactivation of several tumor suppressor genes.[79] Studies analyzing genome-wide DNA methylation in SS have not yet been performed.

MicroRNAs (miRNAs) are a group of small non-coding single-strand RNA molecules that regulate gene expression by inhibiting protein translation.[80] MicroRNAs can play a role in cancer by targeting proteins with a tumor suppressor function.[81] Studies investigating the miRNome in SS found that miR-21, miR-486 and miR-214 were frequently up-regulated and play a possible role in cell survival.[82;83]

DIFFERENTIAL DIAGNOSIS

Especially in the early stages of the disease, it can be challenging to differentiate SS from erythrodermic inflammatory dermatoses (EID). The clinical presentation is generally not discriminative and histology may show reactive changes in up to one third of the cases.[37;38]

Recent immunohistochemical studies suggested that expression of programmed death-1 (PD-1) by more than 50% of skin-infiltrating T cells and expression of CD7 by less than 20% or by less than 50% of the skin-infiltrating T cells are useful additional criteria to differentiate between SS and EID.[84;85] Other studies reported increased expression of thymocyte selection-associated high mobility group box protein (TOX) by the malignant CD4+ T cells in MF and SS, while skin-infiltrating T cells in benign inflammatory dermatoses did not.[22;68;86;87] However, TOX expression has not been studied in patients with EID.

Since clinicopathologic features are often not decisive, the diagnosis of SS relies heavily on demonstration of neoplastic cells in the peripheral blood. Because atypical T cells can also be observed in the peripheral blood of patients with EID and even in normal controls, an expanded CD4+ T-cell population resulting in a CD4/CD8 ratio above 10 and demonstration of clonal T-cell receptor gene rearrangements were included as additional criteria for the diagnosis of SS.[88-92] For Sézary patients who do not fulfill the current immunophenotypic criteria for SS, CD4+CD7− cells of at least 40% and CD4+CD26− cells of 30% or more have been suggested as tentative diagnostic criteria.[9;46;93;94] However, an important drawback of the current diagnostic criteria is lack of specific SS biomarkers that would facilitate diagnosis and quantification of tumor cells.

PROGNOSIS AND PROGNOSTIC FEATURES

Sézary patients have been reported to have a poor prognosis with a 5-year disease specific survival (DSS) of 24−31%.[2;8] Prognostic factors associated with a worse survival reported in SS include advanced age, short duration of skin lesions before diagnosis of

SS, previous history of MF, elevated levels of serum lactate dehydrogenase (LDH) and (the degree of) lymph node involvement.[6;8;25;26;95-101] Other prognostic factors described in SS mostly reflect the blood tumor burden, such as increased leukocyte count and high Sézary cell count.[26;98-103] However, the results of these various studies are inconsistent, which may be due to the use of different diagnostic criteria of SS, for instance inclusion of patients without a T-cell clone in the peripheral blood, and analysis of mixed populations of patients with SS and MF. Whether immunophenotypic and molecular biomarkers diagnostic for SS have prognostic value has not been investigated.

AIMS AND OUTLINE OF THIS THESIS

The studies presented in this thesis were aimed to identify useful diagnostic and prognostic markers in tumor stage MF and SS. The first four studies focused on SS, and in particular its differentiation from EID.

In **chapter 2** the sensitivity and specificity of several previously reported immunophenotypic and molecular biomarkers for SS were investigated in a European multicenter study in 59 well-defined SS patients compared to 19 EID patients. Standard operating procedures were used to allow comparison of experimental results from different centers.

Chapter 3 evaluates the prognostic significance of the molecular biomarkers diagnostic for SS that were identified in **chapter 2** (*MYC* gain, *MNT* loss, up-regulation of *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and down-regulation of *STAT4*) and previous reported prognostic markers in 64 Sézary patients.

Two potential useful additional immunohistochemical markers to discriminate between SS and EID are TOX and C-MYC. In **chapter 4** we investigated the expression of TOX and C-MYC on skin biopsies of 15 patients with SS compared to 17 patients with EID.

To define patterns of aberrant DNA methylation with potential relevance for the pathogenesis of SS and to identify epigenetic biomarkers that can be used in the differential diagnosis of SS and EID we performed in **chapter 5** whole-genome sequencing in 15 SS patients and a validation group of 20 SS patients compared to 3 EID patients.

Chapter 6 was focused on tumor stage MF. In this chapter the variability in tumor development of 46 MF patients with stage IIB was quantified by calculating a frailty score, based on both the number of tumors developed during follow-up and the time interval between each tumor occasion, and investigated the correlation with survival.

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2

Evaluation of immunophenotypic and molecular biomarkers for Sézary syndrome using standard operating procedures: a multicenter study of 59 patients

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ABSTRACT

Differentiation between Sézary syndrome and erythrodermic inflammatory dermatoses can be challenging and a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells that could be useful as additional diagnostic criteria. In this European multicenter study, the sensitivity and specificity of these immunophenotypic and recently proposed but unconfirmed molecular biomarkers in Sézary syndrome were investigated. Peripheral blood CD4+ T cells from 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses were analyzed for cell surface proteins by flow cytometry and for copy number alterations and differential gene expression using custom-made quantitative PCR plates. Experiments were performed in duplicate in two independent centers using standard operating procedures with almost identical results. Sézary cells showed *MYC* gain (40%) and *MNT* loss (66%); up-regulation of *DNM3* (75%), *TWIST1* (69%), *EPHA4* (66%) and *PLS3* (66%); and down-regulation of *STAT4* (91%). Loss of CD26 (≥ 80% CD4+ T cells) and/ or CD7 (≥ 40% CD4+ T cells) and combination of altered expression of *STAT4*, *TWIST1*, and *DNM3* or *PLS3* could distinguish, respectively, 83% and 98% of patients with Sézary syndrome from patients with erythrodermic inflammatory dermatoses with 100% specificity. These additional diagnostic panels will be useful adjuncts in the differential diagnosis of Sézary syndrome versus erythrodermic inflammatory dermatoses.

INTRODUCTION

Sézary syndrome (SS) is a rare and aggressive type of cutaneous T-cell lymphoma that is derived from CD4+ skin-homing memory T cells and characterized by erythroderma, generalized lymphadenopathy and neoplastic T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood.[1]

Differentiation between SS and erythrodermic inflammatory dermatoses (EID) may be extremely difficult, both clinically and histopathologically, but is very important from therapeutic and prognostic perspectives. For a long time the diagnosis was based on demonstration of atypical T cells, so-called Sézary cells, in blood smears.[2;3] However, it was demonstrated that Sézary cells can also be observed in the peripheral blood of patients with EID and even in healthy control subjects.[4;5] Demonstration of at least 1000 Sézary cells per mm^3 was often used as a decisive criterion, but this was not generally agreed on.[1] To prevent patients with EID being misclassified as having SS and being treated as such, in 1997 the European Organization for Research and Treatment of Cancer group proposed the demonstration of clonal T cells and the presence of an expanded CD4+ T-cell population resulting in a CD4/CD8 ratio above 10 as additional criteria for a diagnosis of SS.[6]

At present, the diagnosis of SS is based on clinical presentation (erythroderma and lymphadenopathy) and demonstration of a T-cell clone in the peripheral blood (preferably the same clone in the skin), in combination with one or more of the following criteria: an absolute Sézary cell count greater than 1000 cells per mm^3; loss of T-cell markers CD2, CD3, CD4, and /or CD5; and /or an expanding population of CD4+ T cells leading to a CD4/CD8 ratio of more than 10.[7;8] However, distinction between SS and EID can still be difficult, because T-cell clonality can be observed in a substantial proportion of patients with EID as well, and not all SS patients have a CD4/CD8 ratio of greater than 10 at first presentation.[9;10]

To solve this diagnostic problem, a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells. Flow cytometry studies reported loss of CD7 and CD26 expression by Sézary cells and suggested CD4+CD7− cells of at least 40% and CD4+CD26− cells of at least 30% as tentative diagnostic criteria in those difficult cases.[10-22]

In addition, recent studies described expression of killer cell immunoglobulin-like receptors CD158a, CD158b, and CD158k and the “central memory” T cell phenotype (CD27+, CD45RA−, CD45RO+) as characteristic features of Sézary cells.[14;16;17;23-30] Molecular investigations identified gain of *JUNB*, *MYC*, and loss of *MYC* antagonists *MNT* and *MXI1* as recurrent genetic lesions in the SS genome.[31-33] Gene expression studies showed increased expression of *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *JUNB*, *TWIST1*, *EPHA4*, and *MYC* and decreased expression of *STAT4* in Sézary cells.[32;34-39]

However, the diagnostic value of these biomarkers in diagnosing SS has not been investigated thoroughly. Moreover, most biomarkers were identified in small, single-center studies with a limited number of patients and controls and have not been confirmed in large independent studies. In addition, flow cytometry studies have used widely differing protocols, which impedes interpretation and comparison of results from

different studies.

The goal of this European Organization for Research and Treatment of Cancer multicenter study was to investigate the sensitivity and specificity of these biomarkers for SS in a large group of well-defined SS patients compared with EID patients using standard operating procedures (SOPs).

METHODS

DESIGN OF THE STUDY

To achieve sufficient power for the study, a consortium of six European Organization for Research and Treatment of Cancer centers with extensive experience with SS was formed including centers from Helsinki, Finland; London, United Kingdom; Leiden, The Netherlands; Mannheim, Germany; Turin, Italy; and Paris, France. At time of inclusion peripheral blood samples were collected for investigation of (i) expression of cell surface proteins by flow cytometry, (ii) copy number variation (CNV), and (iii) gene expression (GE) profiles. The markers were selected based on the literature and are presented in **Supplementary Table S1**.

To optimize standardization and to prevent interdepartmental differences, SOPs were produced for the workflow of blood sampling, isolation of peripheral blood mononuclear cells (PBMCs), and enrichment for CD4+ T cells (SOP 001), DNA isolation (SOP 002), RNA isolation (SOP 003), complementary DNA synthesis (SOP 004), CNV and GE assays (SOP 005), flow cytometry experiments (SOP 007), and the freezing and shipment of samples (SOP 008) (**Supplementary Figure S1** and **Supplementary Materials** online). Much effort was put into standardizing flow cytometry analysis, because this technique has been shown to have limited reproducibility in multicenter studies because of limited standardization of laboratory procedures, instrumental settings, and interpretation of results.[40;41]

To test if the use of SOPs leads to increased reproducibility, the flow cytometry experiments were performed in duplicate in Leiden and Paris on all samples, and assays for CNV and GE were performed in Leiden and repeated in London for a selected number of samples.

In all participating centers the study was approved by the local institutional ethical review boards, and written informed patient consent was obtained. Consensus meetings to compare experimental results were held on August 31, 2012, in Paris and October 31, 2013, in Leiden.

PATIENT SELECTION AND CLINICAL CHARACTERISTICS

Between September 2009 and October 2013 a total of 103 subjects were enrolled with the following diagnosis: SS (n = 72), EID (n = 27), and healthy controls (n = 4).

Inclusion criteria for the SS patients were diagnosis of SS based on the recent World Health Organization - European Organization for Research and Treatment of Cancer criteria and available complete clinical data. Inclusion criteria for patients with EID were

presentation with erythroderma and blood test results not meeting the SS blood criteria.

From the initial 72 patients with SS 13 were excluded because of inferior sample quality (n = 10) or insufficient clinical data (n = 3). From the initial 27 EID patients eight were excluded because of inferior sample quality (n = 3) or insufficient clinical data (n = 5).

The final study group consisted of 59 patients with SS, 19 patients with EID, and 4 healthy controls. The SS group consisted of 32 patients with newly diagnosed SS and 27 patients with known SS. Thirty-six SS patients received treatment at the time of blood sampling (10 newly diagnosed with SS and 26 with known SS). The treatment consisted of psoralen plus UVA therapy (n = 2), extracorporeal photopheresis as monotherapy or combined with immunomodulatory agents (n = 12), prednisone alone or in combination with chlorambucil (n = 9), monotherapy with interferon alfa, bexarotene, methotrexate, or acitretin (n = 11), and polychemotherapy (n = 2).

The EID group included nine patients with atopic erythroderma, five patients with erythrodermic psoriasis, two patients with erythrodermic drug eruption, two patients with idiopathic erythroderma, and one patient with paraneoplastic erythroderma secondary to a cholangiocarcinoma. None of the EID patients developed a lymphoma during follow-up (median follow-up = 22 months, range = 8-38 months).

WORKUP BLOOD SAMPLES

PBMCs were isolated from peripheral blood and stored in liquid- or vapour-phase nitrogen. Part of the fresh PBMCs were enriched for CD4+ T helper cells by depletion of non-CD4+ T cells, resulting in greater than 95% purity for the CD4+ T-cell population, using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) for CNV and GE assays.

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at -20 °C.

RNA was isolated using the RNeasy Mini Kit (Qiagen), which included on-column DNase digestion. Two µg of RNA was reverse-transcribed in triplicate with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA), using random priming in a final volume of 20 µL. After synthesis, complementary DNA samples were stored at -20 °C. A detailed description of this workup of blood samples is found in the **Supplementary Materials** (SOPs 001-004 and 008).

FLOW CYTOMETRY

In Leiden and Paris flow cytometry was performed for the following antigens: CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD27, CD45RA, CD45RO, CD158a, CD158b, and CD158k, including isotype-specific controls (**Supplementary Table S2** and SOP 007 in the **Supplementary Materials**). Lymphocytes were gated from forward and sideward scatter patterns; next, antigen expression was assayed from CD4+ gated lymphocytes. Specific antigen expression was observed relative to autofluorescence and (non-)specific signals obtained from each individual patient and a PBMC control sample. This control sample was derived from two healthy donors and functioned as internal reference sample each

flow cytometry session. Samples were analyzed in a blinded setting.

Specific antigen expression in the population of gated cells (expression or loss) was displayed in percentages (**Figure 1a and b**). Antigen expression was considered dim if

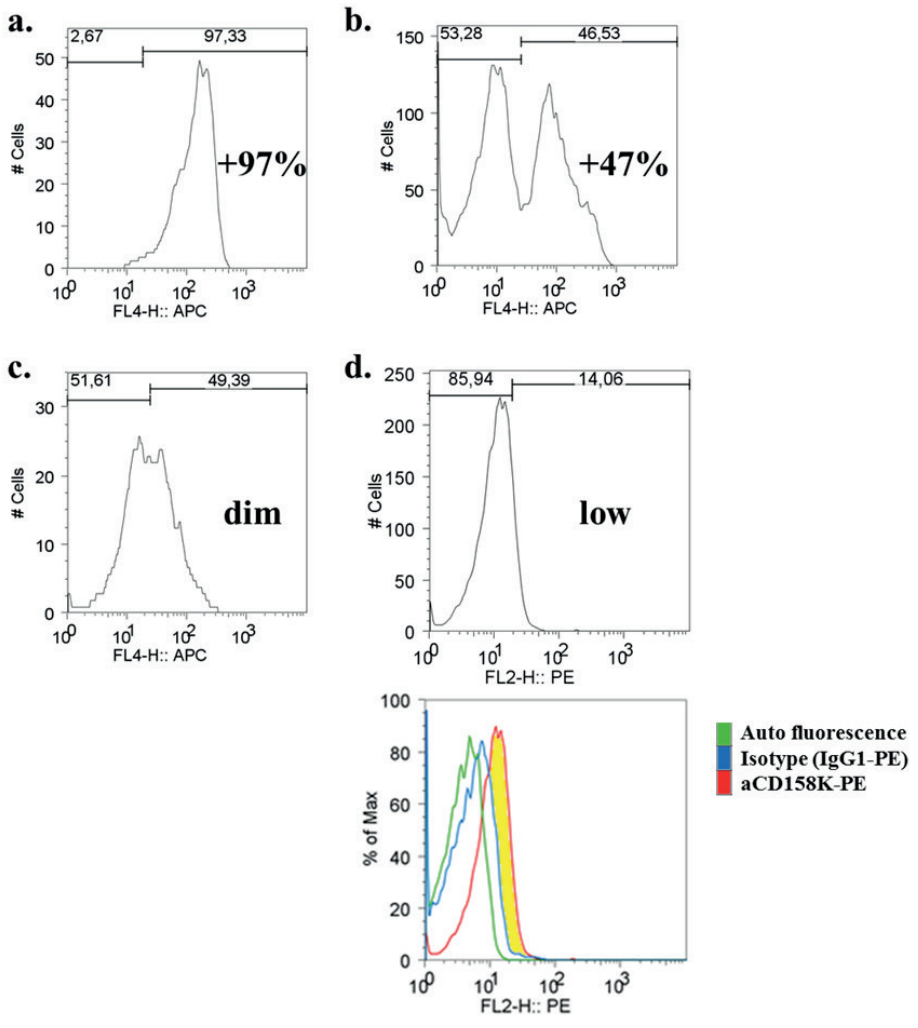

Figure 1. Interpretation of flow cytometry results.
a. A single peak located on the right side of the determined threshold represents a single population with positive staining for CD7 in 97% of the gated cells; b. Two distinct populations of the gated cells, in which 47% of the cells show positive expression for CD7 and 53% show negative staining; c. Diminished expression for CD7 of a single population of gated cells, surrounding a determined threshold, is indicated as "dim". d. The specific CD158k antigen expression signal (indicated in red) is slightly shifted to the right compared to its auto fluorescence and isotype control signals (green and blue lines), as is indicated in yellow. This implicates that the gated cells do express CD158k but at very low level, indicated as "low".

all gated cells showed diminished expression around the determined threshold (**Figure 1c**). For CD158a, CD158b, and CD158k, expression below 5% of the gated cells was considered as no expression, but when intermediate expression of a single population of gated cells, surrounding a determined threshold, was found, this was characterized as low-expressing antigen (for example, CD158k^{low}) (**Figure 1d**).

COPY NUMBER VARIATION ASSAY

Quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies, Carlsbad, CA) were developed for target genes *JUNB*, *TWIST1*, *MYC*, and *MNT* and reference genes *ABT1*, *ARG2*, and *DNM3* (**Supplementary Table S3**). Reference genes were selected from different large copy number-stable chromosomal regions in SS, selected from array-based comparative genomic hybridization experiments on 20 SS samples.[33] Amplification efficiency was evaluated in triplicate, using eight 4-fold serial dilution points ranging from 3 ng/µL to 183 fg/µL DNA concentration, under optimised primer and hydrolysis probe concentrations. Assays with amplification efficiency value between 90% and 100% and a correlation coefficient above 0.98 were accepted for CNV analysis. Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005 (**Supplementary Materials** online).

Data were normalized against reference genes and relative to the common reference using the ΔΔCq method and are presented as relative copy number, where 2 stands for diploid DNA.[42] The following thresholds were maintained for the CNV data: 1.5-2.5 was considered as diploid (normal) DNA, greater than 2.5 as gain in copy number, and less than 1.5 as loss in copy number.

GENE EXPRESSION ASSAY

GE quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies) were developed and validated, as described for CNV quantitative PCR assays, for target genes *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC*, *JUNB*, *TWIST1*, *EPHA4*, and *STAT4* and reference genes *ARF5*, *ERCC3*, and *TMEM87A* (**Supplementary Table S3**). Stably expressed reference genes were selected from microarray experiments on SS samples and validated in SS and EID samples according to the GeNorm method.[37;38;43] Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005. Receiver operating characteristic curve analysis was used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. An one-tailed Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant.

RESULTS

PATIENT CHARACTERISTICS

Clinical characteristics at diagnosis of the 59 SS patients and 19 EID patients are summarized in **Table 1.**

The patients with SS had a T-cell clone in the peripheral blood (59 of 59), a CD4/CD8 ratio above 10 (53 of 57) and/or a Sézary cell count above 1000 per mm^3 (34 of 43, including all four patients with a CD4/CD8 ratio lower than 10).

One EID patient showed a T-cell clone in the peripheral blood, and another EID patient had a CD4/CD8 ratio above 10 because of very low numbers of CD8+ T cells, but none had a Sézary cell count above 1000 per mm^3 (**Table 1**).

FLOW CYTOMETRY

Flow cytometry experiments were performed both in Leiden and Paris for all 59 SS patients and 19 EID patients. Differences in flow cytometry results between Leiden and Paris were less than 20% in 99.8% of individual assays; and in these cases an average was used in further analysis. In only 2 of 1027 assays (0.2%) did the differences in results exceeded 20%, and these were therefore excluded from further analysis.

In this study, 87% of the SS patients (46 of 53) had a CD4/CD8 ratio above 10 at inclusion, compared with 8% of the EID patients (1 of 12) (sensitivity = 87%, specificity = 92%).

The CD4+ gated lymphocytes were CD3+ and CD8−. In the CD4+ T-cell population, 7 of 59 (12%) SS patients showed loss for CD2 (median = 45%, range = 32-100%) and 4 (7%) patients showed diminished expression for CD2 ($CD2^{dim}$), whereas this was never observed in the 19 EID cases. One SS patient showed 90% CD5 loss, compared with none of the EID patients.

In the CD4+ T-cell population, a percentage of CD4+CD7− cells above 40% was found in 32 of 59 (54%) SS patients but never in EID patients (sensitivity = 54%, specificity =

Table 1. Clinical characteristics at diagnosis of the 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses.

Values at diagnosis SS versus erythroderma EID	SS, n=59	EID, n=19
Male-female ratio	37:22	16:3
Age in years, median (range)	65 (32-89)	67 (29-86)
Erythroderma	46/52 (88%)	19/19 (100%)
Pruritus	45/52 (87%)	13/19 (68%)
Ectropion	6/52 (12%)	1/19 (5%)
Hyperkeratosis hand/feet	21/52 (40%)	3/19 (16%)
Palpable lymphadenopathy	21/52 (40%)	4/19 (21%)
Lymphadenopathy confirmed by CT scan	17/41 (41%)	1/7 (14%)
Leukocytes ≥10.0 $x10^9$/L	40/56 (71%)	5/14 (36%)
CD4/CD8 ratio ≥10.0	53/57 (93%)	1/12 (8%)
Absolute Sézary cell count ≥1000 per mm^3	34/43 (79%)	0/10 (0%)
T-cell clone in peripheral blood	59/59 (100%)	1/17 (6%)
Identical T-cell clone in blood and skin	32/38 (84%)	0/3 (0%)

EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome

100%). In addition, $CD7^{dim}$ was found in 2 of 59 (3%) SS patients, compared with none of the EID patients.

In the CD4+ T-cell population a percentage of 30% or more CD4+CD26− cells was found in 51 of 59 (86%) SS patients but also in 10 of 19 (53%) EID patients (sensitivity = 86%, specificity = 47%). When shifting the percentage to 80%, 39 of 59 (66%) SS patients but none of the EID patients had CD4+CD26− cells of 80% or more in the CD4+ T-cell population (sensitivity = 66%, specificity = 100%). In addition, $CD26^{dim}$ was found in 5 of 59 (8%) SS patients and in 1 of 19 (5%) EID patients.

Loss of CD26 by more than 80% and/ or loss of CD7 by more than 40% of CD4+ T cells was found in 49 of 59 (83%) SS patients but was never observed in the EID patients (sensitivity = 83%, specificity = 100%).

Investigations on CD158k expression showed that more than 5% of these CD4+ T cells expressed CD158k or $CD158k^{low}$ in 19 of 58 (33%) SS patients, compared with 1 of 19 (5%) EID patients (sensitivity = 33%, specificity = 95%). The results (including expression of CD158a and CD158b) are summarized in **Table 2**.

No major difference was observed in the expression of CD27, CD45RA and CD45RO, by CD4+ T cells between SS and EID patients (data not shown).

COPY NUMBER VARIATION

CNV experiments were performed in 58 SS patients and 17 EID patients in Leiden. Duplicate experiments for 14 samples were performed in London, which gave identical results (**Supplementary Figure S2**).

In 47 of 58 (81%) SS patients, alterations in copy number were found compared with none of the 17 EID patients. Gain of *MYC* was observed in 23 of 58 (40%) SS patients (sensitivity = 40%, specificity = 100%). *MNT* loss was found in 38 of 58 (66%) SS patients (sensitivity = 66%, specificity = 100%) and one (2%) patient showed gain of *MNT* (**Figure 2**). Gain of *MYC* and/ or loss of *MNT* was found in 76% (44 of 58) of SS patients (sensitivity = 76%, specificity = 100%). Copy number alterations of *JUNB* and *TWIST1* were found in only a minority of SS patients (**Figure 2**).

Table 2. Overview of the tested flow cytometry markers in 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses at inclusion in the study.

Markers for SS described in literature	SS, n=59	EID, n=19	Sensitivity (%)	Specificity (%)
CD4/CD8 ratio ≥ 10	46/53	1/12	87	92
CD4+CD7− ≥ 40%	32/59[1]	0/19	54	100
CD4+CD26− ≥ 30%	51/59[2]	10/19	86	47
CD158a*	2/58	0/19	3	100
CD158b*	13/59	1/19	22	95
CD158k*	19/58	1/19	33	95

EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome
*Low expression and expression of 5% or more of the CD4+ lymphocytes.
[1]Including 4/7 SS patients with a CD4/CD8 ratio below 10.
[2]Including 5/7 SS patients with a CD4/CD8 ratio below 10.

GENE EXPRESSION

GE analyses were performed on 55 SS, 19 EID, and 4 healthy control cases in Leiden. Thirty samples were also analyzed in London, with identical results in 28 samples (**Supplementary Figure S3**); two samples could not be analyzed because of a technical error.

