Diagnostic and prognostic markers in tumor stage mycosis fungoides and Sézary syndrome

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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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