DNM3, *TWIST1, EPHA4*, *PLS3*, and *STAT4* were the most differentially expressed genes in SS patients compared with EID patients and healthy controls with 100% specificity ($P < 0.001$) (**Table 3, Supplementary Figure S4**). Up-regulation of *DNM3*, *TWIST1, EPHA4*, and *PLS3* was found in 66−75% of SS patients, and *STAT4* was down-regulated in 91% of SS patients (**Figure 3**). Up-regulation of *DNM3*, *TWIST1, EPHA4*, and *PLS3* was found in 41 of 55 (75%), 38 of 55 (69%), 36 of 55 (66%), 36 of 55 (66%) of SS patients, respectively, and *STAT4* was down-regulated in 50 of 55 (91%) of SS patients. Combining alterations in gene expression (*STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3*), we could

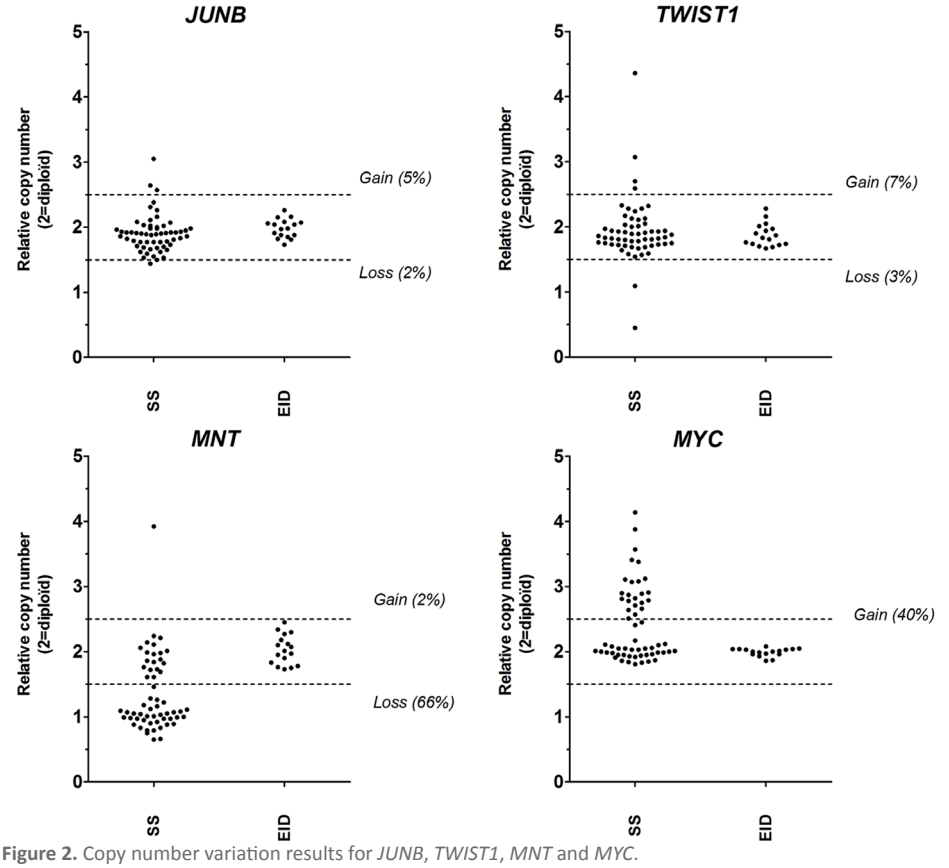

Figure 2. Copy number variation results for *JUNB*, *TWIST1*, *MNT* and *MYC*.
The gains and losses in copy number in 58 SS compared to 17 EID cases are shown as normalized relative copy number, where 2 represents diploid DNA. The dotted lines signifies the chosen thresholds for gain and loss, 2.5 and 1.5, respectively. EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome

distinguish 54 of 55 (98%) SS patients from all EID patients (sensitivity = 98%, specificity = 100%).

Aberrant gene expression of *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC* and *JUNB* was found in only a minority of SS patients (**Table 3**).

DISCUSSION

In the present multicenter study, we investigated the diagnostic sensitivity and specificity of immunophenotypic and molecular biomarkers in SS using SOPs. We show that by using SOPs, it is possible to obtain highly reproducible results for flow cytometry, CNV, and GE analysis and show that loss of CD7 and CD26 by CD4+ T cells; gain in copy number of *MYC* and loss of *MNT*; increased expression of *DNM3, TWIST1, EPHA4*, and *PLS3*; and decreased expression of *STAT4* are highly characteristic for Sézary cells.

In the current study the most SS patients have a CD4/CD8 ratio above 10, but we also show that a significant minority of patients (13%) does not fulfil this diagnostic criterion. For these patients additional immunophenotypic markers are clearly needed.

Comparison of results from previous flow cytometry studies is hindered by the use of different protocols and cell populations. In the present study we focused on CD4+ gated T cells because CD4 is rarely lost by Sézary cells, facilitating the comparison of expression levels of different immunophenotypic markers.

Previous studies reported that a CD4+CD26− cell population greater than 30% had a sensitivity of 97% and specificity of 100% in diagnosing peripheral blood involvement

Table 3. Results of aberrant gene expression in all tested genes in 55 patients with Sézary syndrome relative to 19 patients with erythrodermic inflammatory dermatoses and 4 healthy control subjects.
With the receiver operating characteristic curve analysis a threshold was established at a specificity of 100% and an accuracy above 0.80. *PLS3*, *DNM3*, *TWIST1*, *EPHA4* and *STAT4* and were found to be useful diagnostic markers in Sézary syndrome.

Up-/down-regulation	SS, n=55	Sensitivity (%)
PLS3	36/55	66
DNM3	41/55	75
CDO1	20/55	36
TRAIL	4/55	7
CD1D	6/55	11
GATA3	2/55	4
MYC	0/55	0
JUNB	9/55	16
TWIST1	38/55	69
EPHA4	36/55	66
STAT4	50/55	91

SS, Sézary syndrome

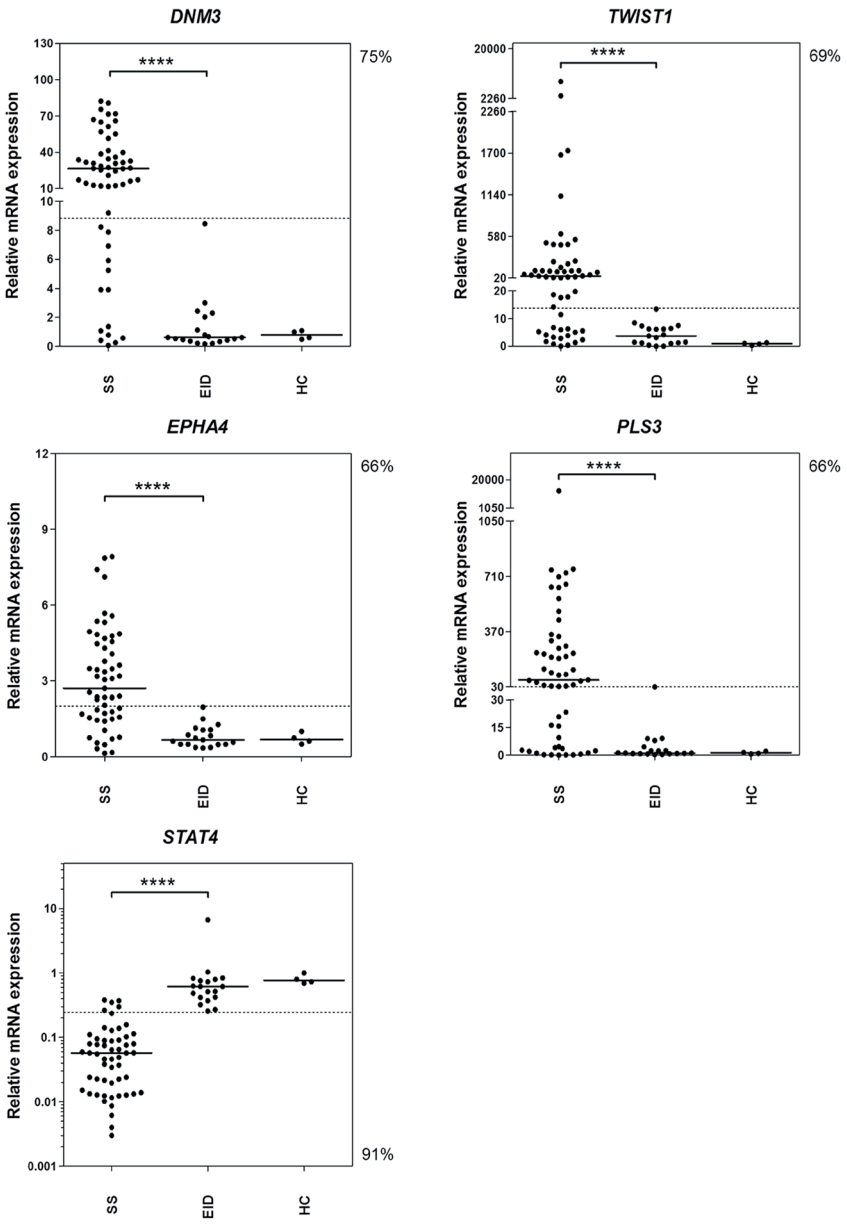

Figure 3. Gene expression results for *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4*.
The differential gene expression is shown as relative normalized mRNA levels in 55 SS compared to 19 EID and 4 HC cases. **** represents the statistical significant difference in gene expression in SS compared to EID and HC ($P < 0.001$). The dotted lines represent the thresholds for differential expression, determined with receiver operating characteristic curves with a specificity of 100%. The Y-axis represents the relative mRNA expression with varying scale in all figures. EID, erythrodermic inflammatory dermatoses; HC, healthy control; mRNA, messenger RNA; SS, Sézary syndrome

and suggested this cut-off point as tentative diagnostic criterion for SS.[10;18] Indeed, loss of CD26 in more than 30% of CD4+ T cells was found in 86% of SS patients but also in 53% of EID patients, resulting in a specificity of 47%. However, when using a percentage of 80% as the cut-off point, we found that 39 of 59 (66%) SS patients but none of the EID patients met this criterion.

These discrepant results can be explained by different flow cytometry protocols. We evaluated CD26 expression on CD4+ gated T cells, whereas Bernengo et al looked at CD4+CD26− cells on gated total lymphocytes. Indeed, when looking at the CD4+CD26− cells of 30% or more on total lymphocytes, similar results were found (sensitivity = 80%, specificity = 95%; data not shown).

A level of CD4+CD7− cells of more than 40% has also been suggested as tentative criterion in the diagnosis of SS.[21] Consistent with literature, we found that loss of CD7 above 40% of the CD4+ T cells is highly specific (100% specificity) but not a sensitive marker (sensitivity = 54%).[10;11;44] Similar results were found for 40% or more CD4+CD7− cells on total lymphocytes (sensitivity = 42%, specificity = 100%; data not shown).

Flow cytometry results show that in 83% of SS patients, CD4+ T cells display loss of CD26 by more than 80% and/or loss of CD7 by more than 40%, whereas this was never observed in EID patients. These observations are relevant because they can readily be included in immunophenotypic testing of erythrodermic patients.

Previous studies reported expression of killer cell immunoglobulin-like receptor CD158k in 65–97% of SS patients.[16;25;26] Flow cytometry analysis, performed in both Leiden and Paris, showed expression of CD158k in only 33% of SS patients (19 of 58 cases), and in most of these SS patients (18 of 19) the CD158k antigen was expressed at low levels. This discrepancy can be explained by the fact that the present study was performed on frozen PBMCs instead of freshly isolated PBMCs. Indeed, a recent study on freshly isolated PBMCs from SS patients showed high CD158k expression in Sézary cells.[45]

For CNV and GE analysis the use of SOPs and custom made PCR platforms led to highly reproducible results as well. Gain of *MYC* and/ or loss of its antagonist *MNT* was found in 76% of SS patients but never in EID. Gain of *TWIST1* and *JUNB* was detected in only a small minority of SS patients.

In line with the literature, we found up-regulation of *DNM3*, *TWIST1, EPHA4*, and *PLS3* and down-regulation of *STAT4* in most of SS patients.[34-39] In contrast, only a minority of SS patients showed up-regulation of *GATA3*, *CD1D*, *TRAIL*, *CDO1*, *JUNB*, and *MYC*, implying that these genes are not useful diagnostic markers. Why gain of *MYC* and loss of *MNT* which is observed in most of patients, does not lead to up-regulation of *MYC* expression is as yet unexplained.

Combined alterations in gene expression of *STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3* could distinguish 98% of SS patients from EID patients, suggesting that this diagnostic panel will be useful as additional molecular criterion in the diagnostic differentiation between SS and EID.

In the present study 27 patients were diagnosed with SS before inclusion in the study. However, no significant differences were found in the prevalence of the previously

described markers between 27 patients already diagnosed with SS before inclusion in the study and the 32 SS patients newly diagnosed at time of inclusion (**Supplementary Table S4**). Similarly, the prevalence of investigated markers was similar in 36 SS patients who received treatment during sample collection at inclusion and the 23 SS patients who did not receive any form of treatment (**Supplementary Table S5**). These observations argue that the observed immunophenotypic and molecular changes are stably expressed in Sézary cells.

We show that standardization of flow cytometry, CNV, and GE procedures leads to strong reproducibility of results. We argue that to facilitate comparison of results from different centers, it will be important to closely define the subset of cells that was investigated, and based on the present study we suggest gating on CD4+ T cells in future studies.

For patients in whom the distinction between SS and EID still cannot be made using the current diagnostic criteria, we propose that these two additional diagnostic panels can be used: (i) loss of CD26 (≥ 80% CD4+ T cells) and/ or loss of CD7 (≥ 40% CD4+ T cells) for immunophenotypic analysis and (ii) combination of altered gene expression of *STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3* for molecular analysis.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Biomarkers for Sézary syndrome selected from literature that are investigated in the present study. The arrows indicate the reported expression in Sézary syndrome.

Protein expression	Copy number variation	Gene expression
CD7↓	*JUNB*↑	*PLS3*↑
CD26↓	*TWIST1*↑	*DNM3*↑
CD27+	*MYC*↑	*CDO1*↑
CD45RA−	*MNT*↓	*TRAIL*↑
CD45RO+		*CD1D*↑
CD158a↑		*GATA3*↑
CD158b↑		*MYC*↑
CD158k↑		*JUNB*↑
		TWIST1↑
		EPHA4↑
		STAT4↓

Supplementary Table S2. List of monoclonal antibodies.

CD marker	Clone	Isotype	Fluorochrome	Source
CD2	S5.2*/ RPA-2.10#	mouse IgG2a	APC*/ PE-Cy5#	BD Pharmingen
CD3	SK7*/ UCHT1#	mouse IgG1	APC*/ FITC#	BD Pharmingen
CD4	SK3*/ SFCI12T4D11#	mouse IgG1	PerCP-Cy5.5*/ PC7#	BD Pharmingen/Beckman Coulter
CD5	UCHT2	mouse IgG1	PE*/ PE-Cy5#	BD Pharmingen
CD7	eBio124-1D1*/ M-T701#	mouse IgG1	APC*/ PE-Cy5#	eBioScience/ BD Pharmingen
CD8	SK1	mouse IgG1	FITC	BD Pharmingen
CD26	M-A261	mouse IgG1	FITC	BD Pharmingen
CD27	M-T271	mouse IgG1	APC*/ PE#	BD Pharmingen
CD45RA	L48	mouse IgG1	FITC	BD Pharmingen
CD45RO	G155-178*/ UCHL1#	mouse IgG2a	PE*/ PE-Cy5#	BD Pharmingen
CD158a	HP-3E4	mouse IgM	FITC*/ PE#	BD Pharmingen
CD158b	CH-L	mouse IgG2b	PE	BD Pharmingen
CD158k	AZ158	mouse IgG1	PE	Innate Pharma

*Antibody used in Leiden

#Antibody used in Paris

Supplementary Table S3. List of optimised copy number variation and gene expression primers and hydrolysis probes used in quantitative PCR assays.

Assay	Gene	Reference	Forward primer (5'-3')	(nM)
CNV	*TWIST1*		CCGCTAGGGAGAGCAGTCTC	900
	MNT		CACGCCTGTCCTGACCATAG	300
	MYC		GCCGCATCCACGAAACTTTG	900
	ABT1	YES	TGCATGTCCTGTTGCTTCGC	900
	ARG2	YES	AAGAGAAGCAAAGTGGGGAGTAG	900
	DNM3	YES	CTAAACACCTCTGCTGATTTCTGC	300
	JUNB			
GE	*PLS3*		CGGGCTGGCAAAAAATTAAC	900
	DNM3		GGATGACTCCTGGATACAGCACT	300
	CDO1		GCAATGTACGCCAAGTTCGA	300
	TRAIL		GCTCACATAACTGGGACCAGAGGAAG	900
	CD1D		ACCAGGGACAAGCAGGTAAGAG	300
	GATA3		ACAACCTGGTCCCGTTTTATTCTGC	300
	STAT4		CGTGTCAACCAACGATTCCCAGAA	900
	MYC		GGTGCTCCATGAGGAGACAC	900
	ERCC3	YES	ATATCCAAGGTAGGTGACACTTCG	900
	TMEM87A	YES	CATCTGGACAACCATGAAGTTCAG	300
	ARF5	YES	TGCTGATGAACTCCAGAAGATGC	900
	JUNB			
	TWIST1			
	EPHA4			

CNV, copy number variation; GE, gene expression

Reverse primer (5'-3')	(nM)	Probe (5'-3')	(nM)
TGACCCTGGGTGTCTCTGTC	900	CCCTCCTGTCACGCACACTCACGC	200
TTCACTGGATTTGACTTCTTCAGC	900	TGCCAACTTCAGGGTCCCCAGCGT	150
GTCCTTGCTCGGGTGTTGTAAG	900	CAAAGTGCCCGCCCGCTGCTATGG	250
CCAGCATCCTCACAGACTGATTC	900	CCAGCCAGGAGCCAAGCACCGC	250
GTGTGATCAAACATACAGCCTCAG	900	AGCCGTGGTCCCAGGTCTAACCCC	250
CCGCCTTTCATGATGCCAATG	900	TGAGCCACCCCTTGCGAATCACCT	250
Assay: Hs00357891_s1 (Life Technologies)			
GCGATTTGATTGAGAAGATGGAA	900	TTTAGTGCTGACATCAAGGATTCCAAAGCCTA	250
GCCGGATGTGGGCCTT	900	AGGTCACCTCCTCCAAGCCCCAC	200
TGTCCTTCACCCCAACAGAGA	900	CGAAATCTTGTGGATCAAGGAAATGGAAA	200
AGTTGCTCAGGAATGAATGCCCAC	900	AAGGCTCTGGGCCGCAAAATAAACTCCTG	250
ACGGTTAAAGCCAAGCCAGG	900	CTGGAACACACATGTCCTATCCAAAGGAATCAGC	250
GCTGGGAAGCAAAGGTGAGCAAAG	900	CCCTTCTTCTCTTTGCTAAACGACCCCTCC	250
AAACTGCCAGCTCATCACCTCCAG	900	TTCTTTAATAATCCTCCACCTGCCACATTGAGTCA	250
CAGCAGAAGGTGATCCAGACTC	900	CCACCACCAGCAGCGACTCTGAGG	250
TTGTACTCTTCTGCAACCATCCC	900	CGAAGCACCCGCCCTAGCCTTTGG	200
AGGATCATGGAGAACAGCAAGC	900	ATCGTCTACCCACAGCTCCCGCCA	200
CGGCTGCGTAAGTGCTGTAG	900	TGTCAGTCAGCTCGCTCACGGGCA	200
Assay: Hs00357891_s1 (Life Technologies)			
Assay: Hs00361186_m1 (Life Technologies)			
Assay: Hs00953175_m1 (Life Technologies)			

Supplementary Table S4. Overview of the biomarkers in patients who were diagnosed with Sézary syndrome before inclusion in the study compared with patients who were newly diagnosed at time of inclusion of the study. The Chi-squared test was performed to test for statistical significant differences in marker distribution between these two groups.

	Diagnosis SS before inclusion, n=27		**New diagnosis SS at inclusion, n=32**		***P*-value**
Flow cytometry markers					
CD4/CD8 ratio ≥ 10	16/21	(76%)	30/32	(94%)	0.065
CD4+CD7− ≥ 40%	14/27	(52%)	18/32	(56%)	0.735
CD4+CD26− ≥ 30%	21/27	(78%)	30/32	(94%)	0.074
CD158a*	1/26	(4%)	1/32	(3%)	0.881
CD158b*	4/27	(15%)	9/32	(28%)	0.219
CD158k*	10/26	(38%)	9/32	(28%)	0.404
Copy number variation markers					
JUNB gain	2/26	(8%)	1/32	(3%)	0.383
TWIST gain	3/26	(12%)	1/32	(3%)	0.212
MYC gain	9/26	(35%)	14/32	(44%)	0.479
MNT loss	17/26	(65%)	21/32	(66%)	0.650
Gene expression markers					
DNM3 up-regulation	17/23	(74%)	24/32	(75%)	0.927
EPHA4 up-regulation	15/23	(65%)	21/32	(66%)	0.975
PLS3 up-regulation	14/23	(61%)	22/32	(69%)	0.544
TWIST1 up-regulation	16/23	(70%)	22/32	(69%)	0.949
STAT4 down-regulation	21/23	(91%)	29/32	(91%)	0.931

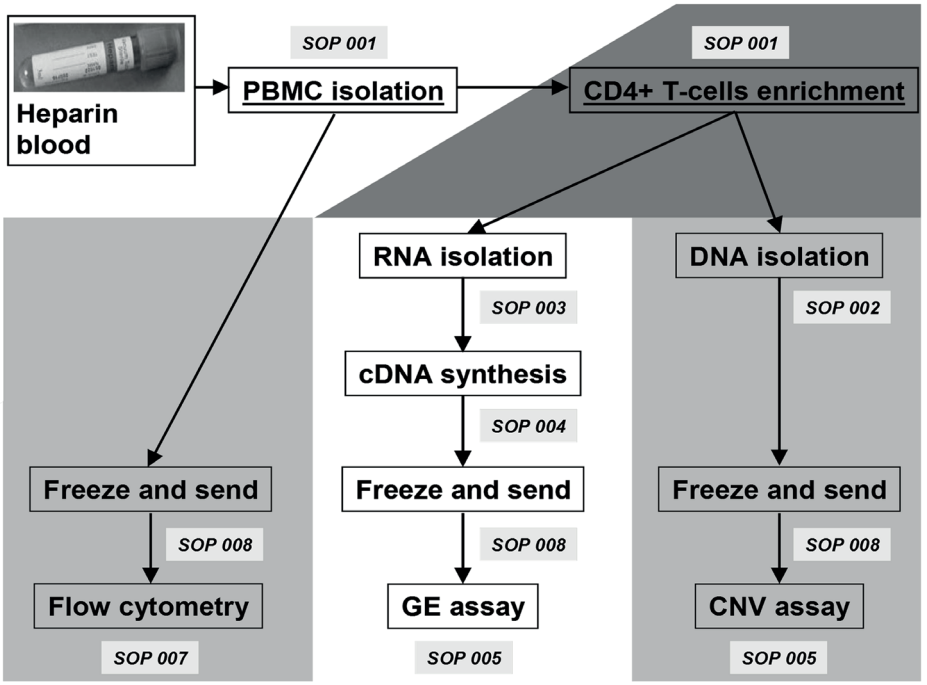
SS, Sézary syndrome

*Low expression and expression of 5% or more of the CD4+ lymphocytes.

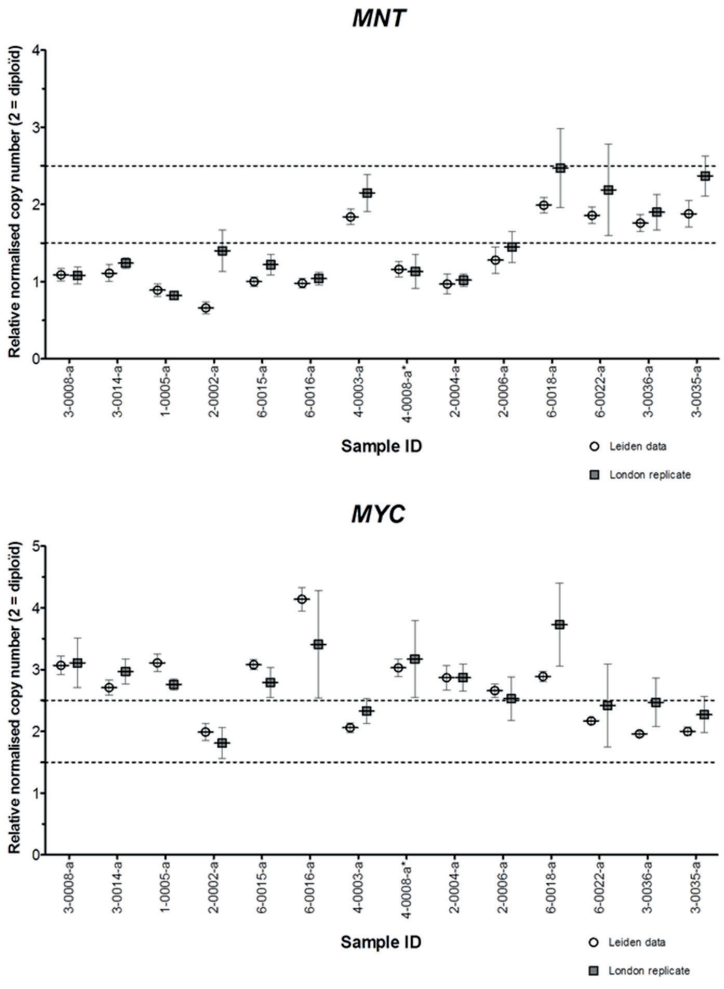
Supplementary Table S5. Overview of the biomarkers in patients with Sézary syndrome without any treatment at time of the inclusion in the study compared with those patients who had treatment at time of inclusion. The Chi-squared test was performed to test for statistical significant differences in marker distribution between these two groups.

	No treatment at inclusion, n=23		**Treatment at inclusion, n=36**		***P*-value**
Flow cytometry markers					
CD4/CD8 ratio ≥ 10	22/23	(96%)	24/30	(80%)	0.095
CD4+CD7− ≥ 40%	12/23	(52%)	20/36	(56%)	0.799
CD4+CD26− ≥ 30%	21/23	(91%)	30/36	(83%)	0.383
CD158a*	0/23	(0%)	2/35	(6%)	0.243
CD158b*	6/23	(26%)	7/36	(19%)	0.548
CD158k*	6/23	(26%)	13/35	(37%)	0.380
Copy number variation markers					
JUNB gain	1/22	(5%)	2/36	(6%)	0.719
TWIST gain	1/22	(5%)	3/36	(8%)	0.165
MYC gain	8/22	(36%)	15/36	(42%)	0.689
MNT loss	16/22	(73%)	22/36	(61%)	0.222
Gene expression markers					
DNM3 up-regulation	17/22	(77%)	24/33	(73%)	0.705
EPHA4 up-regulation	13/22	(59%)	23/33	(70%)	0.418
PLS3 up-regulation	17/22	(77%)	19/33	(58%)	0.132
TWIST1 up-regulation	18/22	(82%)	20/33	(61%)	0.095
STAT4 up-regulation	20/22	(91%)	30/33	(91%)	1

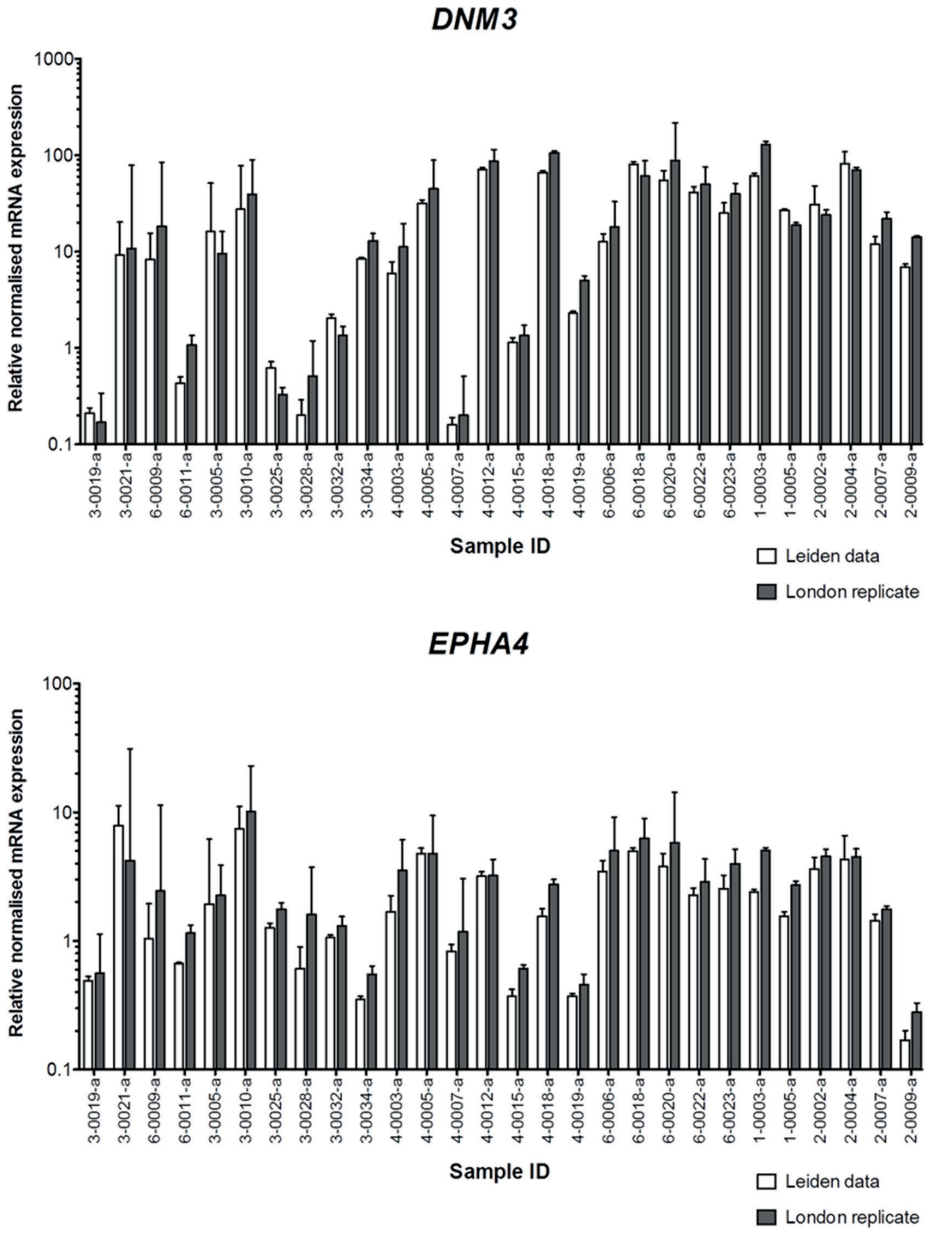
*Low expression and expression of 5% or more of the CD4+ lymphocytes.



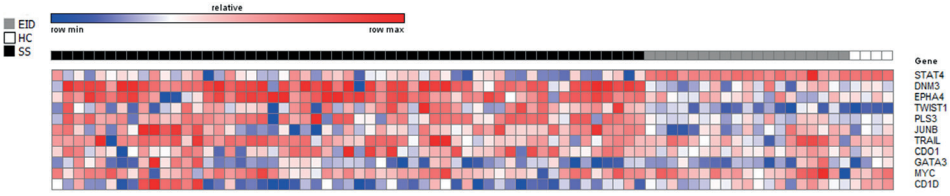
Supplementary Figure S1. Workflow of sample preparation.
This figure gives an overview of the workflow from blood sample collection to the eventual experiments using standard operating procedures (SOPs). CNV, copy number variation; GE, gene expression; PBMC, peripheral blood mononuclear cell



Supplementary Figure S2. Results for copy number analysis performed in Leiden and London. Examples are given of *MNT* and *MYC* for 14 samples.



Supplementary Figure S3. Results for gene expression analysis performed in Leiden and London. Examples are given for *DNM3* and *EPHA4* for 28 samples.



Supplementary Figure S4. Heatmap depicting relative normalized gene expression of evaluated biomarkers in 55 patients with Sézary syndrome, 19 patients with erythrodermic inflammatory dermatoses and 4 healthy control subjects.
Values are visualized as relative gene expression for each gene, normalized against the stably expressed reference genes *ARF5*, *ERCC3* and *TMEM87A* and log2 transformed. Red represents high expression and blue represents low expression. Gene ranking has been performed based on receiver operating characteristic curve analysis accuracy values. Varying from the highest accuracy ranked at top to the lowest placed at the bottom of the heatmap. EID, erythrodermic inflammatory dermatoses; HC, healthy control; SS, Sézary syndrome

Supplementary materials containing all the SOPs are available online at www.jidonline.org.

3

Increased expression of *PLS3* correlates with better outcome in Sézary syndrome

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TO THE EDITOR

Patients with Sézary syndrome (SS), a rare erythrodermic and leukemic form of cutaneous T-cell lymphoma, have a poor prognosis with a 5-year overall survival (OS) of 20-42% and a median OS between 2.5 and 5 years.[1-6] Prognostic factors associated with a worse survival reported in SS include advanced age, short duration of skin lesions before diagnosis of SS, previous history of mycosis fungoides (MF), elevated serum lactate dehydrogenase levels, degree of nodal involvement, and factors reflecting blood tumor burden, such as increased leukocyte counts or high Sézary cell counts.[1-8]

However, the results of these studies are not consistent, which may be due to different diagnostic criteria for SS, such as inclusion of patients without a T-cell clone in the peripheral blood, and analysis of mixed populations of patients with SS and erythrodermic MF.

Recently, we investigated the diagnostic significance of a large number of immunophenotypic and molecular biomarkers for SS in a group of patients with SS that fulfilled the diagnostic criteria of the World Health Organization - European Organization for Research and Treatment of Cancer classification.[9;10] None of these patients had a history of MF. Molecular biomarkers diagnostic for SS were copy number alterations in *MYC* (gain) and/ or *MNT* (loss); increased expression of *DNM3*, *TWIST1*, *EPHA4*, and *PLS3* and decreased expression of *STAT4*.

We investigated the prognostic significance of these molecular biomarkers and previously reported clinical prognostic markers using the same cohort of SS patients. Between September 2009 and October 2013, 64 SS patients from six European Organization for Research and Treatment of Cancer centers, including Helsinki, Finland; London, United Kingdom; Leiden, The Netherlands; Mannheim, Germany; Turin, Italy; and Paris, France were included and followed up until January 30, 2015. At the inclusion of the study, clinical variables (sex, age at diagnosis, duration of skin lesions before diagnosis of SS, lymph node involvement, leukocyte count, absolute CD4 count, and Sézary cell count) were recorded, and peripheral blood samples were collected for copy number variation and gene expression quantitative PCR analysis, as described previously.[9] Lymph node involvement was defined by presence of enlarged lymph nodes of 1.5 cm or larger in the longest transverse diameter on computed tomography scan or histologically confirmed lymph node involvement.

Aberrant gene expression in the SS samples was compared with samples from patients with erythrodermic inflammatory dermatoses (EID) and healthy control samples. Receiver operating characteristic curve analysis was used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. An one-tailed Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant. The results of aberrant expression of the *DNM3*, *TWIST1*, *EPHA4*, *PLS3*, and *STAT4* genes were included in the statistical analysis. A more detailed Methods section including these thresholds is included in the **Supplementary Materials** and **Supplementary Table S1**.

Survival was calculated with the Kaplan-Meier method from the date of diagnosis

until the patient's death or date of last follow-up. The median follow-up time after diagnosis was 45 months (range = 1-129 months). Twenty-seven patients died during follow-up, including 21 SS-related deaths. The disease-specific survival (DSS) after 1, 2, 3 and 5 years was 89%, 82%, 76% and 59%, respectively, and OS was 86%, 79%, 72% and 49%, respectively.

Univariate analysis of parameters with possible prognostic significance for DSS and OS was performed using Cox proportional hazards regression analysis, and parameters that were significant at the 0.1 level were included in a multivariate analysis model. *P*-values below 0.05 were regarded as statistically significant.

Both in univariate and multivariate analyses, up-regulation of *PLS3* was associated with a significantly better outcome for DSS and OS (multivariate $P = 0.006$ and $P = 0.002$, respectively). Patients with up-regulation of *PLS3* had a median survival of 71 months (range = 9-129 months), compared with only 33 months (range = 1-72 months) in SS patients with normal expression of *PLS3* (**Figure 1**).

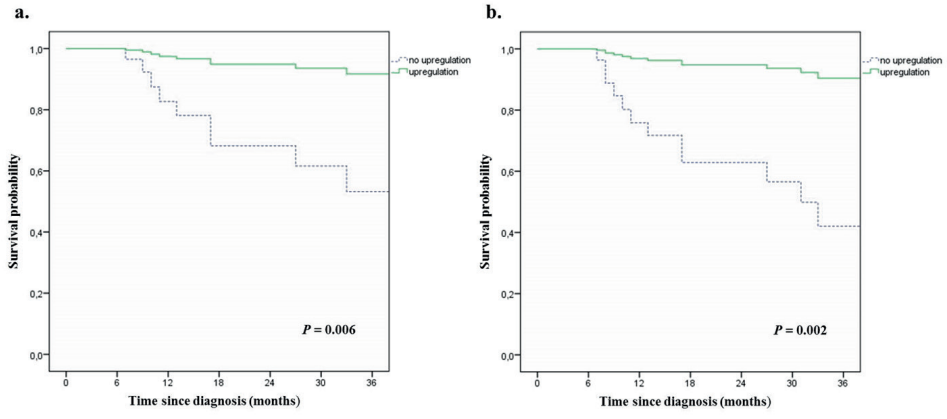

Figure 1. Disease-specific survival (a) and overall survival curve (b) according the groups with and without up-regulation of *PLS3.*

Up-regulation of *DNM3* and *TWIST1* were associated with a better OS in univariate analysis ($P = 0.008$ and $P = 0.043$, respectively) but not in multivariate analysis ($P = 0.658$ and $P = 0.342$, respectively). Gain of *MYC*, loss of *MNT*, up-regulation of *EPHA4* and down-regulation of *STAT4* showed no association with DSS and OS (**Table 1**).

Of the clinical parameters, both univariate and multivariate analyses showed that leukocyte count was a significant prognostic factor for DSS and OS (multivariate $P = 0.005$ and $P = 0.005$, respectively), whereas sex, age, duration of skin lesions before diagnosis, lymph node involvement, absolute CD4 count and Sézary cell count were not (**Table 1**).

PLS3 (T-plastin) is an actin-binding protein that is expressed in all normal cells of solid tissues that have a replicative role, but it is normally not expressed in T cells.[11] Expression of *PLS3* has been described as a specific marker of Sézary cells.[12;13] Studies

Table 1. Results of univariate and multivariate analyses for variables at Sézary syndrome diagnosis. Parameters significant at the 0.1 level were included in multivariate analysis.

Variables	Median survival	Univariate analysis DSS	
	(months)	HR (95% CI)	*P*-value
Gain in copy number of *MYC*			0.843
Yes, n=21	72 (1-129)	0.90 (0.32-2.54)	
No, n=31	49 (7-86)	1	
Loss in copy number of *MNT*			0.355
Yes, n=35	68 (1-129)	0.61 (0.22-1.74)	
No, n=17	49 (7-80)	1	
Up-regulation of *DNM3*			0.337
Yes, n=36	71 (9-129)	0.56 (0.17-1.83)	
No, n=13	33 (1-72)	1	
Up-regulation of *TWIST1*			0.197
Yes, n=32	71 (1-129)	0.48 (0.16-1.46)	
No, n=17	31 (7-80)	1	
Up-regulation of *EPHA4*			0.312
Yes, n=32	49 (7-129)	1.95 (0.53-7.12)	
No, n=17	68 (1-80)	1	
Up-regulation of *PLS3*			0.027
Yes, n=32	71 (9-129)	0.29 (0.10-0.87)	
No, n=17	33 (1-72)	1	
Down-regulation of *STAT4*			0.356
Yes, n=44	68 (1-129)	#	
No, n=5			
Sex			
Female, n=25	49 (1-129)	1.21 (0.51-2.85)	
Male, n=39	68 (1-115)	1	
Age at SS diagnosis in years, n=64		1.01 (0.96-1.05)	0.827
Duration skin lesions, n=60		0.98 (0.96-1.00)	0.075
Lymph node involvement			0.356
Yes, n=18	49 (1-74)	1.82 (0.51-6.53)	
No, n=23	Not reached	1	
Leukocyte count, n=61		1.04 (1.01-1.06)	0.007
Absolute CD4 count, n=59		1	0.146
Sézary cell count, n=47		1	0.123

CI, confidence interval; DSS, disease-specific survival; HR, hazard ratio; OS, overall survival; SS, Sézary syndrome. # means no statistics can be computed.

Multivariate analysis DSS		Univariate analysis OS		Multivariate analysis OS	
HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
			0.366		
		0.66 (0.26-1.63)			
		1			
			0.388		
		0.68 (0.28-1.65)			
		1			
			0.008		0.658
		0.29 (0.12-0.72)		0.74 (0.20-2.75)	
		1		1	
			0.043		0.342
		0.39 (0.16-0.97)		0.57 (0.18-1.81)	
		1		1	
			0.311		
		1.70 (0.61-4.74)			
		1			
	0.006		0.001		0.002
0.14 (0.03-0.56)		0.19 (0.07-0.49)		0.12 (0.03-0.46)	
1		1		1	
			0.250		
		3.28 (0.43-24.77)			
		0.92 (0.43-1.98)			
		1			
		1.00 (0.97-1.04)	0.850		
0.99 (0.97-1.02)	0.447	0.99 (0.97-1.00)	0.152		
			0.420		
		1.55 (0.53-4.52)			
		1			
1.06 (1.02-1.10)	0.005	1.03 (1.01-1.06)	0.011	1.05 (1.01-1.08)	0.005
		1	0.265		
		1	0.204		

investigating the mechanism underlying dysregulation of *PLS3* expression in SS cells found no evidence for *PLS3* mutations within coding or promoter regions but showed significant hypomethylation of CpG dinucleotides 95-99 within the *PLS3* CpG island, which was restricted to the *PLS3*+ cells.[13] Reanalysis of recently published DNA methylation profiles from nine patients with SS and four healthy control subjects included in this study confirmed this correlation between DNA methylation and *PLS3* expression (data not shown).[14] A recent study found that constitutive *PLS3* expression was associated with apoptotic resistance to etoposide and suggested a role for cell survival in SS.[15] How T-plastin expression is linked to a better outcome in patients with SS is not known and should be the subject of further study.

Although for a disease as rare as SS the number of included patients is relatively high, a limitation of this study is a small sample size, which yielded wide confidence intervals, and these observations should be confirmed in an independent study.

In conclusion, we show that up-regulation of *PLS3* is associated with a favorable disease outcome in patients with SS and that increased leukocyte count is a significant adverse prognostic factor for survival.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

METHODS

Peripheral blood mononuclear cells were isolated from peripheral blood from 59 patients with Sézary syndrome (SS), 19 patients with erythrodermic inflammatory dermatoses (EID) and 4 healthy controls (HC) and enriched for CD4+ T helper cells, by depletion of non-CD4+ T cells, using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) for copy number variation (CNV) and gene expression (GE) assays, as described previously.[1]

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). RNA was isolated using the RNeasy Mini Kit (Qiagen), which included on-column DNase digestion. RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) using random priming. In order to quantify copy number changes accurately, quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies, Carlsbad, CA) were developed for target genes *JUNB*, *TWIST1*, *MYC*, *MNT* and reference genes *ABT1*, *ARG2* and *DNM3*. For gene expression analysis, quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies) were developed for target genes *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC*, *JUNB*, *TWIST1*, *EPHA4*, *STAT4* and reference genes *ARF5*, *ERCC3* and *TMEM87A*. Optimisation of both copy number as gene expression assays and sample analysis were performed as described previously.[1]

To compare aberrant gene expression in SS samples, relative to EID and HC samples, receiver operating characteristic curve analysis was used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. An one-tailed Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant. The results of aberrant expression of genes *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4* were included in the statistical analysis (**Supplementary Table S1**).

All statistical calculations were performed using IBM SPSS Statistics 20.0. Survival was calculated from the date of diagnosis of SS until the patient's death or date of last follow-up. The Kaplan-Meier method was used to estimate survival curves and comparison between curves was performed using the log-rank test. Median follow-up was calculated

Supplementary Table S1. This table shows the thresholds determined by receiver operating characteristic curve analysis with an accuracy above 0.8 and specificity of 100% for the genes *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4.*

Gene	Accuracy (>0.8)	Std. error	Threshold	Sensitivity at 100% specificity
DNM3	0.919	0.032	8.826	75%
TWIST1	0.86	0.041	13.7865	69%
EPHA4	0.88	0.039	2.0035	66%
PLS3	0.842	0.045	31.602	66%
STAT4	0.99 (= 1-0.01)	0.008	0.24611	91%

using the reverse Kaplan-Meier method.

Univariate analysis of parameters with possible prognostic significance for disease-specific survival and overall survival was performed using Cox proportional hazards regression analysis. Parameters that were analyzed for their prognostic significance were gain in copy number of *MYC* (yes vs no), loss in copy number of *MNT* (yes vs no), up-regulation of *DNM3* (yes vs no), up-regulation of *TWIST1* (yes vs no), up-regulation of *EPHA4* (yes vs no), up-regulation of *PLS3* (yes vs no), down-regulation of *STAT4* (yes vs no), sex (male vs female), age at diagnosis (continuous variable), duration of skin lesions before diagnosis SS (continuous variable), lymph node involvement (yes vs no), leukocyte count (continuous variable), absolute CD4 count (continuous variable) and Sézary cell count (continuous variable). The parameters that were significant at the 0.1 level were included in a multivariate analysis model. *P*-values below 0.05 were regarded as statistically significant.

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4

Differential expression of TOX by skin-infiltrating T cells in Sézary syndrome and erythrodermic dermatitis

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ABSTRACT

Background The histopathologic differentiation between Sézary syndrome (SS) and erythrodermic dermatitis may be extremely difficult. In this immunohistochemical study, it was investigated if thymocyte selection-associated high mobility group box protein (TOX) and C-MYC can be used as additional diagnostic markers to differentiate between SS and erythrodermic dermatitis.

Method Paraffin-embedded skin biopsies from 15 SS patients and 17 erythrodermic dermatitis patients were stained and scored for TOX or C-MYC expression.

Results Strong nuclear staining for TOX in more than 50% of skin-infiltrating T cells was observed in 13 of 15 (87%) SS cases, whereas erythrodermic dermatitis cases showed weak nuclear staining in 11–50% (median = 25%) of the T cells; strong nuclear staining as found in SS was never observed in erythrodermic dermatitis. No significant differences in C-MYC expression between SS and erythrodermic dermatitis were found. In most patients of both groups, percentages of C-MYC positive cells varied between less than 10 and 25% of skin-infiltrating T cells.

Conclusion Our results suggest that strong expression of TOX in more than 50% of skin-infiltrating T cells in erythrodermic skin is a useful marker in the differentiation between SS and erythrodermic dermatitis, whereas staining for C-MYC does not contribute to differential diagnosis.

INTRODUCTION

Sézary syndrome (SS) is a rare and aggressive type of cutaneous T-cell lymphoma (CTCL), characterized by a pruritic erythroderma and the presence of clonal neoplastic T cells (Sézary cells) in skin and peripheral blood.[1]

The microscopic differentiation between SS and erythrodermic dermatitis may be extremely difficult.[2;3] The histopathologic findings of SS are similar to that of mycosis fungoides (MF), but more commonly the entity shows a monotonous band-like or perivascular infiltrate in the papillary dermis that is mainly composed of lymphocytes with atypical or cerebriform nuclei. Epidermotropism is present and Pautrier microabscesses may be found. However, in up to one third of SS cases, microscopic sections may show non-specific features of chronic dermatitis.[4;5]

In a recent study, blind evaluation of hematoxylin and eosin-stained sections from skin biopsies of 18 patients with a CTCL, including 14 SS patients and 29 patients with erythrodermic dermatitis, correct differentiation between CTCL and erythrodermic dermatitis was made in approximately 50% of the cases.[3] These observations indicate that there is an urgent need for diagnostic biomarkers for SS.

In a recent study of our group both programmed death-1 (PD-1; CD279) and CD7 proved valuable immunophenotypic markers in the differentiation between SS and erythrodermic dermatitis.[6] Expression of PD-1 by more than 50% of the skin-infiltrating T cells was found in 23 of 25 (92%) SS cases and only in 4 of 30 (13%) erythrodermic dermatitis cases. In SS, PD-1 was expressed by the neoplastic CD4+ T cells, whereas in erythrodermic dermatitis PD-1 was predominantly expressed by dermal and epidermal CD8+ T cells. Loss of CD7 by more than 50% of the skin-infiltrating T cells was found in 16 of 24 (66%) SS cases but also in 4 of 30 (13%) erythrodermic dermatitis cases. However, expression of CD7 by 20% or less of infiltrating T cells was found only in SS (13 of 24 cases).

In this study, we used the same cohort of erythrodermic patients to investigate the differential diagnostic value of two other potentially useful biomarkers, namely thymocyte selection-associated high mobility group box protein (TOX) and C-MYC.

TOX belongs to a large family of chromatin-associated proteins. In T-cell development, TOX is highly expressed in the thymus during the transition of CD4+CD8+ precursors to CD4+ T cells but is normally not expressed by mature CD4+ T cells once they leave the thymus.[7;8]

Recent studies have reported increased TOX expression by malignant CD4+ T cells in MF and SS skin biopsies, but not in benign inflammatory dermatoses.[9-12] In addition, Huang et al showed that TOX mRNA expression is significantly enhanced in primary CD4+CD7– cells from peripheral blood of SS patients, as compared to those cells from patients with benign inflammatory dermatosis.[13] The increase of TOX mRNA expression was correlated with increased risk of disease progression and disease-specific mortality. It was suggested that TOX might contribute to the development of CTCL.[13] However, its biological effects on CTCL pathogenesis have not been explored. Studies focusing on TOX expression in skin of patients with extensive erythrodermic dermatitis have not been performed.

The rationale to consider C-MYC as a potential diagnostic marker results from previous studies showing a gain of 8q24 harboring the *MYC* gene in 41–75% of SS patients.[14;15] Immunohistochemical studies for C-MYC expression in MF or SS are limited. Kanavaros et al reported expression of C-MYC by 5–25% of the skin-infiltrating lymphoid cells in cases of early stage MF and SS, whereas higher percentages (25–50%) were found in one-third of patients with advanced MF.[16] In a recent study, 12 of 13 (92%) patients with erythrodermic MF/SS showed positive staining for C-MYC in at most 15% of the dermal lymphocytic infiltrate cells.[17]

In this study, we therefore investigated if TOX and C-MYC can be used as additional markers to differentiate between SS and erythrodermic dermatitis.

METHODS

PATIENTS

Paraffin-embedded skin biopsies from 15 patients with SS and 17 patients with erythrodermic dermatitis were selected for this study. The diagnosis of SS was based on the recent criteria of the World Health Organization – European Organization of Research and Treatment of Cancer classification.[1] The diagnosis of erythrodermic dermatitis in each patient was based on clinical and histopathological criteria, supplemented by immunophenotyping and clonality analysis of peripheral blood to exclude peripheral blood involvement by CTCL. The erythrodermic dermatitis group included four patients with atopic erythroderma, three patients with erythrodermic psoriasis, nine patients with idiopathic erythroderma, and one patient with paraneoplastic erythroderma. Review of clinical records revealed that none of the patients developed a lymphoma after a median follow-up of 46 months (range = 7–332 months). The study complied with the Declaration of Helsinki and was performed in accordance with the Dutch Code and Leiden University Medical Center guidelines on leftover material.

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Sections from all skin biopsies had routinely been stained with hematoxylin-eosin and with monoclonal antibodies against T cell-associated antigens (CD2, CD3, CD4, CD5, CD7, CD8), B cell-associated antigens (CD20 and/or CD79a), and CD68 and CD1a to differentiate between CD4+ T cells and CD4+ histiocytes and Langerhans cells/dendritic cells, respectively. For the purpose of this study, sections from all patients were stained for TOX and C-MYC.

Immunohistochemical staining was performed on 4-µm sections using standard procedures. After antigen retrieval by heating for 10–12 minutes in 1.0 mmol/L ethylenediaminetetraacetic acid (pH 8.0) or 10 mmol/L citrate buffer (pH 6.0), tissue sections were incubated overnight with antibodies against TOX (Sigma-Aldrich, Zwijndrecht, The Netherlands) and C-MYC (Abcam, Cambridge, UK). Sections were then incubated for 30 min with BrightVision Poly-horseradish peroxidase, and subsequently incubated with diaminobenzidin (DAB) solution (Sigma-Aldrich) for 10 min. Finally, all slides were counterstained with Mayer hematoxylin.

The percentages of T cells expressing TOX or C-MYC were scored, as less than 10, 11–25, 26–50, 51–75 and more than 75%. These percentages had been estimated independently by three observers (FC, PMJ and RW). In the few cases in which there was disagreement, sections were viewed jointly by all authors and consensus was reached.

RESULTS

SÉZARY SYNDROME

The results of the immunohistochemical stainings are summarized in **Table 1**. This table also contains the results of PD-1 staining and the presence or absence of antigen loss from our previous study.[6]

Skin biopsies from patients with SS characteristically showed perivascular to band-like infiltrates in the papillary dermis. More diffuse infiltrates extending into the reticular dermis were observed in four cases. Epidermotropic neoplastic CD4+ T cells with (n = 5) or without (n = 5) Pautrier microabscesses were found in 10 cases. The dermal infiltrates were predominantly composed of small to large atypical CD4+ T cells with hyperconvoluted nuclei (Sézary cells) and variable numbers of blast cells. In 2 of 15 cases (nos. 6 and 15) the cellular atypia was minimal, and a diagnosis of CTCL was at most suspected. Percentages of admixed CD8+ T cells varied between less than 5% and almost 50%, but a percentage more than 30% was observed in only two cases (nos. 6 and 15).

Strong nuclear staining for TOX was observed in more than 50% of the skin-infiltrating T cells in 13 of 15 (87%) with percentages more than 75% in 10 of them (**Table 1**). Intraepidermal neoplastic T cells consistently showed strong nuclear staining for TOX (**Figure 1**). In two cases (nos. 6 and 15), weak nuclear staining in 25–30% of the skin-infiltrating T cells was found (**Figure 2**). C-MYC was expressed by less than 10% of the skin-infiltrating T cells in 5 of 15 cases, by 11–25% in eight cases, and in 26–50% in two cases. Strong nuclear C-MYC staining was particularly observed in large Sézary cells and blast cells (**Figure 1**). In addition, epidermal basal cells showed strong nuclear C-MYC staining, serving as a useful internal control.

ERYTHRODERMIC DERMATITIS

Biopsy specimens of erythrodermic dermatitis generally showed a sparse to moderately dense perivascular to band-like infiltrate in the superficial dermis, and the infiltrate was generally much less pronounced than observed in SS. Intraepidermal T cells were few or absent, and in some cases could only be recognized in immunostained sections. The superficial dermal infiltrate was mainly composed of small lymphocytes admixed with variable numbers of histiocytes, and in cases of atopic and idiopathic erythroderma with eosinophils. In three cases of atopic erythroderma (nos. 16, 18 and 19), the dermal infiltrate showed a considerable number of slightly atypical small to medium-sized pleomorphic T cells and scattered blast cells, and a diagnosis of suspected CTCL had initially been made. Percentages of CD8+ T cells in the dermal infiltrate varied between 15 and 75% (median = 30%) of the dermal CD3+ T cells.

Weak nuclear staining for TOX was observed in 11–25% of the infiltrating T cells in

Table 1. Summary of the immunohistochemical stainings.

	Diagnosis	TOX (%)	C-MYC (%)	PD-1 (%)	Antigen loss
1	SS	++++	+	++++	–
2	SS	++++	+	++++	CD7, CD2
3	SS	++++	+	++++	CD7
4	SS	+++	–	++++	CD7
5	SS	++++	+	++++	CD7
6	SS[1]	++ (w)	–	+++	–
7	SS	++++	++	++++	CD7
8	SS	+++	–	+++	–
9	SS	++++	++	++++	CD7
10	SS	++++	+	++++	CD7
11	SS	++++	+	+	–
12	SS	++++	+	+++	CD7, CD2
13	SS	+++	–	++++	CD7
14	SS	++++	+	++++	CD7
15	SS[1]	++ (w)	–	+++	CD7
16	AE[2]	+ (w)	–	+	–
17	AE	+ (w)	–	++	–
18	AE[2]	+ (w)	–	+++[#]	–
19	AE[2]	++ (w)	–	+++[#]	CD7
20	PSOR	+ (w)	+	++	–
21	PSOR	+ (w)	–	+	–
22	PSOR	+ (w)	–	–	–
23	IE	+ (w)	+	–	–
24	IE	++ (w)	–	–	–
25	IE	++ (w)	+	–	–
26	IE	+ (w)	+	–	–
27	IE	++ (w)	–	–	–
28	IE	+ (w)	+	+	–
29	IE	+ (w)	+	+	CD7
30	IE	+ (w)	+	+	–
31	IE	+ (w)	–	+	–
32	PARA	+ (w)	+	+	–

AE, atopic erythroderma; IE: idiopathic erythroderma; PARA: paraneoplastic erythroderma; PD-1, programmed death-1; PSOR: psoriatic erythroderma; SS: Sézary syndrome; TOX, C-MYC and PD-1: –, <10%; +, 11–25%; ++, 26–50%; +++, 51–75%; ++++, >75%. Antigen loss: loss of CD2, CD3, CD4, CD5 or CD7 expression by more than 50% of the (neoplastic) T cells; –: no loss

[1] SS cases showing no or minimal cellular atypia.

[2] Cases showing considerable numbers of small to medium-sized atypical T cells.

[#] CD8+ T cells; (w): weak staining.

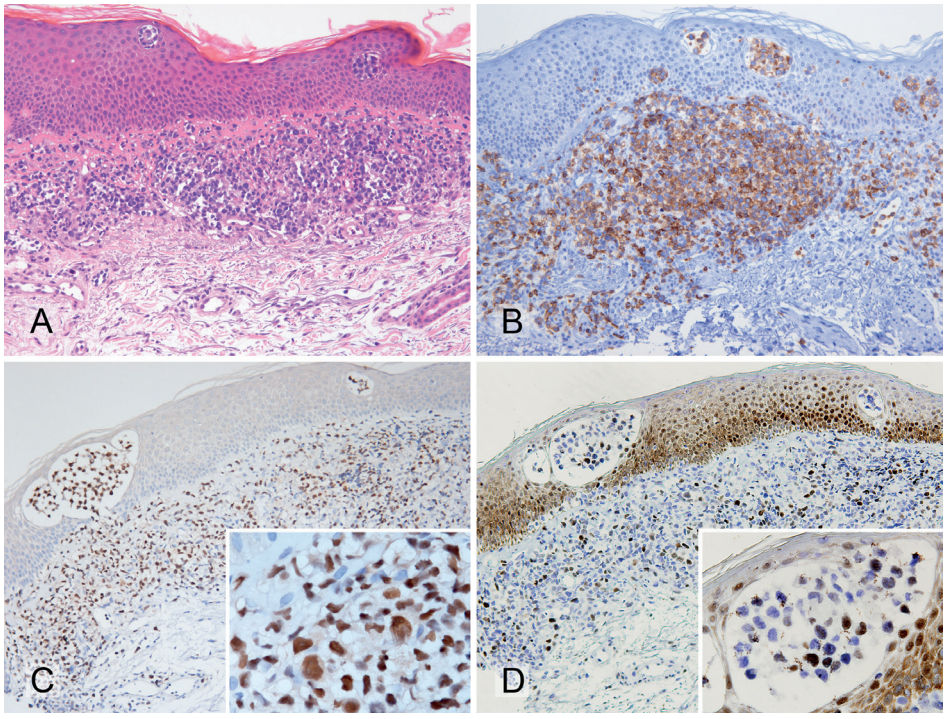

Figure 1. Histopathologic features of a representative Sézary syndrome patient with strong TOX expression.
A) Hematoxylin-eosin staining showed an infiltrate in the superficial dermis with epidermal Pautrier microabscesses. B) Expression of CD3. C) More than 50% of the skin-infiltrating T cells show strong nuclear staining for TOX, particularly in large Sézary cells and blast cells. D) Less than 25% of the skin-infiltrating T cells expressed C-MYC. The insets in (C) and (D) show higher magnifications of corresponding areas. (A – D: ×100, inset in C: ×630, inset in D: ×400).

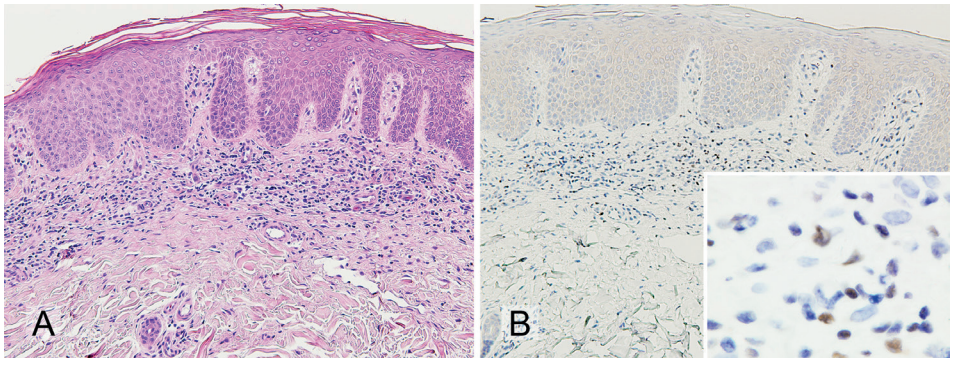

Figure 2. Histopathologic features of a Sézary syndrome patient with weak TOX expression.
A) Hematoxylin-eosin staining showed a perivascular to band-like infiltrate in the papillary dermis. B) Weak expression of TOX by 25–30% of the skin-infiltrating T cells. (A and B: ×100 and inset in B: ×630).

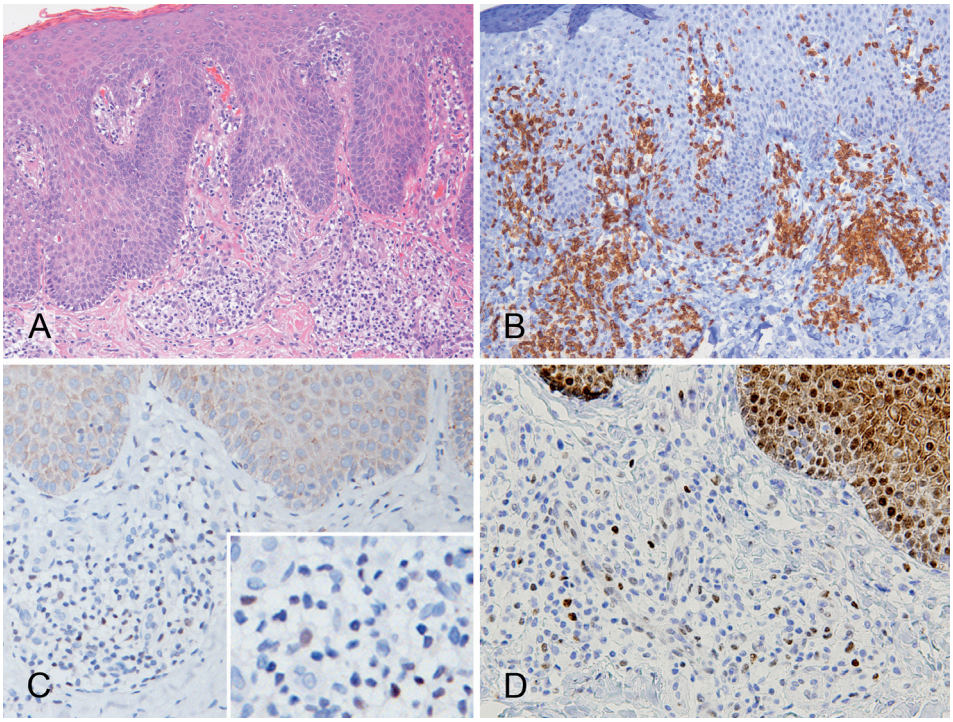

Figure 3. Histopathologic features of a representative patient with erythrodermic psoriasis.
A) The hematoxylin-eosin staining of the lesion showed acanthosis and a dermal lymphocytic infiltrate. B) Expression of CD3. C) Weak nuclear staining for TOX in a small proportion of the infiltrating T cells. D) Nuclear staining for C-MYC in scattered infiltrating T cells. (A and B: ×100, C and D: ×200, inset in C: ×630).

13 of 17 cases, and in 26–50% in four cases (**Figure 3**). Strong nuclear staining for TOX as found in SS was never observed in erythrodermic dermatitis.

Nuclear staining for C-MYC was detected in 11–25% of both small and scattered larger T cells in 8 of 17 cases and by less than 10% in nine cases. In three cases, they were completely lacking (nos. 17, 18 and 19), whereas the epidermal basal cells still showed strong nuclear staining.

DISCUSSION

In this study, strong nuclear staining for TOX by more than 50% of the skin-infiltrating T cells was found in 13 of 15 (87%) Sézary cases, whereas all erythrodermic dermatitis cases showed weak nuclear staining of TOX varying between 11 and 50% of the T cells. The two remaining SS cases (nos. 6 and 15) showed weak nuclear TOX expression at a similar level as seen in erythrodermic dermatitis patients. Interestingly, both cases showed minimal atypia and had high numbers of admixed CD8+ T cells. It should however be noted that in our previous study both cases had shown expression of PD-1 by more than 50% of the skin-infiltrating T cells. Alternatively, in one SS patient with expression

of PD-1 in only 10% of neoplastic T cells (no. 11), more than 75% of the neoplastic T cells showed strong nuclear TOX staining, suggesting that combining both markers may be diagnostically helpful.

In a previous study, we observed that – in contrast to SS – PD-1 is uncommonly expressed by the neoplastic T cells in skin biopsies from erythrodermic MF. Expression of PD-1 by more than 50% of the neoplastic T cells was found in only one of eight cases.[18] Examination of skin biopsies from seven of these cases showed strong nuclear staining for TOX by more than 50% of the malignant T cells in six of them (Willemze et al; unpublished observations 2015). These results are similar to those of this study in SS and indicate that TOX is a useful biomarker to differentiate erythrodermic dermatitis from both SS and erythrodermic MF.

Our results are consistent with recent studies showing strong nuclear staining for TOX by atypical CD4+ T cells, both in dermis and epidermis, in SS and MF skin biopsies compared to benign inflammatory dermatoses and normal skin.[9;10;12] In addition, previous studies have shown increased mRNA expression levels for TOX in both SS and MF as well.[9-13]

The high expression of TOX in CTCL is as yet unexplained. In normal T-cell development mature CD4+ T cells do not express TOX after leaving the thymus. Whether the TOX expression found in SS and other types of CTCL may be due to an impaired regulated maturation status or to re-expression during the formation into memory T cell has yet to be determined.[7]

No significant differences in C-MYC expression between SS and erythrodermic dermatitis were found. In both groups about half of the cases showed C-MYC positivity in 11–25% of the skin-infiltrating T cells. Higher percentages (25–50%) were found in only two cases of SS. Similarly, Kavanos et al reported percentages of 11–25% in three of three (100%) SS cases.[16]

Previous studies reported a gain of 8q24 harboring the *MYC* gene in 41–75% of SS patients.[14;15] In a recent European multicenter study on 59 Sézary patients gain for *MYC* was found in 23 of 58 (40%) SS cases as well (Boonk et al. 2015, submitted manuscript). However, using the quantitative PCR technique, no differences in *MYC* gene expression levels were found between Sézary cells and CD4+ T cells from erythrodermic dermatitis patients. Three of the 15 SS cases (nos. 2, 10 and 11) from this study had been included in the previous study and showed a gain for *MYC*. However, all of them showed C-MYC staining in only 11–25% of the skin-infiltrating T cells, similar to the other SS cases. Why the gain for *MYC* does not lead to increased gene and protein expression is as yet unexplained.

In conclusion, strong expression of TOX, but not C-MYC, can be another useful adjunct in the differentiation between SS and erythrodermic dermatitis.

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5

Epigenomic analysis of Sézary syndrome defines patterns of aberrant DNA methylation and identifies diagnostic markers

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ABSTRACT

Sézary syndrome (SS) is a malignancy of skin-homing CD4+ memory T cells that is clinically characterized by erythroderma, lymphadenopathy, and blood involvement. Distinction of SS from erythroderma secondary to inflammatory skin diseases (erythrodermic inflammatory dermatoses [EID]) is often challenging. Recent studies identified recurrent mutations in epigenetic enzymes involved in DNA modification in SS. Here we defined the DNA methylomes of purified CD4+ T cells from patients with SS, EID, and healthy controls subjects. SS showed extensive global DNA methylation alterations, with 7.8% of 473,921 interrogated autosomal CpG sites showing hypomethylation and 3.2% hypermethylation. Promoter CpG islands were markedly enriched for hypermethylation. The 126 genes with recurrent promoter hypermethylation in SS included multiple candidate tumor suppressors that showed transcriptional repression, implicating aberrant methylation in the pathogenesis of SS. Validation in an independent sample set showed promoter hypermethylation of *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, and *PAM* in 94-100% of SS samples but not in EID samples. Notably, promoter hypermethylation of a single gene, the chemokine-like factor *CMTM2*, was sufficient to accurately distinguish SS from EID in all cases. This study shows that SS is characterized by widespread yet distinct DNA methylation alterations, which can be used clinically as epigenetic diagnostic markers.

INTRODUCTION

Sézary syndrome (SS) is an aggressive type of cutaneous T-cell lymphoma (CTCL) that is characterized clinically by erythroderma, generalized lymphadenopathy, and the presence of malignant T cells in the skin, lymph nodes, and peripheral blood.[1] SS is a malignant clonal proliferation of mature CD4+ skin-homing central memory T cells.[2] Malignant T cells of patients with SS commonly display a Foxp3+, CD25+ phenotype, and it has been postulated that SS represents a malignancy of regulatory T cells in a subset of patients.[3;4] Differentiation of SS from erythroderma secondary to inflammatory skin diseases (EID) can be challenging, but it is important for the therapeutic management of patients. Especially in early stages of the disease, clinical, cytological, and histopathological findings in SS are often nonspecific. Because circulating atypical lymphocytes with immunophenotypic alterations and T cell clonality can be found in EID as well as SS, there is a need for molecular markers to reliably diagnose SS. The use of combined flow cytometry markers and combined gene transcript analysis has been proposed to aid in diagnosing SS.[5-8]

The molecular pathogenesis of SS is not fully understood, and targets for directed therapeutic intervention remain to be defined. Recent exome sequencing studies in SS have described a broad spectrum of genetic alterations, including mutations in genes involved in DNA methylation, histone modification, and chromatin remodelling.[9-12] Epigenetic modifier genes with recurrent mutations in SS include *DNMT3a*, *TET1*, and *ARID1A*. These observations are in line with experimental evidence showing that epigenetic mechanisms, such as aberrant DNA methylation, have a causative role in various hematopoietic malignancies.[13;14] In SS, promoter CpG island hypermethylation of several tumor suppressor genes, including *CDKN2A* and *FAS*, has been found.[15;16] The therapeutic efficacy of histone deacetylase inhibitors provides additional evidence for a causative role of epigenetic alterations in the molecular pathogenesis of SS.[17;18]

In this study we report on distinct genome-wide DNA methylation characteristics of CD4+ T cells isolated from patients with SS as compared with those from patients with EID and healthy individuals. This analysis enabled us to define patterns of aberrant DNA methylation with potential relevance for the pathogenesis of SS and to identify epigenetic markers that can be used in the diagnosis of this T-cell malignancy.

METHODS

SELECTION OF PATIENTS

DNA isolated from peripheral blood CD4+ T cells of 15 patients with SS, three patients with EID, and four age-matched healthy volunteers was subjected to Illumina 450k (Illumina) DNA methylation analysis. Differential methylation was validated in a second patient series of 20 patients with SS, 10 patients with EID, and seven healthy volunteers. Diagnosis of SS was based on criteria defined in the World Health Organization - European Organization for Research and Treatment of Cancer (WHO-EORTC) classification.[1] Patients with SS presented with erythroderma, highly elevated CD4/CD8 ratios, and

clonality of malignant T cells in peripheral blood (**Table 1**). EID was secondary to atopic dermatitis, psoriasis, and paraneoplastic or idiopathic syndromes (**Supplementary Table S1**). CD4+ T cells were isolated by negative selection from Ficoll density centrifuged peripheral blood mononuclear cells (CD4+ T-cell Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany). DNA methylation profiling was additionally performed on the SeAx and HuT78 cell lines, derived from patients with SS. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Approval for these studies was obtained from the institutional review board of Leiden University Medical Center. Written informed consent was provided according to the Declaration of Helsinki.

GENOME-WIDE DNA METHYLATION DATA GENERATION AND ANALYSIS

The Infinium methylation assay was performed at a certified Illumina service provider (Service XS, Leiden, The Netherlands). DNA was bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Orange, CA) and hybridized onto Illumina 450k

Table 1. Clinical characteristics of the 15 patients with Sézary syndrome subjected to genome-wide DNA methylation analysis and 20 patients analyzed to validate epigenetic diagnostic biomarkers.

Characteristics at diagnosis of SS	Confirmation SS, n=15	Validation SS, n=20
Male:female ratio	6:9	16:4
Age in years, median (range)	64 (46-78)	68 (32-89)
Findings, n/total		
Erythroderma	14/15	17/20
Pruritus	13/15	17/20
Ectropion	2/14	3/20
Hyperkeratosis hands/feet	8/13	3/20
Palpable lymphadenopathy	6/15	5/20
Lymphadenopathy confirmed by CT scan	5/11	3/14
Leukocytes ≥ 10.0 x10^9/L	12/15	14/20
CD4/CD8 ratio ≥ 10.0	14/15	20/20
Absolute Sézary cell count ≥ 1000 per mm^3	5/5	13/16
T-cell clone in peripheral blood	15/15	20/20
Identical T-cell clone in blood and skin	6/6	9/9
Status at last date of follow-up, n		
Alive with disease	9	15
Died of disease	5	4
Died of other cause than linked to SS	1	0
Died of unknown cause	0	1
Duration of follow-up in months, median (range)	49 (8-120)	20 (1-33)

CT, computed tomographic; SS, Sézary syndrome

arrays (Illumina, San Diego, CA). Analyses were performed using R Statistics, version 2.15.1. Data were normalized for color bias using Lumi package. CpG sites on the sex chromosomes were excluded for analysis. After quality control, 473,921 CpG sites were used to test for differences between healthy T cells and SS T cells. Individual CpGs were tested for an association between SS and benign T-cell samples using a linear model, and *P*-values were adjusted for multiple testing using the false discovery rate. CpG sites with *P*-value adjusted for multiple testing using the false discovery rate less than 0.05 and absolute β-value difference greater than 0.2 were considered significant. Statistical testing using the Welch *t* test and multiple testing correction using Bonferroni correction (P_{BON}) for promoter CpG islands were performed using the Limma package. For promoter CpG islands, methylation was calculated as average β-values of all CpG sites located in CpG islands of proximal promoter regions. Promoters with P_{BON} less than 0.05 and absolute average β-value difference greater than 0.2 between SS and benign T cells were considered differentially methylated. CpG sites were annotated to a combined gene- and CpG island-centric annotation. For the gene-centric annotation, CpGs were classified as distal promoter (-10 kilobase pairs to -1.5 kilobase pairs), proximal promoter (-1.5 kilobase pairs to +500 base pairs), gene body (+500 base pairs to 3'), or downstream region (3' to +5 kilobase pairs).[19] The β-value denotes the ratio of fluorescence intensities between methylated and unmethylated alleles and was used to estimate the methylation level at each interrogated CpG site. For promoter CpG islands the methylation was calculated as average β-values of all CpG sites located in CpG islands of the proximal promoter region.

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COMPARATIVE ANALYSIS OF METHYLATION DATA

For comparison with Illumina 450k array data generated by The Cancer Genome Atlas Research Network (http://cancergenome.nih.gov/), cancer and control IDAT files were obtained for thyroid carcinoma (509 tumor biopsy samples, 56 normal tissue samples), stomach adenocarcinoma (427, 2), kidney renal clear cell cancer (312, 160), bladder cancer (266, 19), lung adenocarcinoma (434, 32), colon adenocarcinoma (297, 38), and melanoma (427, 2). Quality control was performed using the R package MethylAid, and data were normalized using functional normalization in the Minfi package.[20;21] Average DNA methylation and their difference per CpG site were calculated for tumor and normal tissues, with absolute β-value difference greater than 0.2 considered as differentially methylated. To allow comparison of DNA methylation data of SS with published data from T-cell subsets, obtained using similar 450k arrays (Gene Expression Omnibus accession: GSE49667), quality control and normalization were performed as for The Cancer Genome Atlas samples.[22] Raw methylation data from T-cell subsets were kindly provided by Leonard C. Harrison. Average methylation of 1,689 CpG sites, identified as discriminative between activated regulatory T cells and activated naïve T cells, was calculated for SS and external T-cell samples.

METHYLATION-SPECIFIC MELTING CURVE ANALYSIS

Bisulfite primers were designed to amplify CpG islands located in the promoter region of the genes of interest, encompassing the corresponding significant probe sequences on the Illumina 450k array (**Supplementary Materials** online). Amplification was

performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands) using a touchdown PCR protocol followed by melting curve analysis.[23] A promoter was considered methylated if melting temperatures exceeded half of the maximum temperature difference as determined for the reference control samples.

5-AZA-2'-DEOXYCYTIDINE TREATMENT

The SeAx cell line was treated with 0.5µmol/L 5-aza-2'-deoxycytidine (Sigma, St. Louis, MO) for 3 weeks, during which multiple cell divisions occurred. Culture medium containing 5-aza-2'-deoxycytidine was replaced every 3-4 days. Cells were harvested for RNA and DNA extraction.

QUANTITATIVE REAL-TIME PCR

Expression of differentially methylated genes was examined in 32 SS and nine EID patients and the SeAx cell line using quantitative real-time PCR. RNA was isolated using the RNeasy Mini Kit (Qiagen). Complementary DNA synthesis was performed by using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed with SYBR Green Supermix (Bio-Rad). Data were normalized according the ΔΔCq method and presented as relative messenger RNA expression.[24] Primer sequences are given in **Supplementary Materials** online.

RESULTS

DEFINING THE SÉZARY SYNDROME METHYLOME

DNA samples from CD4+ T cells of 15 patients with SS, three patients with EID, and four healthy control subjects were subjected to genome-wide DNA methylation analysis using the Illumina 450k array that interrogates cytosine positions in 99% of annotated genes (**Table 1** and **Supplementary Table S1**). Multidimensional scaling analysis showed substantial differences in global DNA methylation patterns between samples from SS patients and samples from EID patients and healthy volunteers (**Figure 1a**). Hypomethylation was more common in the SS samples (37,139 CpG sites; 7.8%; β-value difference > 0.2; *P*-value adjusted for multiple testing using the false discovery rate ≤ 0.05) than hypermethylation (15,056 CpG sites, 3.2%). The methylation patterns of the SS cell lines SeAx and HuT78 differed substantially from those of our SS patient samples. In patients with lower CD4+ T-cell counts, indicative of peripheral blood tumor burden, the frequency of aberrant methylation events was not significantly higher (data not shown). All individual differentially methylated CpG sites are listed in **Supplementary Materials** online. The location of differentially methylated CpG sites across all human autosomes showed an even distribution, with no preferential hyper- or hypomethylation at specific chromosomes (**Figure 1b** and **Supplementary Figure S1**). Comparison of DNA methylation data of SS with that of solid tumor types generated by The Cancer Genome Atlas Research Network using the same array platform showed that both hypermethylation and hypomethylation were strikingly more common in SS (**Figure 1c**).

To further capture the distribution of aberrant methylation in SS, we visualized

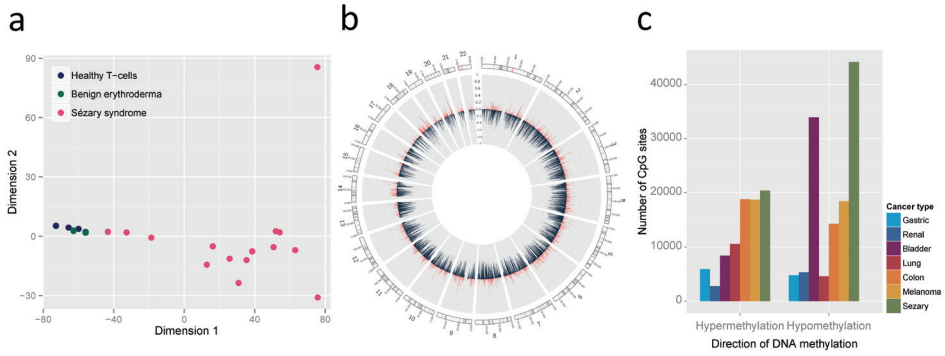

Figure 1. Genome-wide distribution of DNA methylation in Sézary syndrome (SS) patients, erythrodermic inflammatory dermatoses (EID) patients and, healthy control subjects.
a. Multidimensional scaling of all normalized methylation data for all autosomal CpG sites among all patient samples. The largest differences were found between SS samples and benign T-cell samples (dimension 1), and among SS patients variation was found as well (dimension 2). b. Circos representation of the location of differential DNA methylation between SS and benign T cells from EID patients and healthy volunteers across all autosomes. Red indicates hypermethylation and black hypomethylation. Median difference in DNA methylation values were calculated per 100 kilobase pair bins. c. Number of CpG sites with hypermethylation or hypomethylation (absolute β-value difference > 0.2, no *P*-value threshold) between healthy and tumor tissue of thyroid, stomach, kidney, bladder, lung, colon and skin melanoma and SS. CpG, 5'-cytosine-phosphate-guanine-3'; EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome.

the correlation between average methylation in the SS samples and the benign T-cell samples, considering CpG sites located in a CpG island context and non-CpG islands separately (**Figure 2a**). This showed a contrast between CpG sites within CpG islands that were predominantly hypermethylated and CpG sites not located in CpG islands that were more frequently hypomethylated. CpG sites at various specific loci showed selective hyper- and hypomethylation in SS, as illustrated for the *GNMT* and *SPON2* loci (**Figure 2b**). The differentially methylated CpG sites were then assigned to CpG islands, shores and non-CpG islands (NCs) in different regions of a gene, namely the intergenic (IG), distal promoter (DP), proximal promoter (PP), gene body (GB), or downstream (DS) region. This gene-centric analysis showed that CpG sites located in CpG islands in the proximal promoter region of genes showed striking enrichment of hypermethylation in SS (odds ratio = 38, $P \leq 0.0001$; **Figure 2c**). Unsupervised clustering of promoter CpG island methylation separated SS and benign T-cell samples, as evidenced by multidimensional scaling analysis of CpG sites located in promoter CpG islands (**Supplementary Figure S2**).

Given the strong enrichment of hypermethylation in promoter CpG islands in SS and because this epigenetic alteration is often associated with transcriptional repression, we focused on aberrant methylation of promoter CpG islands in SS when compared with EID patients and healthy volunteers. In total, 126 protein-coding genes showed significant and frequent promoter CpG island hypermethylation in SS, whereas only the *LCN6* gene had a hypomethylated promoter CpG island (Welch *t* test, adjusted $P \leq 0.05$ and β-value difference > 0.2) (**Supplementary Materials**). A heat map showing the methylation patterns of 46 promoter CpG islands illustrates the specificity of these methylation alterations for SS (**Figure 2d**). The promoter CpG islands with the highest average β-value

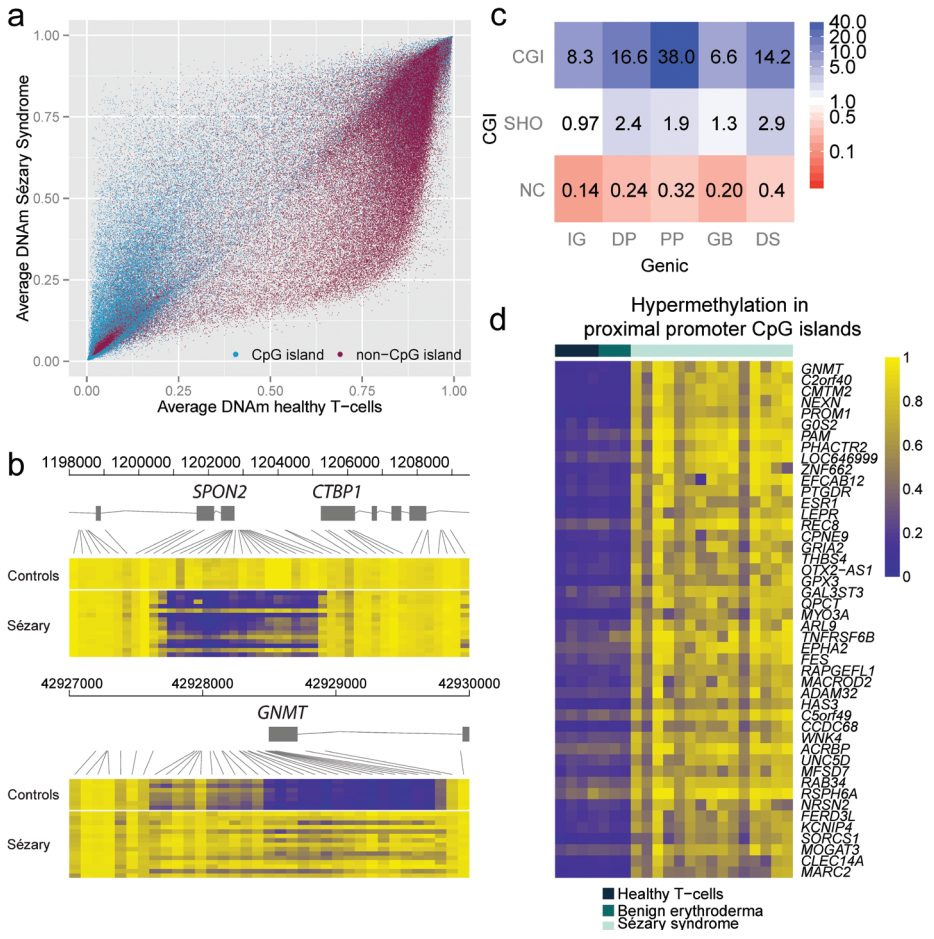

Figure 2. Gene-centric analysis of aberrant DNA methylation of CpG sites within and outside CpG islands. a. Scatterplot illustrating the relation between average DNA methylation in Sézary syndrome (SS) T cells and benign T cells for CpG sites located in a CpG island and CpG sites not located in a CpG island context, that is, not in a CpG island or 4-kilobase pair flanking region. b. Top: promoter of *SPON2*, which is hypomethylated in SS T cells and methylated in healthy T cells. Bottom: promoter of *GNMT*, which is hypermethylated in SS T cells and hypomethylated in healthy T cells. c. Enrichment (as odds ratio) of hypermethylation over hypomethylation in CpG island- and gene-centric annotations. CpG island shores were located within a 2-kilobase pair distance of CpG islands. d. Heat map highlighting the top promoter CpG islands identified as hypermethylated in SS T cells ($P \leq 0.05$, β-value difference ≥ 0.4) when compared with benign T cells, ranked according to average β-value difference. Genes are clustered based on euclidean distance. CGI, CpG island; CpG, 5′-cytosine-phosphate-guanine-3′; DP, distal promoter; DS, downstream; GB, gene body; IG, intergenic; NC, non-CpG island; PP, proximal promoter; SHO, CpG island shore.

difference, indicative of the level of hypermethylation, were those of *GNMT*, *C2orf40*, *CMTM2*, *NEXN*, *PROM1*, and *G0S2*. The hypermethylated promoter CpG islands in SS included those of the tumor suppressor genes *GNMT*, *C2orf40*, *GPX3*, *FES*, *NRSN2*, *THRB*, *TGFBI*, *IRX1*, *ASCL1*, and *CDK2AP1*, listed in the TSGene database.[25] Assessment of genes previously reported as hypermethylated in SS and other CTCL types showed promoter

hypermethylation of *THBS4* (93% of patients), *TP73* (27%), *NEUROG1* (13%), *RARB* (7%), and *PPARG* (7%) in the SS patients (**Supplementary Table S2**). However, we could not confirm promoter hypermethylation of other genes, including *MLH1*, *BCL7A*, and *SOCS1*, previously reported in CTCL. The discrepancies might be related to the fact that particular promoter CpG sequences were not interrogated by the array probes and to differences in promoter methylation patterns between SS and other CTCL types analyzed in the reported studies.

It has been reported that SS T cells show similarity to regulatory T cells.[3] We compared the SS methylomes to those of regulatory T-cell subsets generated using the same array platform.[22] In their study, 1,689 CpG sites were identified that were significantly differentially methylated between activated naïve and activated regulatory T-cell subsets. Joint analysis of DNA methylation of these 1,689 CpG sites in our SS samples and in the published T-cell subsets showed that methylation in most SS samples differed from that of activated naïve T cells and bore more resemblance to that of activated regulatory T cells (**Supplementary Figure S3**). In two SS samples, the methylation pattern appeared more similar to that of activated naïve T cells, suggesting variability of T-cell phenotype among patients with SS.

HYPERMETHYLATED PROMOTER CPG LOCI AS DIAGNOSTIC MARKERS FOR SÉZARY SYNDROME

Among the 126 hypermethylated genes in SS, we selected 12 genes for validation with methylation-specific melting curve analysis. These included the seven gene promoter CpG islands with the highest average β-value difference (>0.6) and the *TFAP2A*, *ID4*, *TGFB2*, *HSPB6*, and *CPEB3* genes based on gene function. Promoter hypermethylation has been shown previously for these genes in other tumor types, except for *NEXN* and *CPEB3*.[26-29] First, we confirmed the Illumina 450k array results by examining promoter methylation using methylation-specific melting curve analysis of the 12 genes in the 15 SS and seven benign control samples previously subjected to genome-wide methylation analysis (**Table 2**). The frequency of methylation of these genes ranged from 40 –100% in SS samples, whereas methylation of these genes was absent in all benign T-cell samples. Next, we investigated the methylation status of these genes in an independent sample set consisting of DNA isolated from CD4+ T cells of 20 patients with SS, 10 patients with EID, and seven healthy volunteers. Detailed results of methylation analysis studies are shown for the *CMTM2* gene as example in **Figure 3**. Methylation-specific melting curve analysis profiles show a clear additional peak corresponding with the melting temperature of the methylated sequence after bisulphite conversion in SS samples, whereas only a peak corresponding to unmethylated DNA is seen in the EID samples (**Figure 3b**). The methylation frequencies in SS samples in the independent sample group were largely similar to those obtained in the primary sample set (**Table 2**). None of the 12 selected genes was affected by promoter CpG island methylation in the benign T-cell samples from patients with EID or from healthy volunteers. The *CMTM2* gene was methylated in all SS samples of the validation group and of the initial group subjected to epigenomic analysis. Detection of promoter CpG island hypermethylation of a single gene, *CMTM2*, therefore allowed accurate diagnosis of SS and distinction from EID with 100% sensitivity

Table 2. Promoter CpG island hypermethylation of selected genes.

Gene		Frequency of aberrant promoter hypermethylation		
		Confirmation		
Symbol	**Function**	**SS, n=15**	**EID, n=3**	**HC, n=4**
CMTM2	Chemokine-like factor gene	**100%**	0%	0%
C2orf40	ECRG4; tumor suppressor gene	**93%**	0%	0%
G0S2	Regulates proliferation and apoptosis; tumor suppressor gene	**93%**	0%	0%
HSPB6	Heat shock protein 20; promotes apoptosis	**93%**	0%	0%
PROM1	CD133; transmembrane protein expressed by tumor stem cells	**100%**	0%	0%
PAM	Multifunctional enzyme; activates neuropeptides	**100%**	0%	0%
CPEB3	Regulates mRNA translation by binding to 3'-UTR	**93%**	0%	0%
GNMT	Metabolic modulator of DNA methylation; tumor suppressor gene	**93%**	0%	0%
TFAP2A	Transcription factor AP-2 alpha; tumor suppressor gene	**93%**	0%	0%
NEXN	Actin-binding protein with role in migration	**87%**	0%	0%
ID4	Transcription factor; tumor suppressor gene	**73%**	0%	0%
TGFB2	Multifunctional cytokine; regulates proliferation and differentiation	**40%**	0%	0%

Boldface is meant to place emphasis on the results of SS.
AP-2, activator protein; CpG, 5'-cytosine-phosphate-guanine-3'; EID, erythrodermic inflammatory dermatoses; HC, healthy control subjects; mRNA, messenger RNA; SS, Sézary syndrome; UTR, untranslated region.

and specificity in these patient groups. Promoter hypermethylation of *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, *PAM*, and the *CPEB3* gene was observed in at least 90% of cases in the validation group as well as in the initial group.

DIMINISHED EXPRESSION OF HYPERMETHYLATED GENES IN SÉZARY SYNDROME

Comparison of promoter methylation and messenger RNA levels in 32 CD4+ T-cell samples from SS patients and nine CD4+ T-cell samples from EID patients showed that the *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, *GNMT*, and *NEXN* genes, hypermethylated in most of the analyzed SS samples, were expressed at significantly lower levels in SS than in benign T-cell samples (**Table 3**). For *CMTM2*, the results of messenger RNA expression analysis using quantitative real-time PCR are depicted in **Figure 3c** and **d**. Treatment of the SeAx cell line with the demethylating agent 5-aza-2'-deoxycytidine and analysis of gene expression further showed an association between promoter methylation and transcriptional repression for the *CMTM2* and *NEXN* genes. Genes previously

Frequency of aberrant promoter hypermethylation				
	Validation		Results (combined)	
SS, n=20	EID, n=10	HC, n=7	SS, n=35	EID/HC, n=24
100%	0%	0%	**100%**	0%
100%	0%	0%	**97%**	0%
95%	0%	0%	**94%**	0%
95%	0%	0%	**94%**	0%
90%	0%	0%	**94%**	0%
90%	0%	0%	**94%**	0%
90%	0%	0%	**91%**	0%
85%	0%	0%	**89%**	0%
85%	0%	0%	**89%**	0%
75%	0%	0%	**80%**	0%
70%	0%	0%	**71%**	0%
40%	0%	0%	**40%**	0%

shown to be epigenetically regulated were not analyzed in 5-aza gene reactivation experiments. These results show that for most of these genes, including the candidate tumor suppressors *GNMT* and *C2orf40*, promoter hypermethylation is associated with transcriptional repression.

DISCUSSION

In this study we characterized the DNA methylomes of CD4+ T cells from patients with SS and from patients with EID and healthy control subjects. The extent of aberrant methylation in SS was high and exceeded that in solid malignancies analyzed by The Cancer Genome Atlas Research Network. This observation fits well with recently described mutations in epigenetic modifier genes such as *DNMT3a* and *TET1* in SS.[11] Although recent DNA methylation studies in cancer have highlighted the occurrence

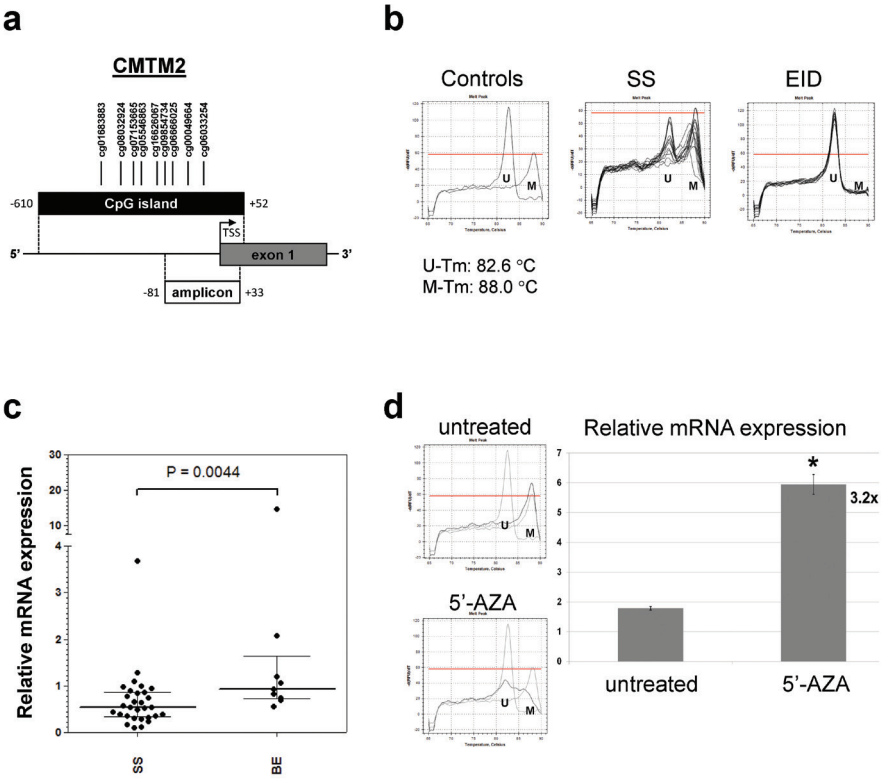

Figure 3. Validation of promoter hypermethylation and transcriptional down-regulation of the *CMTM2* gene in Sézary syndrome (SS).
a. Schematic depiction of the CpG island in relation to the promoter and gene body with location of the significant CpG sites interrogated by the array and amplicon for MS-MCA relative to the transcription start site. b. MS-MCA curve profiles of DNA samples plotted jointly in single graphs. Left panel: fully methylated and unmethylated control samples. The SS samples in the middle panel show a peak in their melting curve patterns at the melting temperature of 88.0°C for methylated DNA, whereas EID samples in the left panel show a peak exclusively at the temperature for unmethylated DNA of 82.6°C. c. Relative mRNA expression data of 32 SS and nine EID patients depicted as dot plots. Median and error bars are generated according to the standard interquartile range where an one-tailed Mann-Whitney test was applied to prove significant differential expression. d. MS-MCA curve plots before and after treatment of the SeAx cell line with 5-aza-2'-deoxycytidine showing relative demethylation (left panel). The effect of demethylation on *CMTM2* expression in the SeAx cell line cultured in the presence or absence of 0.5 mmol/L 5-aza-2'-deoxycytidine (right panel). Data are representative of duplicate treatment experiments; error bars indicate standard deviation from triplicate quantitative real-time PCR experiments. The fold induction in expression upon treatment with 5-aza-2'-deoxycytidine is indicated next to the bars. An one-sided t test was used for statistical analysis. *$P <$ 0.05. 5'-AZA, 5-aza-2'-deoxycytidine; BE, benign erythroderma; CpG, 5'-cytosine-phosphate-guanine-3'; EID, erythrodermic inflammatory dermatoses; M, methylated; mRNA, messenger RNA; MS-MCA, methylation-specific melting curve analysis; SS, Sézary syndrome; Tm, melting temperature; U, unmethylated.

of non-CpG island methylation, this unbiased genome-wide analysis shows that hypermethylation is particularly enriched for in CpG islands located in promoters and to a lesser extent in downstream regions in SS. Hypomethylation occurred predominantly at CpG sites not located in CpG islands in intergenic regions. This high-resolution view of

Table 3. Expression of selected genes.[26-28;34;43;44]

Gene	Differential expression 32 SS vs 9 EID	Up-regulation of demethylated SeAx cell line 5-aza-2'-deoxycytidine	
	P-value	Fold change	*P*-value
CMTM2	0.0044	x3.2	0.0011
G0S2	0.0045	x83.2	0.0021
HSPB6	0.0143	x2.2	ns
PAM	ns	n/a	n/a
CPEB3	ns	x1.9	0.0624
NEXN	0.0450	x2.9	0.0189
C2orf40	0.0003	Yue et al.	
PROM1	0.0005	Pleshkan et al.	
GNMT	0.0002	Huidobro et al.	
TFAP2A	Up-regulated	Douglas et al.	
ID4	Not expressed	Chen et al.	
TGFB2	ns	Hinshelwood et al.	

EID, erythrodermic inflammatory dermatoses; n/a, not applicable; ns, not significant; SS, Sézary syndrome.

DNA methylation showed an array of epigenetic alterations common to SS patients with potential clinical applications. DNA methylation detection is increasingly applied in the clinical diagnosis of malignant tumors, because analysis of this stable epigenetic mark is straightforward and yields binary results.[30] Here we concentrated on the most robustly hypermethylated gene promoters to identify diagnostic markers. In two independent sample sets the promoters of *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, and *PAM* were methylated in almost all SS samples but not in benign T-cell samples. Promoter hypermethylation of these genes was associated with transcriptional down-regulation in most cases. Promoter hypermethylation of a single gene, *CMTM2*, accurately distinguished all SS patients from EID patients and healthy control subjects with 100% sensitivity and specificity. SS-specific methylation of these selectively hypermethylated gene promoters, and *CMTM2* in particular, renders them useful epigenetic markers for the diagnosis of this lymphoid malignancy. The *CMTM2* (chemokine-like factor MARVEL transmembrane domain-containing 2) gene belongs to the chemokine-like factor superfamily and is primarily expressed in testis, bone marrow and peripheral blood cells.[31] *CMTM2* affects the function of the cyclic AMP response element-binding protein (AP-1) and CRE-binding protein (CREB) transcription factors in T cells.[32] Tumor suppressive properties have been reported for other members of this protein family.[33] The specificity of *CMTM2* promoter hypermethylation with concomitant transcriptional down-regulation might signify tumor suppressive functions in SS.

Among the 126 gene promoters with consistent hypermethylation in SS, there were multiple established and potential tumor suppressor genes. We found promoter hypermethylation of the *GNMT* tumor suppressor gene in most SS patients included

in this study, although it was expressed at significantly lower levels in SS than in EID. *GNMT* encodes a methyltransferase that catalyzes the conversion of the DNA methyl donor group *S*-adenosylmethionine to *S*-adenosylhomocysteine. Epigenetic silencing of *GNMT* leads to increased *S*-adenosylmethionine levels and has been found in hepatocellular cancer to promote establishment of DNA hypermethylation.[34] The consistent promoter hypermethylation and repression of *GNMT* in SS cells could indicate that a similar mechanism is operational in SS. Another potential tumor suppressor gene with recurrent promoter hypermethylation in SS is *ID4* (inhibitor of DNA binding protein 4), which is implicated in the development of chronic lymphocytic leukemia.[27] *G0S2* (G0/G1 switch gene 2), methylated and repressed in almost all SS samples, has been reported to regulate the proliferation of hematopoietic stem cells and leukemia cells.[35] In addition, in SS extensive DNA hypomethylation of CpG sites not located in CpG islands was found. DNA hypomethylation can result in chromosomal instability, and accordingly, the SS genome is characterized by gross chromosomal instability with many numerical and structural chromosomal alterations.[36;37] The silencing of multiple tumor suppressor genes and the extensive hypomethylation that is associated with chromosomal instability jointly point to a critical role for epigenetic mechanisms in the molecular pathogenesis of SS. This study therefore provides further rationale for epigenetic therapy of SS with histone deacetylase inhibitors (HDAC) and DNA demethylating agents. Accordingly, recent experiments that showed that exposure of the SS cell line HUT78 to a DNA demethylating agent restored sensitivity to apoptosis and reduced proliferation.[38] HDAC can cause reversal of gene promoter methylation and the clinical efficacy of vorinostat and romidepsin used in the treatment of patients with CTCL might be conferred in part by this property.[39]

SS cells have been proposed to represent a malignant clonal proliferation of the regulatory T cells in a subset of patients.[3] We found that in most patients with SS the methylation pattern differed from that of activated naïve T cells and bore more resemblance to that of activated regulatory T cells. The findings of this comparative analysis, although limited by technical variation, point to an epigenetic imprint in most SS samples with similarity to that of regulatory T cells. It would be worthwhile to perform a direct comparison of DNA methylation patterns of SS T cells with all T-cell subsets.

In summary, genome-wide methylation patterns of CD4+ T cells from patients with SS show extensive aberrant DNA methylation and show highly SS-specific hypermethylation events that can be used as epigenetic markers in the clinic for the diagnosis of this lymphoid malignancy. Several previous studies have tried to define additional immunophenotypic markers, copy number variations, gene, microRNA and long noncoding RNA expression profiles that can aid in the diagnosis of SS.[40-42] Our results indicate that measuring promoter methylation of a single gene, *CMTM2*, is sufficient to distinguish SS from benign conditions, rendering it an epigenetic marker that can readily be implemented in clinical practice. A prospective clinical study is justified to evaluate the diagnostic utility of *CMTM2* and other epigenetic markers in clinical practice. In addition, the widespread aberrant DNA methylation observed in this study strengthens the rationale for epigenetic therapies of SS using HDAC, possibly combined with demethylating agents.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Clinical characteristics of patients with Sézary syndrome and erythrodermic inflammatory dermatoses.
a. Blood cell counts and treatment at time of sample collection for each patient with Sézary syndrome.

Sézary patient, n=15	Leukocytes $x10^9/L$*	Lymphocytes % (including Sézary cells)	CD4 count $x10^6/L$*	CD8 count $x10^6/L$*	CD4/CD8 ratio*	Sézary cells /mm^3	Treatment at sample collection
1	9,70	33,00	3918	182	21,48	2910	prednisone
2	8,60	6,00	272	28	9,58	172	prednisone
3	12,00	28,60	3589	39	93,20	N/A	prednisone
4	14,10	48,00	6809	153	44,51	2820	prednisone
5	6,80	47,00	3277	173	18,90	1292	methotrexate
6	12,10	44,00	5372	313	17,18	1815	chlorambucil
7	8,40	44,10	3807	70	54,11	N/A	no therapy
8	20,40	72,20	15170	113	134,25	N/A	no therapy
9	17,10		11732	113	103,75	N/A	prednisone
10	23,20		17113	276	62,14	N/A	prednisone
11	11,74	55,70	3282	849	3,86	2113	acitretin
12	15,20	28,00	5051	26	194,27	N/A	prednisone
13	13,10	28,00	5110	66	77,42	N/A	prednisone and interferon-alpha
14	13,70	59,00	8612	378	22,78	N/A	no therapy
15	25,20	45,50	10606	122	86,93	N/A	prednisone and chlorambucil

N/A, not available.
*Median blood cell counts for the group of patients with Sézary syndrome: leukocytes 13.1 (range = 6.8-25.2); absolute CD4 count 5110 (range = 272-17113); absolute CD8 count 122 (range = 26-849); CD4/CD8 ratio median 54.11 (range = 3.86-194.27).

b. Clinical characteristics of patients with erythrodermic inflammatory dermatoses (EID).
Of the initial group of three EID patients, who were subjected to Infinium 450k Beadchip methylation analysis, two patients had erythroderma secondary to atopic dermatitis and one patient had an idiopathic erythroderma. The validation group, consisting of 10 patients with EID who were analyzed for methylation of 12 selected genes, included five patients with erythroderma secondary to atopic dermatitis, three patients with erythrodermic psoriasis, one patient with erythrodermic drug eruption and one patient with paraneoplastic erythroderma (colorectal cancer).

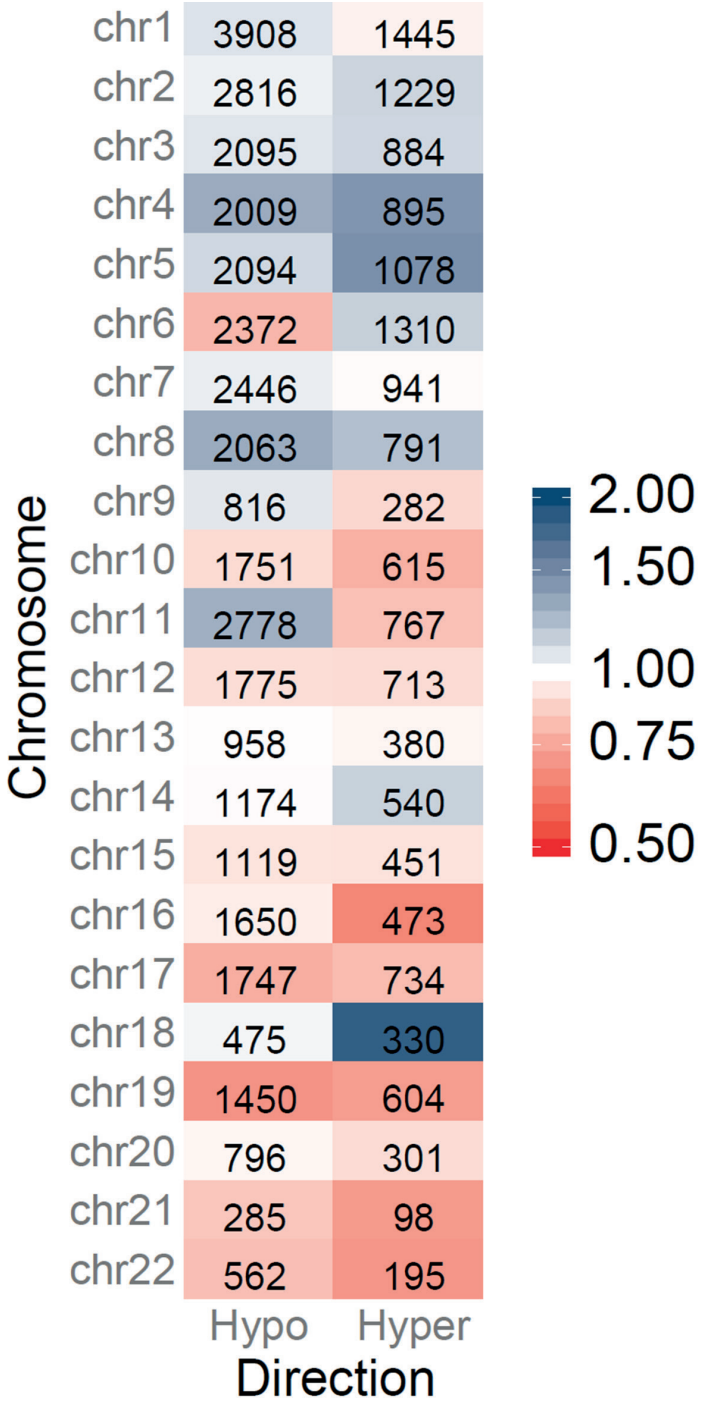
Characteristics of EID	**Confirmation EID, n=3**	**Validation EID, n=10**
Male:female ratio	2:1	9:1
Age in years, median (range)	41 (31-74)	71 (37-86)
Erythroderma	3/3	10/10
Pruritus	3/3	5/10
Ectropion	0/3	1/10
Hyperkeratosis hands/feet	1/3	0/10
Palpable lymphadenopathy	0/3	0/10
Lymphadenopathy confirmed by CT scan	0/0	0/1
Leukocytes ≥ 10.0 $x10^9$/L	0/2	4/6
CD4/CD8 ratio ≥ 10.0	0/0	0/4
Absolute Sézary cell count ≥ 1000 per mm^3	0/0	0/2
T-cell clone in peripheral blood	0/3	0/10
Identical T-cell clone in blood and skin	0/0	0/0
Status last date of follow-up		
Alive with disease	3	10
Died of disease		
Died of other cause than linked to SS		
Died of unknown cause		

EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome

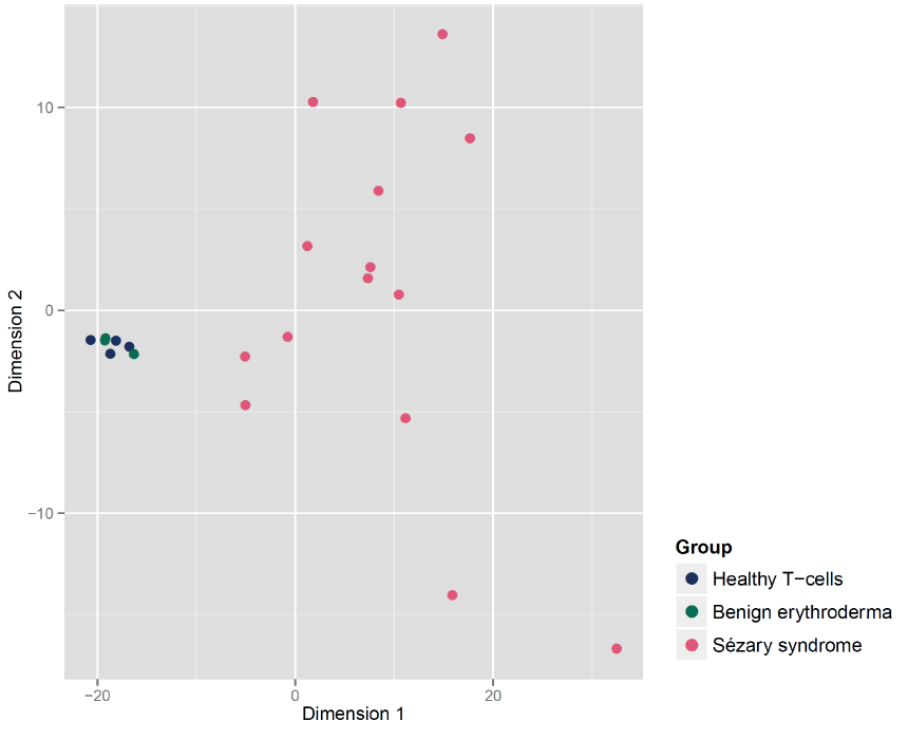
Supplementary Table S2. Promoter hypermethylation of genes reported in previous studies.[1-11]

Gene	Frequency of aberrant promoter hypermethylation					
	SS, n=15	EID, n=3	HC, n=4	reported as	in CTCL type	reference
SAMHD1	**0%**	0%	0%	hypermethylated	Sezary syndrome	de Silva et al.
FAS	**0%**	0%	0%	hypermethylated	Sezary syndrome, CTCL	Jones et al., 2010; Wu et al.
CDKN2B	**0%**	0%	0%	hypermethylated	Sezary syndrome, CTCL	Scarisbrick et al., 2002; Gallardo et al.; van Doorn et al.
CDKN2A	**0%**	0%	0%	hypermethylated	Sezary syndrome, CTCL	Navas et al.; Scarisbrick at al., 2002; Gallardo et al,; van Doorn et al.; Ferrara et al.
MGMT	**0%**	0%	0%	hypermethylated	CTCL	Gallardo et al.; van Doorn et al; Ferrara et al.
MLH1	**0%**	0%	0%	hypermethylated	Mycosis fungoides	Scarisbrick et al., 2003; Ferrara et al.
BCL7A	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
PTPRG	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
THBS4	**93%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
TP73	**27%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
CHFR	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
PYCARD	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
CDKN1B	**0%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
IGF2	**80% (Hypomethylation)**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
NEUROG1	**13%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
PPARG	**7%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
RARB	**7%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
SOCS1	**0%**	0%	0%	hypermethylated	Mycosis fungoides	van Doorn et al.; Ferrara et al.
CDKN1C	**0%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
PLS3	**7% (Hypermethylation)**	0%	0%	hypomethylated	Sezary syndrome	Jones et al., 2012
FOXP3	**0%**	0%	0%	hypomethylated	Sezary syndrome, CTCL	Heid et al.

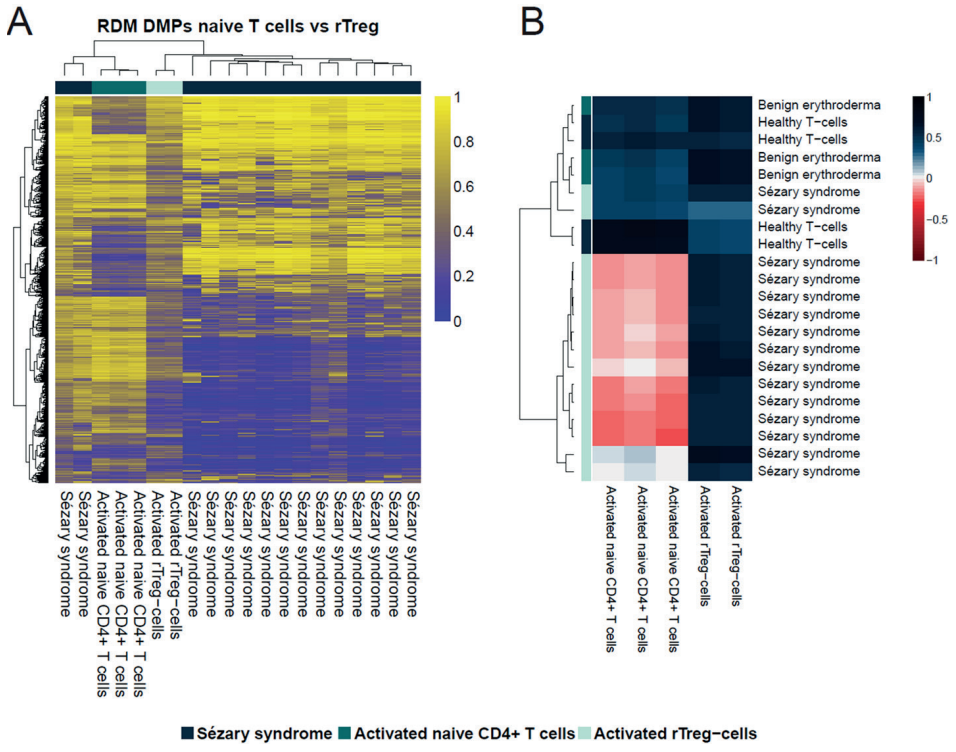
CTCL, cutaneous T-cell lymphoma; EID, erythrodermic inflammatory dermatoses; HC, healthy control; SS, Sézary syndrome



Supplementary Figure S1. Enrichment of hyper- and hypomethylation over chromosomes (absolute number of CG sites and odds ratios).



Supplementary Figure S2. Multidimensional scaling analysis of CpG sites located in CpG islands of proximal promoter regions of autosomal genes in Sézary syndrome, erythrodermic inflammatory dermatoses and healthy control subjects.



Supplementary Figure S3. Comparative analysis of DNA methylation with T-cell subsets.
a. DNA methylation for 1,689 CpG sites previously identified to be discriminative between activated naïve T cells and activated regulatory T cells in Sézary syndrome (SS) samples and external T-cell samples. The DNA methylation profile of SS resembles the methylation profile of activated regulatory T cells to a larger extent than that of activated naïve T cells. b. Correlation between DNA methylation of SS patients and activated naïve T cells and activated regulatory T cells. A high positive correlation was found between SS methylation profiles and regulatory T cells, suggesting it is more similar to regulatory T cells than activated naïve T cells.

Supplementary materials containing the remaining tables are available online at www.jidonline.org

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6

Quantitation of tumor development correlates with prognosis in tumor stage (stage IIB) mycosis fungoides

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ABSTRACT

Background Patients with mycosis fungoides (MF) tumor stage IIB disease show considerable variation in the number of tumors and time interval between each tumor occasion.

Objectives To quantify the extent of tumor formation in patients with stage IIB MF and correlation with survival.

Methods The variability in tumor development of 46 patients with stage IIB MF was quantified by calculating a frailty score with the use of a statistical frailty model, based on both the number of tumors developed during follow-up and the time interval between each tumor occasion. The prognostic value of the frailty scores and the number of tumors at 6 and 12 months after first tumor development were studied.

Results Frailty scores varied between 0.05 en 6.94. Patients with high frailties (> 1.0, n = 14) had the worst disease-specific survival (DSS) and overall survival (OS), compared with patients in the low frailty group (0-0.35, n = 17) and medium frailty group (0.35-1.0, n = 15). Differences in DSS and OS between the three frailty groups were highly significant (both $P < 0.001$). The number of tumors that developed within 6 months after the diagnosis of MF stage IIB was prognostic for subsequent DSS and OS ($P < 0.001$ and $P = 0.021$, respectively).

Conclusions The number of tumors and time interval between tumor formation differs greatly among patients with stage IIB MF and these differences correlate with survival. Patients with an adverse prognosis can be identified by quantifying the number of tumors that develop within 6 months after diagnosis of MF stage IIB.

INTRODUCTION

Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma, accounting for almost 50% of all cutaneous lymphomas. The disease course is characterized clinically by progression through defined clinical stages − from patches to plaques to tumors − and in a minority of patients to extracutaneous localisations.[1]

The staging of MF is based on the tumor-node-metastasis-blood (TNMB) staging system, which classifies both type and extent of skin lesions, the presence and degree of lymph nodes and visceral and blood involvement. In the latest revisions to the staging and classification of MF by the International Society for Cutaneous Lymphomas (ISCL) and the European Organization for Research and Treatment of Cancer (EORTC), tumor stage disease is defined by the presence of at least one tumor ≥ 1 cm in diameter.[2]

The prognosis of patients with MF is closely correlated with disease stage. While the survival in MF stage IA is comparable with an age-, race- and sex-matched control population, the prognosis deteriorates with progression of disease.[3-6] Patients with tumor stage MF without extracutaneous disease, stage IIB, have a reduced 5-year disease-specific survival (DSS) of 56−80% and are at risk for progression to extracutaneous sites.[7;8] Apart from clinical stage, folliculotropic MF and large cell transformation have been associated with adverse prognosis in MF.[6-11]

Although patients with stage IIB MF are categorized as a homogeneous group, clinical observations show considerable variation in patients with MF stage IIB disease. Some patients present with a solitary tumor whereas others present with extensive tumor formation. Also, the time interval between the development of new tumors differs greatly among patients with stage IIB disease. Previous studies addressing the relationship between tumor formation and prognosis focused on tumor distribution at diagnosis. Two studies reported that patients who have generalized skin tumors at presentation have a reduced survival compared with those who present with only a solitary tumor.[9;12] In contrast, Agar et al did not find an association between the extent of skin tumors at diagnosis and survival.[8] However, in these studies the exact number of tumors was not quantified and the number of tumors developing during follow-up was not investigated.

The aim of the current retrospective follow-up study was to investigate the relationship between tumor formation and survival in more detail. We quantified the extent of tumor development in patients with MF stage IIB disease using a statistical frailty model fitted on the basis of both the number of tumors that developed during follow-up and the time interval between each tumor occasion, and correlated results with survival. In addition, we evaluated whether the number of tumors that developed within 6 and 12 months after the appearance of the first tumor is prognostic for subsequent survival.

METHODS

PATIENT SELECTION

Forty-six patients, who were managed at the Department of Dermatology of the Leiden University Medical Center (LUMC) between 1984 and 2012, were selected from the cutaneous lymphoma database of the Dutch Cutaneous Lymphoma Group (DCLG).

Inclusion criteria were (i) clinical and histological features of MF, as assessed by a panel of dermatologists and pathologists during one of the quarterly meetings of the DCLG; (ii) stage IIB disease at first diagnosis of MF or development of stage IIB disease during follow-up; and (iii) under regular surveillance in the LUMC. Stage IIB was defined according to the classification, proposed by the ISCL and EORTC, requiring at least one tumor ≥ 1 cm in diameter.[2]

CLINICAL EVALUATION

Medical records and photographs taken at the time of first tumor presentation and during follow-up were reviewed for all patients. Each occasion of tumor growth was registered with dates, and clinical information was obtained about the number of tumors (categorized as 1, 2, 3, 4, 5 or more tumors), therapy and response to therapeutic regime.

Other variables recorded for each patient were sex, duration of skin lesions before diagnosis MF, age at diagnosis of MF, stage at diagnosis of MF, age at progression to stage IIB, response to initial therapy, progression towards stage IV, the presence of folliculotropic MF, duration of follow-up and survival status.

Additionally, from available skin biopsies from tumors at first development of stage IIB disease, large cell transformation (presence of large T cells exceeding 25% of the total lymphoid infiltrate or forming microscopic nodules) was recorded.[13]

FRAILTY SCORE

The frailty model is a statistical approach to test for and quantify differences in the occurrence of a specific event between individuals of a defined group.[14] We used this method to investigate the propensity to develop tumors in patients with MF stage IIB disease. This frailty model takes into account both the number of tumors developed during follow-up (including tumors at the time of the diagnosis of stage IIB) and the time interval between each tumor occasion. The frailty model specifies that the tumor recurrence rate of an individual equals a baseline rate multiplied by a random effects term, called the frailty. These unmeasured effects are assumed to follow a given statistical distribution (γ) with mean equal to 1 and unknown variance σ^2. The frailty variance σ^2 quantifies the heterogeneity in recurrence rates between the patients. After having estimated the unknown parameters in the model, the clinical heterogeneity was translated into a numerical score for each individual, called the frailty score, using this formula:

$$\text{Frailty score}_i = \frac{n_i(t) + \frac{1}{\sigma^2}}{H_i(t) + \frac{1}{\sigma^2}}$$

Here t denotes the total follow-up time from first diagnosis of MF stage IIB disease of the individual, $H_i(t)$ the cumulative recurrence rate and $n_i(t)$ the total number of tumors developed in individual i. To correlate the frailty scores with survival we divided the patients into three categories of approximately the same size, namely the low, medium and high frailty scores.

STATISTICAL ANALYSIS

All statistical calculations were performed using SPSS Statistics 20.0 (IBM, Armonk, NY, USA), with the exception of the frailty and landmark analyses (below), which were performed in R, version 2.15.0 (http://www.r-project.org). For comparison between groups the ANOVA test or, in case of discrete data, the χ^2 test, was used. Survival was calculated from the date of development of stage IIB disease until the patient's death or date of last follow-up. The Kaplan-Meier method was used to estimate survival curves, and comparison between curves, was performed using the log-rank test. Median follow-up was calculated using the reverse Kaplan-Meier method.

In order to study whether the number of tumors during follow-up was predictive of survival, a landmark analysis was performed, where the number of tumors that developed in the first 6 and 12 months of follow-up was used as a predictor of subsequent survival in all individuals who were still at risk after 1 year. In the analysis the number of tumors present at first development of stage IIB was included. Analysis with DSS was performed accounting for death of other causes as a competing risk.[15]

Univariate analysis of parameters with possible prognostic significance for DSS and overall survival (OS) was performed using Cox proportional hazards regression analysis. Parameters that were analyzed for their prognostic significance were sex (male vs female), age at development of stage IIB (continuous variable), time interval between first diagnosis of MF and development of stage IIB disease (continuous variable), folliculotropic MF (absent vs present) and large cell transformation at development of stage IIB (yes vs no). These parameters were combined with (i) the frailty score (continuous; after log-transformation), or (ii) the number of tumors at the first development of stage IIB, and (iii) the first 6 months or (iv) the first 12 months after development of stage IIB (continuous variable). The parameters that were significant at the 0.25 level were included in a multivariate analysis model. *P*-values below 0.05 were regarded as statistically significant.

RESULTS

CLINICAL CHARACTERISTICS AND FOLLOW-UP

The main clinical characteristics and follow-up data of the 46 patients with stage IIB disease are summarized in **Table 1**. There was a male predominance (male/female ratio of 2.5) and the median age at development of stage IIB was 69 years (range = 39-90 years). Twenty-three patients had stage IIB at the time of first diagnosis of MF, the other 23 patients developed stage IIB during follow-up, varying from 1 to 181 months after first diagnosis of MF. Of the 46 patients, 20 patients (43%) were diagnosed with folliculotropic MF. Forty-two patients had skin biopsies available from tumors at development of stage IIB disease, of whom 23 patients showed large cell transformation.

Previous treatments before development of stage IIB disease included local steroids, psoralen plus ultraviolet A (PUVA) therapy, local radiotherapy, interferon alfa and total skin electron beam therapy. None of these patients received (poly)chemotherapy prior to or during development of stage IIB disease. The first therapy after development of stage IIB disease consisted of skin-directed therapies other than local radiotherapy (local steroids, PUVA therapy, excision, topical nitrogen mustard) (n = 9), local radiotherapy (n = 31) or total skin electron beam therapy (n = 6). During follow-up a total of 11 patients received (poly)chemotherapy because of rapid and massive tumor formation and/ or progression to stage IV disease.

The median follow-up time calculated from diagnosis of MF was 96 months (range = 3-323 months) compared with 88 months (range = 2-241 months) when measured from development of stage IIB disease. During follow-up 28 patients died, including 18 MF-related deaths. After progression to stage IIB, DSS after 1, 2 and 5 years was 97%, 81% and 60%, respectively, and OS was 93%, 73% and 46%, respectively.

TIMELINE TEMPLATES

For each patient a timeline template was constructed in Excel to get a clear understanding of the tumor formation during follow-up. In these timelines the development of new tumors and the number of tumors developed at that moment are plotted against follow-up time in months, as demonstrated in **Figure 1** for three selected patients. The smaller the interval between every tumor occasion, the higher the rate of tumor recurrence. These timeline templates visualize different patterns of tumor formation in individual patients, illustrating the clinical heterogeneity in patients with stage IIB disease.

FRAILTY SCORE AND CORRELATION WITH SURVIVAL

The frailty model revealed a highly significant ($P < 0.001$) variability in tumor recurrence rates between patients, with an estimated frailty variance of 1.64. The individual frailty scores, calculated for each patient, varied from 0.05 to 6.94 with a median frailty score of 0.64.

To analyse further the correlation of the tumor recurrence rates with survival, we divided the patients into three groups of equal size, namely the low, medium and high frailty categories, defined by frailty scores from 0 to 0.35, 0.35 to 1.0 and > 1.0, respectively. The main clinical characteristics of the low (n = 17), medium (n = 15) and

Table 1. Clinical characteristics and outcomes of 46 patients with mycosis fungoides (MF) stage IIB disease.

Male:female ratio	33:13
Duration of skin lesions before MF diagnosis in months, median (range)	24 (1-480)
Age in years, median (range)	
At diagnosis of MF	66 (39-90)
At stage IIB	69 (39-90)
Clinical stage at diagnosis of MF, n	
IA-IIA	23
IIB	23
Folliculotropic MF, n	
Absent	26
Present	20
Large cell transformation at development of stage IIB	
Yes	23
No	19
No biopsy available	4
Duration of follow-up in months, median (range)	
After diagnosis of MF	96 (3-323)
After stage IIB	88 (2-241)
Progression to stage IV during follow-up, n	
Yes	14
No	29
Unknown	3
Status at date of last follow-up, n	
Alive without disease	4
Alive with disease	14
Died of other cause	9
Died of unknown cause	1
Died of MF	18
Disease-specific survival after development of stage IIB, %	
At 1 year	97
At 2 years	81
At 5 years	60
Overall survival after development of stage IIB, %	
At 1 year	93
At 2 years	73
At 5 years	46

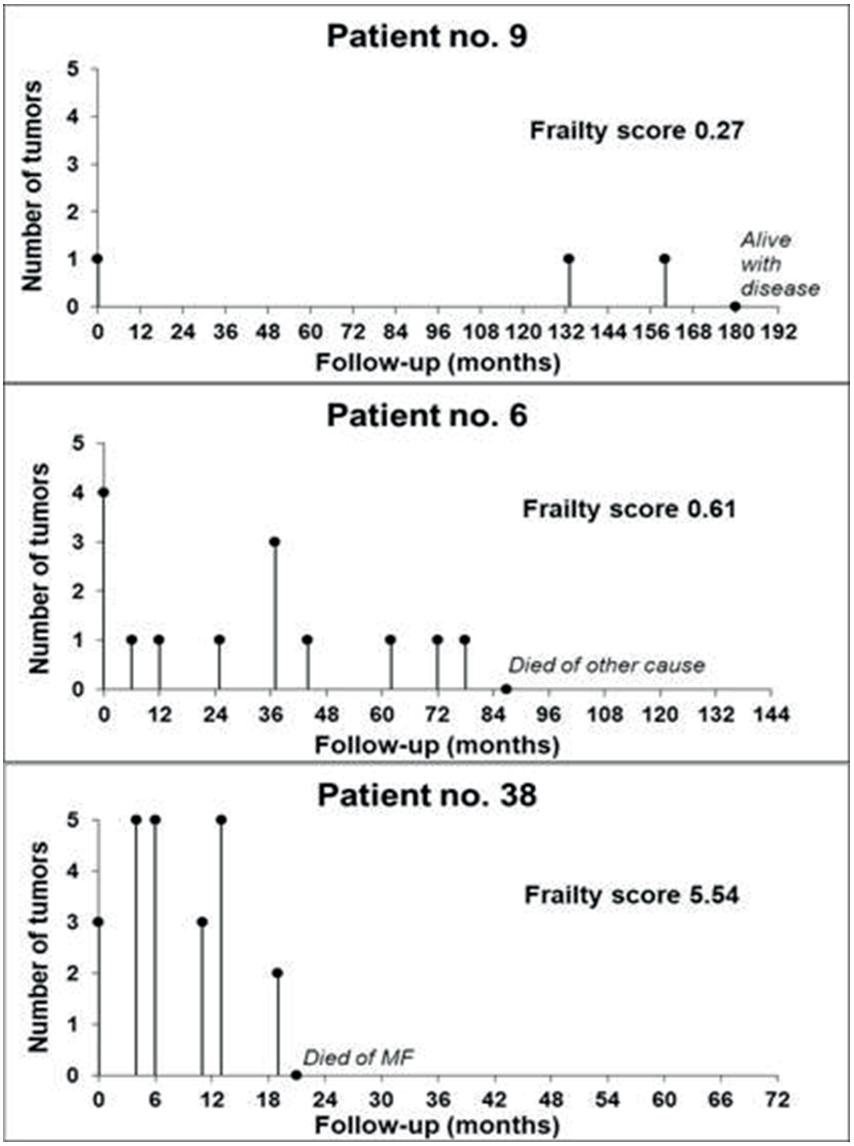

Figure 1. Three examples of the variability in number of tumors and time interval between each tumor recurrence per patient and accompanying frailty score. The dots represent a tumor occasion. Follow-up (month 0) starts at development of mycosis fungoides (MF) stage IIB.

high (n = 14) frailty groups are summarized in **Table 2.** The three groups were comparable in sex, median age at development of stage IIB disease and presence of folliculotropic MF.

The low frailty group had a DSS after 1, 2 and 5 years of 100%, 100% and 88%, respectively, and OS was 94%, 94% and 76%, respectively. The patients in the medium frailty group had a DSS after 1, 2 and 5 years of 100%, 79% and 64%, respectively, and OS

was 93%, 71% and 43%, respectively. The high frailty group had the worst survival with a DSS after 1, 2 and 5 years of 93%, 64% and 36%, respectively, and OS was 93%, 50% and 14%, respectively (**Figure 2**).

FRAILTY SCORE AND OTHER PROGNOSTIC PARAMETERS

In univariate analysis for DSS, time interval between first diagnosis of MF and development of stage IIB disease and folliculotropic MF were selected for subsequent multivariate analysis (univariate $P = 0.190$ and $P = 0.190$, respectively). Both univariate and multivariate analyses established that the differences in DSS between the three frailty groups were highly significant (multivariate $P < 0.001$; hazard ratio (HR) = 3.28; 95% confidence interval (CI) 1.69-6.38), while the time interval between first diagnosis of MF and development of stage IIB disease and presence of folliculotropic MF were not significant ($P = 0.730$ and $P = 0.670$, respectively).

In univariate analysis for OS, age at development of stage IIB was selected for subsequent multivariate analysis (univariate $P = 0.024$). Both univariate and multivariate analyses established that differences in OS between the three frailty groups were highly significant (multivariate $P < 0.001$; HR = 2.06; 95% CI 1.39-3.05). In addition, in both univariate and multivariate analyses age at development of stage IIB disease was significant for subsequent OS (multivariate $P = 0.050$; HR = 1.03; 95% CI 1.00-1.07).

Table 2. Clinical characteristics and outcomes of the low, medium and high frailty groups.

	Low frailty	Medium frailty	High frailty
Frailty score (range)	0-0.35	0.35-1.0	> 1.0
Number of patients	17	15	14
Male:female ratio	10:7	12:3	11:3
Age at stage IIB in years, median (range)	68 (39-90)	73 (46-86)	65 (54-87)
Folliculotropic MF, n	7	6	7
Large cell transformation at stage IIB, n	4	9	10
Disease-specific survival, %			
At 1 year	100	100	93
At 2 years	100	79	64
At 5 years	88	64	36
Overall survival, %			
At 1 year	94	93	93
At 2 years	94	71	50
At 5 years	76	43	14

MF, mycosis fungoides.

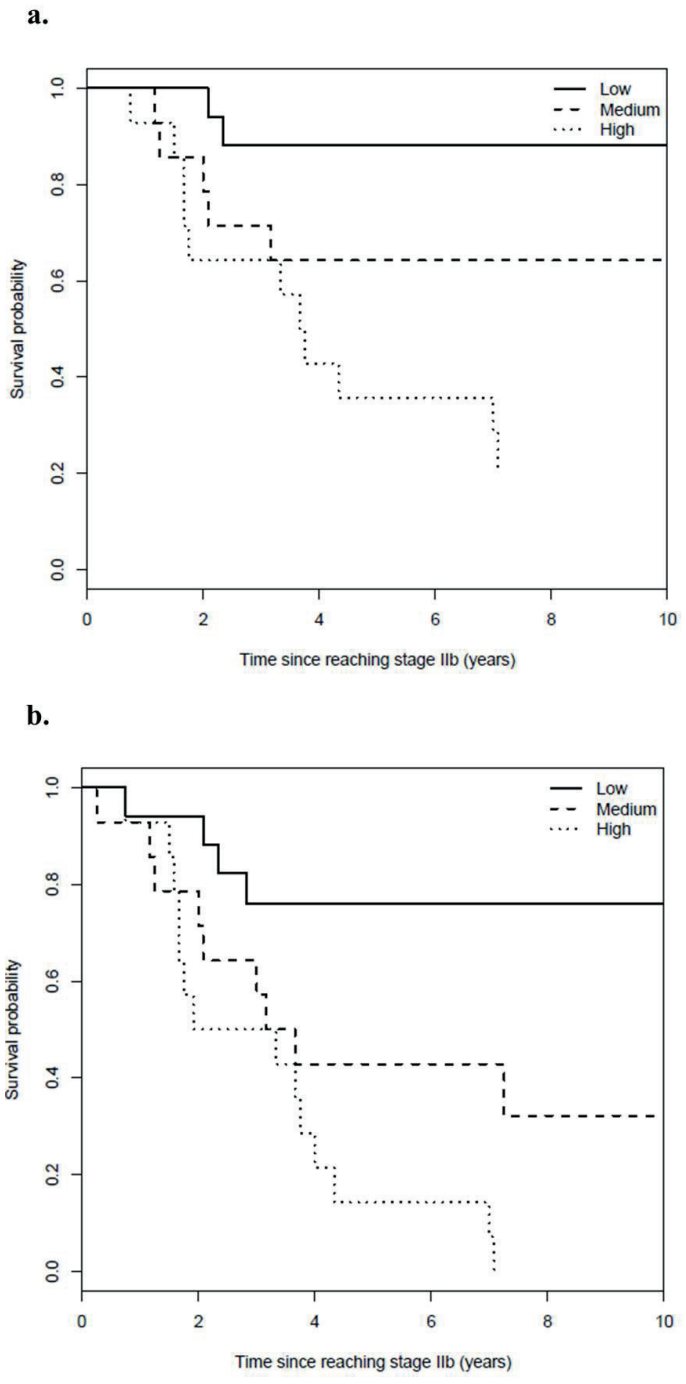

Figure 2. Disease-specific survival (a) and overall survival curve (b) according the low, medium and high frailty groups.

NUMBER OF TUMORS AT 0, 6 AND 12 MONTHS AFTER DEVELOPMENT OF STAGE IIB DISEASE AND OTHER PROGNOSTIC PARAMETERS

In univariate analysis for both DSS and OS, the number of tumors present at first presentation of MF stage IIB disease was not significant at the 0.25 level and was therefore not included in multivariate analysis ($P = 1$ and $P = 0.66$, respectively).

For the multivariate analysis with the number of tumors developed within the first 6 months after development of stage IIB disease (univariate $P = 0.002$) the same parameters were selected as described for the frailty score (see above).

Both univariate and multivariate analyses established that the number of tumors developed within the first 6 months after development of MF stage IIB disease was highly prognostic for subsequent DSS ($P < 0.001$; HR = 1.92; 95% CI 1.11-1.50). In multivariate analysis, folliculotropic MF was associated with an increased disease-specific death rate ($P = 0.044$; HR = 3.36; 95% CI 1.03-10.95), while the time interval between first diagnosis of MF and development of stage IIB disease was not prognostic ($P = 0.476$).

In multivariate analysis, the number of tumors developed within 6 months after development of MF stage IIB was prognostic for subsequent OS ($P = 0.021$; HR = 1.14; 95% CI 1.02-1.27), while age was no longer significant ($P = 0.093$).

Sensitivity analysis, performed using the total number of tumors developed during the first 12 months after diagnosis of stage IIB disease, confirmed its prognostic value for DSS and OS (data not shown).

In order to simplify the previous results, we divided the patients who had completed a 6 months follow-up after development of stage IIB disease into three categories of the same size based on the number of tumors developed within the first 6 months, namely one tumor (n = 17), 2-3 tumors (n = 12) and ≥ 4 tumors (n = 15) and correlation with survival (**Table 3**, **Figure 3** and **Supplementary Figure S1).** This implies that patients who develop four or more tumors during the first 6 months after diagnosis of stage IIB disease have a worse prognosis.

Table 3. Number of tumors developed within the first 6 months after development of stage IIB disease.

	1 tumor	2-3 tumors	≥ 4 tumors
Number of patients	17	12	15
Disease-specific survival, %			
At 1 year	100	100	93
At 2 years	88	92	67
At 5 years	71	75	47
Overall survival, %			
At 1 year	100	92	93
At 2 years	88	75	60
At 5 years	53	58	33

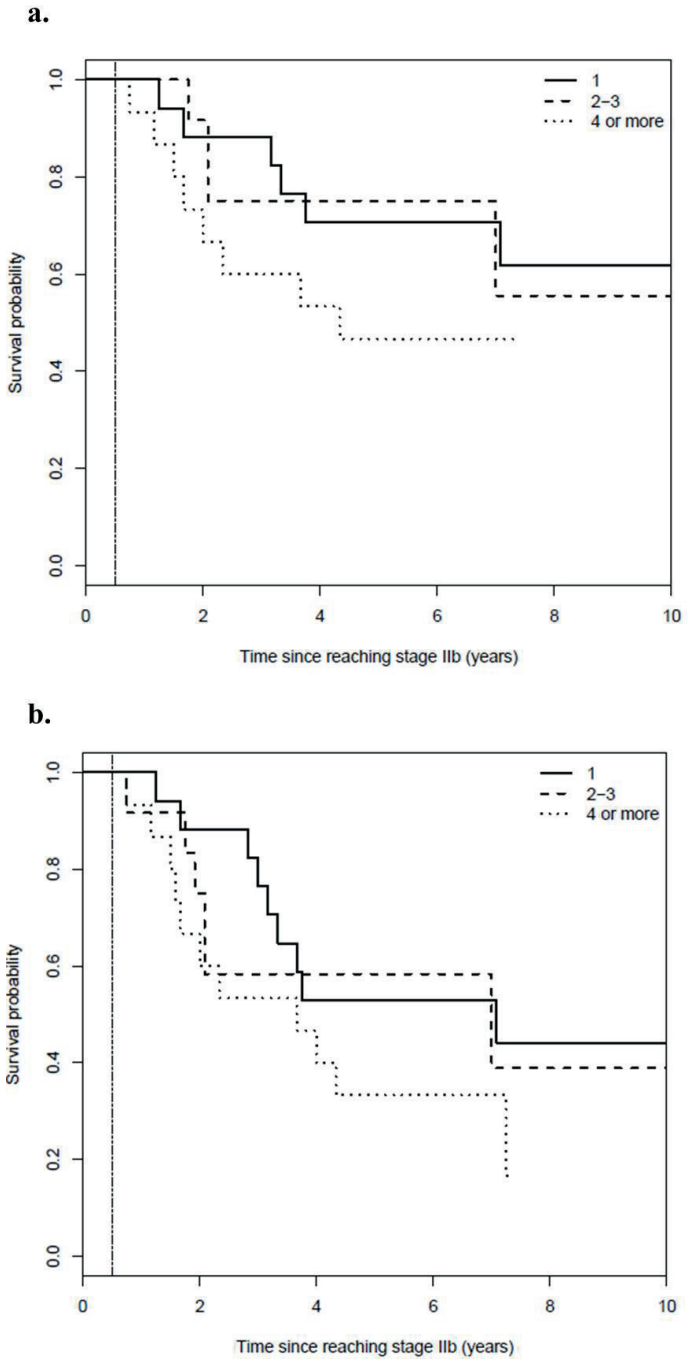

Figure 3. Disease-specific survival (a) and overall survival curve (b) according to number of tumors developed within the first 6 months after diagnosis of stage IIB.

DISCUSSION

Previous studies in MF reported a 5-year DSS varying between 56% and 80% for patients with stage IIB disease. However, patients with stage IIB disease differ greatly in the number of tumors at presentation, time interval between development of new tumors and the number of tumors that develop during follow-up. The significance of this clinical heterogeneity is controversial. In previous studies investigating the correlation between extent of tumor formation at presentation and survival no relationship was found by Agar et al, whereas two other studies reported that patients with generalized tumors had a worse prognosis than patients presenting with a solitary tumor.[8;9;12]

In the present study we quantified the extent of tumor formation in two ways. Firstly, we calculated a frailty score, which takes into account both the number of tumors that developed during follow-up and the time interval between each tumor occasion. This frailty score varied from 0.05 to 6.94 and high frailty scores (> 1.0) correlated with decreased survival. These results show that the total number of tumors that develop during follow-up and the time interval between each tumor recurrence holds prognostic information in patients with MF stage IIB disease.

Although the frailty score is statistically an attractive and sensitive method to quantify the development of tumors in patients with MF, it will be difficult to use in daily practise. Because of the positive results of the frailty score, we decided, in a second line of approach, to calculate the number of tumors that develop within the first 6 and 12 months after diagnosis of stage IIB disease. It was found that these provided prognostic information as well. The major advantage of this latter approach is that careful documentation of the number of tumors is sufficient.

The 5-year DSS and OS of the 46 patients with MF stage IIB disease was 60% and 46%, respectively. These results are similar to those reported by previous studies (5-year DSS and OS, 56−80% and 47−65%, respectively).

Our study showed no association of the number of tumors present at first diagnosis of MF stage IIB disease with survival, which is in line with the findings of Agar et al.[8] A possible explanation for this remarkable observation could be that only 17% of our patients presented with four or more tumors at first tumor development.

A more detailed method for the assessment of skin tumor burden is the modified Severity Weighted Assessment Tool (mSWAT) score, which takes into account not only tumors, but also patches and plaques. The mSWAT score is an established end point for clinical trials.[16] However, in this study, in which clinical photographs had documented all skin tumors, but not all concurrent patches and plaques, it was not possible to calculate mSWAT scores retrospectively.

Consistent with previous studies, our study showed that large cell transformation in patients with stage IIB disease was not associated with a reduced survival.[12;17]

The present study contains a high percentage of patients with folliculotropic MF (20 of 46 patients; 43%). Data from the Dutch Cutaneous Lymphoma Registry indicate that approximately 17% of all patients with MF have folliculotropic MF (R. Willemze, personal communication). Previous studies reported that patients with folliculotropic MF often have a complicated disease course and a greater risk of disease progression.[8;18] Therefore

these patients are more likely to be under continuous surveillance at our department, which may explain the high percentage of folliculotropic MF in our study. Consistent with these previous studies we also observed a reduced DSS in patients with folliculotropic MF.

In conclusion, this study shows that the number of tumors and time interval between tumor formation differs greatly among patients with MF stage IIB disease and that these differences in tumor formation correlate with survival. Patients with an adverse prognosis can be identified by quantifying the number of tumors that develop within 6 months after diagnosis of MF stage IIB. Although these observations need to be repeated in an independent study, our findings suggests that patients who develop more than four tumors within the first 6 months after diagnosis of MF stage IIB have a worse prognosis and should be monitored more carefully. We propose that patients with MF who develop stage IIB disease should be examined every 6 to 8 weeks in the first 6 months and those patients with extensive tumor formation (at least four tumors) should be under monthly surveillance.

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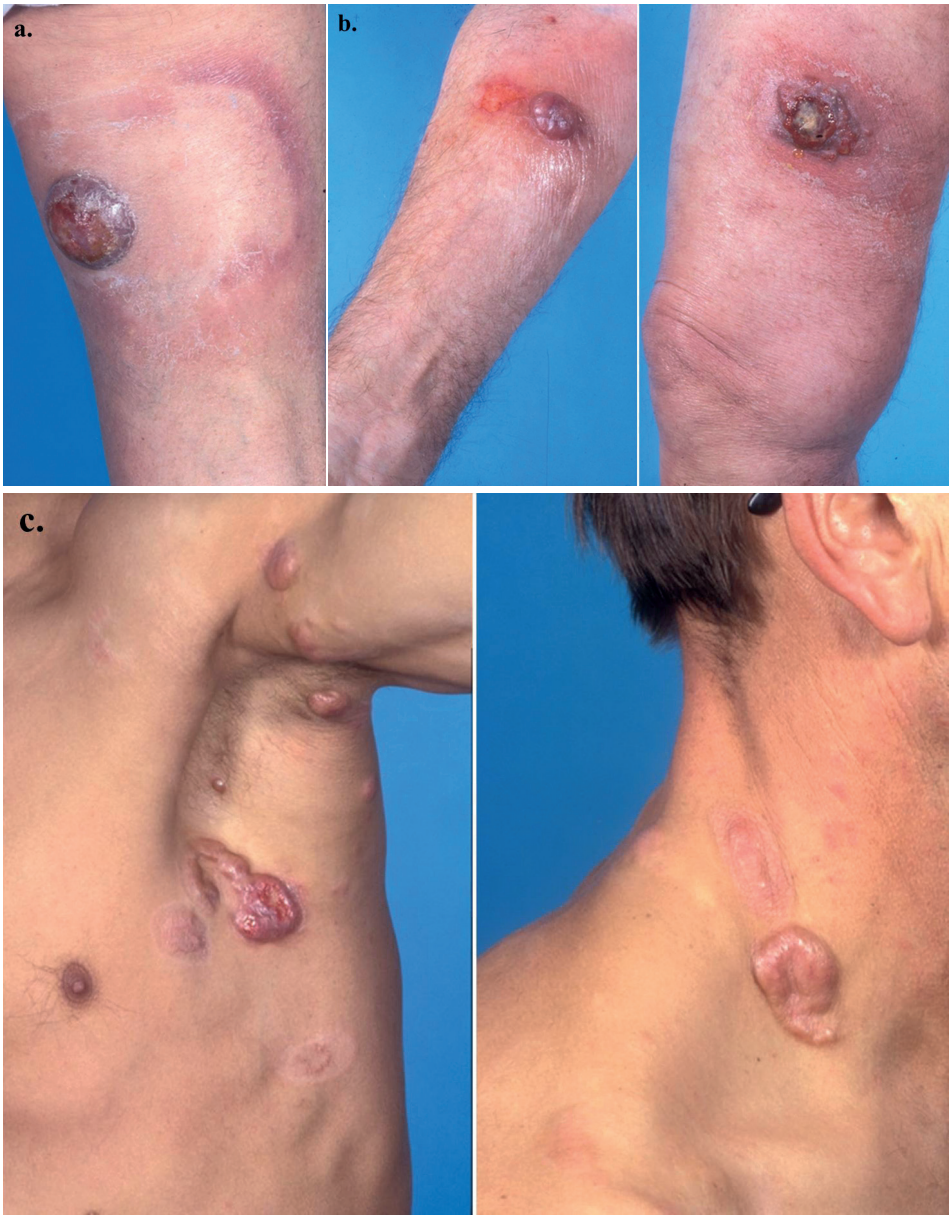
SUPPLEMENTARY MATERIAL



Supplementary Figure S1. Examples of patients with mycosis fungoides stage IIB disease with (a) one tumor on the dorsal side of the right upper leg, (b) two tumors lokalized on the right forearm and on the right upper leg, and (c) more than four tumors lokalized on the upper half of the body.

7

Summary and discussion

The studies presented in this thesis were aimed to identify useful diagnostic and prognostic markers in tumor stage mycosis fungoides (MF) and Sézary syndrome (SS). The first four studies focused on SS, in particular on immunophenotypic and (epi) genetic differences between SS and erythrodermic inflammatory dermatoses (EID) and the prognostic significance of novel biomarkers for SS. The fifth study focused on tumor stage MF and describes attempts to define prognostically different subgroups in this stage of MF. In this last chapter the results of these studies, together with those of recent literature, are summarized and discussed.

EVALUATION OF THE DIAGNOSTIC VALUE OF PREVIOUSLY REPORTED IMMUNOPHENOTYPIC AND MOLECULAR BIOMARKERS FOR SÉZARY SYNDROME

Differentiation between SS, especially in the early stages of disease, and EID may be extremely difficult, both clinically and histopathologically. The clinical presentation is generally not discriminative and histology of SS may show non-specific features of chronic dermatitis in up to one third of cases.[1;2] Since clinicopathologic features are often not decisive, the diagnosis of SS relies heavily on demonstration of neoplastic T cells in the peripheral blood. For many decades the diagnosis was based on demonstration of atypical T cells, the so-called Sézary cells, in blood smears. However, it was demonstrated that Sézary cells can also be observed in the peripheral blood of patients with EID and even in that of normal controls.[3;4] There was however no consensus as to the percentage of circulating Sézary cells required for the diagnosis of SS. Demonstration of at least 1000 Sézary cells per mm^3 was often used as a decisive criterion, but was not generally agreed upon.[5] To prevent that patients with EID would be labeled incorrectly as SS and treated as such, the European Organization for Research and Treatment of Cancer (EORTC) group proposed in 1997 demonstration of clonal T cells and the presence of an expanded CD4+ T-cell population resulting in a CD4/CD8 ratio above 10 as additional criteria for a diagnosis of SS.[6]

In the World Health Organization (WHO) - EORTC and WHO 2008 classifications the diagnosis of SS is based on the clinical presentation (erythroderma and lymphadenopathy) and the presence of a T-cell clone in the peripheral blood (preferably the same clone in skin), in combination with one or more of the following criteria: an absolute Sézary cell count ≥ 1000 cells per mm^3; loss of T-cell markers CD2, CD3, CD4 and /or CD5; and / or an expanding population of CD4+ T cells leading to a CD4/CD8 ratio of more than 10.[7;8] For Sézary patients who do not fulfill the current immunophenotypic criteria for SS, CD4+CD7− cells of at least 40% and CD4+CD26− cells of 30% or more have been suggested as tentative diagnostic criteria.[9-12]

An important drawback of the current diagnostic criteria is the lack of specific SS biomarkers that would facilitate diagnosis and quantification of tumor cells. In the last decade, many studies have reported potentially specific immunophenotypic and molecular biomarkers in SS.[9;13-35]

However, these biomarkers have almost all been identified in small, single center studies with a limited number of patients and controls, and most results have not been confirmed in independent large studies. For this reason, a multicenter European study was designed in order to investigate the sensitivity and specificity of these novel

immunophenotypic and molecular biomarkers for SS (**chapter 2**). Standard operating procedures (SOPs) for a standardized work flow and experiments were developed to increase the reproducibility of results. Immunophenotypic and molecular biomarkers were investigated in 59 patients with SS, defined according to the criteria of the WHO - EORTC classification, and 19 patients with an EID.

Flow cytometry experiments showed loss of CD7 in 40% or more of CD4+ T cells and loss of CD26 in 80% or more of CD4+ T cells in 54% and 66% SS patients, respectively, and never in EID (100% specificity). Loss of CD7 by more than 40% and/ or loss of CD26 by more than 80% of CD4+ T cells was found in 83% of Sézary patients but never in the EID patients. Previous studies described expression of killer cell immunoglobulin (KIR)-like receptor CD158k as a new diagnostic marker for SS with the proportion of patients with Sézary cells expressing CD158k varying from 65% to 97%.[18;22;23] In our study, expression of CD158k was observed in only 33% of SS patients and in 5% of EID cases. Studies have suggested that Sézary cells have a "central memory" T-cell phenotype (CD27+, CD45RA−, CD45RO+).[16;19;24;25;36] However, in our study no major difference in the expression of CD27, CD45RA and CD45RO by CD4+ T cells from SS and EID patients was observed. In line with literature expression of CD158a and CD158b was found in only a minority of SS patients.[16;22]

Copy number variation (CNV) experiments showed gain of *MYC* in 40% and loss of *MNT* in 66% of SS patients, but in none of the EID patients (100% specificity), which confirms the results of previous studies.[28;37] In contrast to two studies that described *JUNB* gain in 18% and 57% of Sézary patients, we found gain of *JUNB* in only 5% of SS patients.[26;27]

Gene expression (GE) experiments showed significant up-regulation of *DNM3* (75%), *TWIST1* (69%), *EPHA4* (66%) and *PLS3* (66%) and down-regulation of *STAT4* (91%) with 100% specificity. When we combined the alterations in gene expression of *STAT4*, *TWIST1* and *DNM3* or *STAT4*, *TWIST1* and *PLS3*, we could distinguish 98% SS patients from all EID cases. Aberrant gene expression of *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC* and *JUNB*, previously found up-regulated in SS, was found in only a minority of the SS patients.[27;29;31;32;35]

Taken together, our results suggest that loss of CD26 (≥ 80% CD4+ T cells) and/ or CD7 (≥ 40% CD4+ T cells) and combination of altered expression of *STAT4*, *TWIST1* and *DNM3* or *PLS3* could be useful as additional diagnostic panels for distinguishing SS from EID.

Michel et al previously reported a diagnostic panel for SS consisting of a combination of *TWIST* and *PLS3* or *CD158k/KIRD3DL2* expression that could diagnose 98% of SS patients.[38] In our study the combination of *TWIST1* and *PLS3* or *TWIST1* and CD158 expression was present in 87% and 80% of SS patients, respectively. An explanation for this difference could be the use of different comparative material, Michel et al compared the altered gene expression in CD4+ T cells from SS samples to CD4+ T cells from healthy donors, while we used CD4+ T cells from EID patients as comparison. Because SS is difficult to distinguish from EID, and not from healthy donors, we prefer our aberrant gene expression combination as additional panel for the diagnosis of SS.

The results of our study also emphasize the importance of SOPs for the reproducibility of results of the flow cytometry, CNV and GE experiments. Especially flow cytometry studies use widely differing protocols that hamper interpretation and comparison of

results from different centers. Due to the use of SOPs the flow cytometry experiments performed in duplicate in Leiden and Paris on all samples led to identical results in 99.8%, while assays for CNV and GE, performed in Leiden and repeated in London for a selected number of cases, showed 100% identical results. The importance of SOPs is further illustrated by the different result for CD158k expression by flow cytometry. Previous studies have described CD158k expression in 65% to 97% of Sézary patients.[18;22;23] In the present study we investigated CD158k expression on frozen peripheral blood mononuclear cells (PBMCs) instead of freshly isolated PBMCs and found expression of CD158k in only 33% of the SS patients. By using SOPs, and therefore using the same settings and interpretation of results, this outcome was found in both Leiden and Paris.

Future studies should investigate if the suggested diagnostic panels can also be helpful in diagnosing erythrodermic patients who are suspected to have SS, but do not (yet) fulfil the current diagnostic criteria for SS, the so called "pre-SS" patients. In those cases the current diagnostic criteria are not helpful and these additional diagnostic panels could be diagnostic.

In addition, it would be interesting to find out if these immunophenotypic and molecular markers are also expressed by CD4+ T cells from patients with erythrodermic MF. Despite the fact that SS and MF are classified as two separate cutaneous T-cell lymphoma (CTCL) entities in recent classifications, SS is often regarded as a leukemic phase or leukemic variant of MF because of the morphologic (atypical cells with cerebriform nuclei) and immunophenotypic (CD3+CD4+CD8− T cells) similarities between both conditions.[1] Apart from the differences in clinical presentation and clinical course, there are also histopathologic, immunophenotypic and genetic differences between SS and MF. Recent evidence suggests that SS and MF originate from distinct functional T-cell subsets.[36;39] Circulating Sézary cells have been shown to express CD27, CCR7 and L-selectin, consistent with a central memory T-cell phenotype, while skin-homing lymphocytes in MF lesions strongly express CCR4 and CLA, consistent with an effector memory T-cell phenotype.[36] Moreover, recent studies showed genomic differences between MF and SS, which further contributes to the view that these conditions are distinct entities.[32;40]

PROGNOSTIC VALUE OF MOLECULAR BIOMARKERS IN SÉZARY SYNDROME

Sézary patients have been reported to have a poor prognosis with a 5-year disease specific survival (DSS) of 24–31%.[7;41] Previous studies have identified several prognostic parameters in SS, including advanced age, short interval before diagnosis of SS, elevated levels of serum lactate dehydrogenase (LDH), increased leukocyte counts, high Sézary cell counts and the degree of lymph node involvement.[41-51] Using the same cohort as **chapter 2** we investigated the prognostic significance of molecular markers *MYC* (gain), *MNT* (loss) and aberrant expression of *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4* in 64 Sézary patients in **chapter 3**. In addition, several clinical parameters reported previously in SS were analyzed as well.

Analysis showed a 5-year DSS and overall survival (OS) of 59% and 49%, respectively, for the Sézary patients. Both in univariate and multivariate analyses patients with up-regulation of *PLS3* had a significantly better DSS and OS (multivariate P = 0.006 and

P = 0.002, respectively). Up-regulation of *DNM3* and *TWIST1* were associated with a better OS in univariate analysis, but not in multivariate analysis. *MYC* gain, *MNT* loss, up-regulation of *EPHA4* and down-regulation of *STAT4* showed no association with survival. Of the clinical parameters we found that increased leukocyte count was a significant prognostic factor for DSS and OS in both univariate and multivariate analyses (multivariate *P* = 0.005 and *P* = 0.005, respectively), while gender, age, duration of skin lesions before diagnosis, lymph node involvement, absolute CD4 count and Sézary cell count were not.

PLS3 (T-plastin) is an actin-binding protein that is expressed in all normal cells of solid tissues that have a replicative role, but is normally not expressed in T cells.[52] Previous studies have described expression of *PLS3* as a specific marker of Sézary cells.[29-33] Functional studies have demonstrated that *PLS3* expression in Sézary cells was induced by calcium entry and regulated by the calcineurin/ nuclear factor of activated T-cell (NFAT) pathway.[53;54] It was also found that constitutive *PLS3* expression in SS was associated with apoptotic resistance to etoposide and a role of *PLS3* for cell survival was suggested.[53] However, the possible protective function of *PLS3* in SS is not yet understood and requires further study.

A limitation of the current study was that the CNV and GE assays, but also flow cytometry experiments, were performed during follow-up in 43% of Sézary patients. For the molecular biomarkers we assumed that they remain constant over time and we feel strengthened in our assumption because we found a similar trend in survival curves and Hazard ratios when we repeated the analysis in 30 Sézary patients in whom the CNV and GE analyses were performed at diagnosis. Because immunophenotypic markers may change due to systemic treatment, these were not included in our analyses.

HISTOPATHOLOGIC DIFFERENTIATION BETWEEN SÉZARY SYNDROME AND ERYTHRODERMIC INFLAMMATORY DERMATOSES

As mentioned before, histopathologic differentiation between SS and EID on skin biopsies can be extremely difficult. This is illustrated by a recent study that performed blind evaluation of hematoxylin-eosin stained sections from skin biopsies of 18 patients with an erythrodermic CTCL, including 14 SS patients, and 29 patients with EID, in which correct differentiation between CTCL and EID was made in only 57% of the cases.[55] In a recent EORTC cutaneous lymphoma task force study, blinded histological and immunohistochemical evaluation of skin biopsies of 57 SS patients and 40 EID patients resulted in a correct diagnosis of SS or EID in 51% and 80% of the cases, respectively.[56] These observations clearly indicate that there is a need for additional immunohistochemical markers that can be useful in the histopathologic differentiation between SS and EID.

A recent immunohistochemical study of our group suggested expression of programmed death-1 (PD-1; CD279) by more than 50% of the skin-infiltrating T cells and expression of CD7 by 20% or less of infiltrating T cells as valuable markers in the differentiation between SS and EID.[57] Using the same cohort of patients we investigated the differential diagnostic value of two other potentially useful biomarkers, namely TOX and C-MYC, in skin biopsies from 15 SS and 17 EID patients in **chapter 4**.

We found strong nuclear staining for TOX in more than 50% of skin-infiltrating T cells in 13 of 15 (87%) SS cases, while all 17 EID cases only showed weak nuclear staining in less than 50% of the T cells. We observed no significant differences in C-MYC expression between SS and EID cases.

The thymocyte selection-associated high mobility group box protein (TOX) belongs to a large family of chromatin associated proteins and plays a role in T-cell development. TOX is transiently expressed in the thymus during the transition of CD4+CD8+ precursors to CD4+ T cells, but is normally not expressed by mature CD4+ T cells.[58;59] Several studies have reported increased TOX expression by malignant CD4+ T cells in MF and SS skin biopsies, but not in benign inflammatory dermatoses.[60-63] In a recent study TOX was not only expressed by malignant CD4+ T cells, but also by malignant CD8+ T cells in MF cases with a CD4−, CD8+ T-cell phenotype.[64] A recent study described the relation between up-regulation of TOX and down-regulation of *RUNX3*, a tumor suppressor gene, suggesting an important role for the TOX-RUNX3 pathway in the pathogenesis of SS.[65] Another study showed that increased TOX mRNA expression in SS was correlated with increased disease-specific mortality.[66] In addition, TOX silencing resulted in reduced growth of MF and SS cells in vitro and in vivo, suggesting that TOX might contribute to MF and SS development. This finding was confirmed in another study that found that overexpression of TOX promotes MF cell proliferation in vitro.[67]

In summary, strong expression of TOX in more than 50% of skin-infiltrating T cells in erythrodermic skin is a useful marker in the differentiation between SS and EID, while staining for C-MYC does not contribute to differential diagnosis.

DNA METHYLATION IN SÉZARY SYNDROME

DNA methylation is the best known epigenetic marker and is commonly disrupted in cancer cells.[68] DNA hypermethylation of CpG islands in promoter regions of tumor suppressor genes is often associated with transcriptional silencing of the gene. Previous studies that investigated aberrant DNA methylation in SS have focused on singles genes only and found that tumor suppressor genes *CDKN2A* and *FAS* among others were found to be frequently silenced by promoter hypermethylation in SS.[69;70] Studies analyzing DNA methylation on a genome-wide scale have not yet been performed in SS.

In **chapter 5** we report the genome-wide DNA methylation patterns of CD4+ T cells from 15 SS patients, 3 EID patients and 4 healthy controls. We found extensive global hypomethylation in the SS samples compared to the samples of the EID and healthy controls. DNA hypermethylation in SS occurred most frequent in CpG islands of the promoter region and gene body of genes. We identified 126 genes with significantly and highly recurrent promoter hypermethylation in SS compared to the benign controls. Among the 126 genes twelve genes were validated in the first sample set and in an independent sample set consisting of 20 Sézary patients and 10 EID cases with similar results. Promoter hypermethylation of *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, *PAM* and *CPEB3* was observed in at least 90% of SS cases in both sample sets, but never in EID samples. Promoter hypermethylation of a single gene, *CMTM2*, was present in 100% of SS samples and in none of the EID samples, allowing accurate discrimination between SS and EID with a 100% sensitivity and specificity. Promoter hypermethylation of *CMTM2*, *C2orf40*, *G0S2*,

HSPB6, *PROM1*, *GNMT* and *NEXN* were associated with diminished expression in SS as compared with benign controls. Treatment of the SeAx cell line with the demethylating agent 5-aza-2'-deoxycytidine resulted in significantly increased expression of *CMTM2*, *G0S2* and *NEXN*, suggesting that these genes are epigenetically silenced in SS.

In summary, genome-wide DNA methylation patterns in CD4+ T cells from patients with SS show extensive differences in DNA methylation compared to those from EID patients and healthy controls. Promoter hypermethylation of a single gene, *CMTM2*, accurately distinguished SS from EID in all cases, making it a useful epigenetic biomarker for the diagnosis of SS.

CMTM2 belongs to the chemokine-like factor gene superfamily and is primarily expressed in testis, bone marrow, and peripheral blood cells, including CD4+ T cells.[71] The overall function of *CMTM2* in T cells is poorly understood. The diagnostic value of *CMTM2* methylation as a potential epigenetic biomarker should be investigated in "pre-SS" and erythrodermic MF patients.

In solid tumors it has been demonstrated that certain promoter hypermethylation events have prognostic value and can be used to predict poor survival.[72] Due to the relatively small size of the cohort of SS samples analyzed we were unable to identify DNA methylation events with prognostic significance. Future studies should investigate the prognostic value of epigenetic biomarkers in SS.

The current study showed that SS is characterized by widespread DNA methylation alterations including promoter CpG island methylation of tumor suppressor genes, which can be partly reversed using demethylating agents. These findings strengthen the rationale for using epigenetic therapies in SS.

PROGNOSTIC VALUE OF NUMBER AND RECURRENCES OF TUMORS IN MYCOSIS FUNGOIDES STAGE IIB

Patients with tumor stage MF, stage IIB, have a reduced 5-year DSS of 56–80% and are at risk for extracutaneous progression.[41;73] Patients with only skin tumors are currently classified as one group, but clinical observations show considerable variation in the number of tumors and time interval between each tumor occasion. Studies that investigated the association between tumor formation and survival found that patients who have generalized skin tumors at diagnosis of MF or at diagnosis of transformed MF have a reduced survival compared to those who present with only a solitary tumor.[44;74] However, the relation between the exact number of tumors that develop during follow-up and survival has not been investigated before.

In **chapter 6** we therefore quantified the extent of tumor development in 46 patients with MF stage IIB by calculating frailty scores with use of a statistical frailty model fitted on the basis of both the number of tumors that developed from the same tumor and the time interval between each tumor occasion, and correlated the results with survival. In addition, we investigated whether the number of tumors that developed within 6 and 12 months after appearance of the first tumor was predictive for survival.

Analysis showed a 5-year DSS and OS of 60% and 46%, respectively, for the patients with MF from development of the first tumor (stage IIB). The individual frailty scores of the MF patients varied between 0.05 and 6.94 and the variability in frailty scores was

highly significant. Patients with high frailties (> 1.0, n = 14) had a significantly worse DSS and OS compared to patients with low frailty scores (0−0.35, n = 17) and medium frailty scores (0.35−1.0, n = 15) (multivariate $P < 0.001$ and $P < 0.001$, respectively). The number of tumors that developed within 6 months after diagnosis of MF stage IIB was prognostic for DSS and OS (multivariate $P < 0.001$ and $P = 0.021$, respectively). Patients that developed four or more tumors during the first 6 months after diagnosis of stage IIB had a poor prognosis.

Based on our results we propose that patients with extensive tumor formation (at least four tumors within the first 6 months after diagnosis of stage IIB) should be monitored more closely. Moreover, if confirmed by larger studies it can be suggested to divide stage IIB into subgroups based on the number of tumors, for example < 4 and ≥ 4 tumors, that develop within 6 months after diagnosis of stage IIB.

Because several genetic aberrations have been described in tumors from MF patients, the question arises if the patients with rapid tumor formation have a different genetic profile compared to those patients that do not. Future studies should reveal if there are genetic differences between patients with and without rapid tumor formation and if these differences are already present in patch/plaque stage or develop within the tumors.

In summary, the number of tumors and time interval between tumor formation differs greatly among patients with MF stage IIB and these differences correlate with survival. Patients with an adverse prognosis can be identified by quantifying the number of tumors that develop in the first 6 months after diagnosis of MF stage IIB.

CONCLUSIONS AND PERSPECTIVES

The studies in this thesis have identified immunohistochemical (TOX), immunophenotypic (CD7, CD26), molecular (*STAT4*, *TWIST1*, *DNM3*/ *PLS3*) and epigenetic (*CMTM2*) markers that are useful as additional diagnostic markers to discriminate SS from EID. Of the molecular markers, *PLS3* expression was an important prognostic factor in SS. Future studies should investigate the diagnostic role of these markers in patients that are suspected to have SS but do not (yet) fulfil the diagnostic criteria for SS and in patients with erythrodermic MF.

In addition, we proved that the number of tumors and time interval between tumor formation differs greatly among patients with MF stage IIB and these differences correlate with survival. Patients that developed four or more tumors during the first 6 months after diagnosis of stage IIB had a poor prognosis. If confirmed by larger studies, it can be proposed to subdivide stage IIB based on the number of tumors that develop within the first 6 months after diagnosis of MF stage IIB.

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8

Appendix

NEDERLANDSE SAMENVATTING

Primair cutane lymfomen omvatten een heterogene groep van non-Hodgkin lymfomen die zich primair in de huid manifesteren zonder aanwijzingen voor lokalisaties elders in het lichaam. Binnen de primair cutane lymfomen zijn twee hoofdcategorieën te onderscheiden: 75% zijn primair cutane T-cel lymfomen (CTCL) en de overige 25% zijn primair cutane B-cel lymfomen (CBCL).

Mycosis fungoides (MF) en Sézary syndroom (SS) zijn de meest bekende typen CTCL. MF kent in het algemeen een indolent ziekte beloop waarbij in de loop der jaren een langzame progressie van patches naar plaques en uiteindelijk tumoren wordt gezien. SS wordt beschouwd als een leukemische variant van CTCL en heeft in het algemeen een veel slechtere prognose. SS wordt klinisch gekarakteriseerd door de trias van een diffuus rode huid (erythrodermie), gegeneraliseerd vergrote lymfeklieren en kwaadaardige T-cellen (Sézary cellen) in huid, lymfeklieren en bloed.

Een centraal onderwerp in dit proefschrift zijn de diagnostische en prognostische parameters in MF en SS.

EVALUATIE VAN DE DIAGNOSTISCHE WAARDE VAN EERDER BESCHREVEN IMMUNOFENOTYPISCHE EN MOLECULAIRE BIOMARKERS IN SÉZARY SYNDROOM

Zeker in de vroege fase van de ziekte, is het moeilijk om een SS te onderscheiden van een erythrodermie op basis van goedaardige inflammatoire huidziekten zoals geneesmiddelenreacties, psoriasis of atopisch eczeem. De diagnostiek wordt bemoeilijkt omdat de klinische presentatie van SS en deze erythroderme inflammatoire dermatosen (EID) sterk op elkaar kunnen lijken. Tevens wordt bij histopathologisch onderzoek van SS in eenderde van de gevallen slechts reactieve veranderingen gezien. Daarom berust de diagnose van SS met name op het aantonen van atypische T-cellen (Sézary cellen) in het bloed. Echter, vergelijkbare atypische T-cellen kunnen ook voorkomen in het bloed van patiënten met EID en worden zelfs in het bloed van gezonde personen gezien. Om te voorkomen dat patiënten met een EID ten onrechte gediagnosticeerd (en behandeld) worden als een SS, werden in 1997 diagnostische criteria voor SS vastgesteld. In de recente classificatie systemen is er sprake van SS bij een klinische presentatie van erythrodermie en gegeneraliseerde lymfadenopathie en de aanwezigheid van een T-cel kloon in het bloed, gecombineerd met één of meer van de volgende criteria: een absoluut aantal Sézary cellen van meer dan 1000 per mm^3; verlies van T-cel markers CD2, CD3, CD4 en/of CD5; en/of een toegenomen absoluut CD4 aantal leidend tot een CD4/CD8 ratio gelijk of groter dan 10. Aanvullende diagnostische criteria welke in latere studies werden gesuggereerd, betreffen de aanwezigheid van ten minste 40% CD4+CD7− cellen en ten minste 30% CD4+CD26− cellen in het perifere bloed.

Een belangrijk nadeel van de huidige diagnostische criteria is het ontbreken van SS specifieke biomarkers waardoor vroege herkenning van patiënten met een SS wordt bemoeilijkt. In de afgelopen 10 jaar hebben verscheidene studies nieuwe, potentieel SS specifieke, immunofenotypische en moleculaire biomarkers beschreven. Echter zijn deze biomarkers bijna altijd in kleine, slechts in één centrum uitgevoerde studies

geïdentificeerd, met vaak een klein aantal patiënten en zijn de meeste resultaten niet bevestigd in onafhankelijke grote studies.

In **hoofdstuk 2** werd de diagnostische waarde van deze nieuwe, immunofenotypische en moleculaire biomarkers geëvalueerd in een Europese multi-centrum studie in 59 SS patiënten, gedefinieerd volgens de huidige diagnostische criteria, en 19 patiënten met een EID. Standaard werkprocedures werden gebruikt zodat alle experimenten gestandaardiseerd plaatsvonden en ook de reproduceerbaarheid van de resultaten kon worden geëvalueerd. De experimenten werden in 2 centra verricht met bijna volledig overeenkomstige resultaten. Op genetisch niveau toonden Sézary cellen een toename in het aantal gen-kopieën van *MYC* (40%), een afname in het aantal gen-kopieën van *MNT* (66%), verhoogde genexpressie van *DNM3* (75%), *TWIST1* (69%), *EPHA4* (66%) en *PLS3* (66%) en een verlaagde genexpressie van *STAT4* (91%).

De resultaten van deze studie toonden aan dat met de immunofenotypische markers CD7 (verlies van ten minste 40% van de CD4+ T-cellen) en/ of CD26 (verlies van ten minste 80% van de CD4+ T-cellen) 83% van de SS patiënten onderscheiden konden worden van de EID patiënten met 100% specificiteit. Met de combinatie van een veranderde genexpressie van de moleculaire markers *STAT4*, *TWIST1* en *DNM3* of *STAT4*, *TWIST1* en *PLS3* kon 98% van de SS patiënten onderscheiden worden van EID patiënten met 100% specificiteit. Deze twee diagnostische panels lijken waardevolle additionele criteria bij de differentiatie van SS en EID.

PROGNOSTISCHE WAARDE VAN MOLECULAIRE BIOMARKERS IN SÉZARY SYNDROOM

Sézary patiënten hebben een slechte prognose met een ziekte-specifieke 5-jaars overleving van 24−31%. Eerdere studies hebben verscheidene prognostische parameters in SS beschreven zoals toegenomen leeftijd, kort tijdsinterval tot diagnose, verhoogde waarde van lactaat dehydrogenase (LDH) in het bloed, toegenomen aantal witte bloedcellen, hoog aantal Sézary cellen in het perifere bloed en de uitgebreidheid van lymfeklier betrokkenheid. Met gebruik van hetzelfde cohort als in **hoofdstuk 2**, werd in **hoofdstuk 3** de prognostische waarde van de moleculaire markers (*MYC*, *MNT*, *DNM3*, *TWIST1*, *EPHA4*, *PLS3* en *STAT4*) onderzocht in 64 patiënten met SS. Tevens werden de eerder beschreven prognostische parameters geanalyseerd.

De resultaten toonden een ziekte-specifieke 5-jaars overleving van 59% voor de Sézary patiënten. De analyses toonden verder dat SS patiënten met een verhoogde expressie van *PLS3* een significante betere overleving hadden. Van de klinische parameters was alleen een verhoogd aantal witte bloedcellen een significant prognostische factor.

HISTOPATHOLOGISCHE DIFFERENTIATIE TUSSEN SÉZARY SYNDROOM EN ERYTHRODERME INFLAMMATOIRE DERMATOSEN

Het onderscheiden van SS en EID op basis van het histologische beeld is erg lastig. In **hoofdstuk 4** werd de diagnostische waarde van de eiwit markers TOX en/of C-MYC onderzocht op huidbiopten van 15 SS patiënten en 17 EID patiënten. De resultaten toonden sterke kernkleuring van TOX in meer dan 50% van de huid-infiltrerende T-cellen bij 87% van de SS patiënten, terwijl er slechts zwakke kernkleuring in minder dan 50%

van de T-cellen werd gezien bij de EID patiënten. Op basis van deze resultaten lijkt TOX expressie een goede additionele marker bij de histopathologische differentiatie tussen SS en EID. Er werd geen verschil in C-MYC aankleuring gezien tussen SS en EID patiënten.

DNA METHYLATIE IN SÉZARY SYNDROOM

DNA-methylering of DNA-methylatie is een epigenetisch proces waarbij een methylgroep aan een DNA-molecuul wordt toegevoegd. DNA methylering kan een belangrijke rol spelen bij het ontstaan van kanker doordat DNA hypermethylatie van CpG eilanden in de promoter regio van een tumor suppressor gen kan leiden tot inactivatie van het betreffende gen, terwijl globale DNA hypomethylatie geassocieerd is met chromosomale instabiliteit. Eerdere studies die hebben gekeken naar DNA methylatie in SS concentreerden zich steeds op één gen. In **hoofdstuk 5** beschrijven wij de genoombrede DNA methylatie patronen van CD4+ T-cellen van 15 SS patiënten, 3 EID patiënten en 4 gezonde controles.

We vonden uitgebreide globale hypomethylatie in SS vergeleken met EID en gezonde controles. Daarnaast identificeerden we bij de SS patiënten 126 genen die significant vaker promoter hypermethylatie toonden dan de benigne controles. Van deze 126 genen werden 12 genen in deze eerste groep patiënten gevalideerd met een onafhankelijke techniek en vervolgens in een tweede onafhankelijke groep patiënten bestaande uit 20 SS patiënten en 10 EID patiënten onderzocht waarbij vergelijkbare resultaten werden gevonden. In beide groepen werd promoter hypermethylatie van *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, *PAM* en *CPEB3* gezien in ten minste 90% van de SS patiënten , maar nooit bij de EID patiënten. Promoter hypermethylatie van één gen, *CMTM2*, was aanwezig in 100% van de SS patiënten en in geen van alle EID patiënten, waarmee dus nauwkeurig onderscheid met 100% sensitiviteit en specificiteit tussen SS en EID gemaakt kon worden. Dit maakt *CMTM2* een sterke kandidaat epigenetische biomarker voor SS.

De promotor hypermethylatie van *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, *GNMT* en *NEXN* bij de SS patiënten was geassocieerd met een verlaagde genexpressie. Behandeling van de van een SS patiënt afkomstige SeAx cellijn met het demethyleringsmiddel 5-aza-2'-deoxycytidine resulteerde in een afname van de promoter methylatie en significant toegenomen expressie van *CMTM2*, *G0S2* en *NEXN*, wat suggereert dat deze genen epigenetisch gesilenced zijn in SS.

PROGNOSTISCHE WAARDE VAN HET AANTAL TUMOREN EN TUMOR RECIDIEVEN IN MYCOSIS FUNGOIDES STADIUM IIB

MF patiënten met huidtumoren zonder extracutane lokalisaties worden geclassificeerd als een MF stadium IIB. Deze patiënten hebben een toegenomen risico op extracutane progressie en een ziekte-specifieke 5-jaars overleving van 56−80%. Hoewel alle MF patiënten met huidtumoren zonder extracutane afwijkingen worden geclassificeerd als een MF stadium IIB wordt binnen deze groep patiënten een grote variatie gezien in het aantal tumoren en het tijdsinterval tussen het ontstaan van nieuwe tumoren. Studies die de associatie tussen tumor vorming en overleving hebben onderzocht vonden dat patiënten met gegeneraliseerd huidtumoren bij de diagnose MF en patiënten waarbij sprake was van blastaire transformatie een slechtere prognose hebben. Echter de relatie

tussen overleving en het exact aantal tumoren dat zich tijdens follow-up ontwikkeld is nog niet eerder onderzocht.

Derhalve hebben wij in **hoofdstuk 6** de uitgebreidheid van tumor ontwikkeling in 46 patiënten met MF stadium IIB gekwantificeerd met behulp van een statistisch model gebaseerd op het aantal tumoren dat gedurende follow-up ontstond (inclusief het aantal tumoren ten tijde van de diagnose MF stadium IIB) en het tijdsinterval tussen elke tumor. Hiermee werd een zogenaamde "frailty score" berekend welke werd gecorreleerd met overleving. Tevens keken we of het aantal tumoren dat binnen 6 en 12 maanden ontstond na de eerste tumor voorspellend was voor de overleving.

Analyses toonde een ziekte-specifieke 5-jaars overleving van 60% voor patiënten met MF vanaf het moment van de eerste tumor (MF stadium IIB). De individuele frailty scores van de MF patiënten was significant verschillend en varieerde tussen 0.05 en 6.94. Patiënten met een hoge frailty score (> 1.0, n = 14) hadden een significant lagere ziekte-specifieke overleving in vergelijking met patiënten met een lage frailty score (0−0.35, n = 17) en gemiddelde frailty score (0.35−1.0, n = 15). Het aantal tumoren dat binnen 6 maanden na de diagnose MF stadium IIB ontstond was significant voorspellend voor de ziekte-specifieke overleving. Patiënten die vier of meer tumoren ontwikkelden binnen 6 maanden na de diagnose MF stadium IIB hadden een slechte prognose.

LIST OF ABBREVIATIONS

CBCL	primary cutaneous B-cell lymphoma
CGI	CpG islands
CMTM2	CKLF-like MARVEL Transmembrane Domain-Containing 2
CNV	copy number variation
CTCL	primary cutaneous T-cell lymphoma
DCLG	Dutch Cutaneous Lymphoma Group
DP	distal promoter
DS	downstream
DSS	disease-specific survival
EID	erythrodermic inflammatory dermatoses
EORTC	European Organization for Research and Treatment of Cancer
G0S2	G0/G1 switch gene 2
GB	gene body
GE	gene expression
HDAC	Histone deacetylase
ID4	inhibitor of DNA binding protein 4
IG	intergenic
ISCL	International Society for Cutaneous Lymphomas
KIR	killer cell immunoglobulin
LDH	lactate dehydrogenase
MDS	Multidimensional scaling
MF	mycosis fungoides
miRNA	microRNA
MS-MCA	methylation-specific melting curve analysis
mSWAT	modified Severity Weighted Assessment Tool
NC	shores and non-CpG islands
NFAT	nuclear factor of activated T-cell
NHL	non-Hodgkin lymphomas
OS	overall survival
PBMC	peripheral blood mononuclear cell
PD-1	programmed death-1
PLS3	T-plastin
PP	proximal promoter
PUVA	psoralen plus ultraviolet A
SAMe	S-adenosylmethionine
SOP	standard operating procedure

SS	Sézary syndrome
TCGA	The Cancer Genome Atlas
TNMB	tumor-node-metastasis-blood
TOX	thymocyte selection-associated high mobility group box protein
WHO	World Health Organization

LIST OF PUBLICATIES

van der Rhee JI, **Boonk SE**, Putter H, Cannegieter SC, Flinterman LE, Hes FJ, de Snoo FA, Mooi WJ, Gruis NA, Vasen HF, Kukutsch NA, Bergman W. Surveillance of second-degree relatives from melanoma families with a CDKN2A germline mutation. Cancer Epidemiology, Biomarkers & Prevention, 2013; 22(10):1771-1777.

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CURRICULUM VITAE

Stéphanie Boonk is geboren op 9 november 1984 te Den Helder. Na het behalen van haar atheneum diploma aan het Studiehuis Molenplein te Den Helder in 2003, begon zij datzelfde jaar met de studie geneeskunde aan de Universiteit Leiden.

Gedurende haar wetenschapsstage verrichte zij onderzoek naar de opbrengst van surveillance in tweedegraads verwanten van melanoompatiënten uit families met een p16-Leiden mutatie onder leiding van mw. prof. dr. W. Bergman en mw. dr. N.A. Kukutsch op de afdeling Dermatologie van het Leids Universitair Medisch Centrum. Na het behalen van haar artsexamen in mei 2011 werd zij op deze afdeling aangesteld als AIOSKO (assistent in opleiding tot specialist en klinisch onderzoeker). Dit leidde tot het in dit proefschrift beschreven onderzoek onder begeleiding van prof. dr. M.H. Vermeer en prof. dr. R. Willemze. In juli 2014 is zij gestart met de opleiding tot dermatoloog (opleider mw. dr. A.P.M. Lavrijsen).

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