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Clinical significance of T-cell clonality in mycosis fungoides and other cutaneous T-cell lymphomas

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General introduction

Chapter 1 - General introduction

1.1 Primary cutaneous T-cell lymphoma

Primary cutaneous T-cell lymphomas (CTCL) represent a heterogeneous group of mature T-cell neoplasm, clinically originating in the skin and subsequently disseminating into lymph nodes, blood, and other visceral organs. After the gastrointestinal tract, the skin is the second most common site of extranodal non-Hodgkin lymphoma, with an estimated annual incidence of 1:100,000¹. The primary cutaneous occurrence of neoplastic cells distinguishes CTCL from histologically similar lymphomas that develop skin lesions as sign of metastatic dissemination and aggressive behavior.

WHO-EORTC classification	%*	5-DS*
Indolent clinical behavior		
Mycosis fungoides	44	88
Folliculotropic MF	4	80
Pagetoid reticulosis	<1	100
Granulomatous slack skin	<1	100
Primary cutaneous anaplastic large cell lymphoma	8	95
Lymphomatoid papulosis	12	100
Subcutaneous panniculitis-like T-cell lymphoma	1	82
Primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma†	2	75
Aggressive clinical behavior		
Sézary syndrome	3	24
Primary cutaneous NK/T-cell lymphoma, nasal-type	<1	NR
Primary cutaneous aggressive CD8+ T-cell lymphoma‡	<1	18
Primary cutaneous γ/δ T-cell lymphoma‡	<1	NR
Primary cutaneous peripheral T-cell lymphoma, unspecified‡	2	16

Table 1. Relative frequency (%) and disease-specific 5-year survival (5-DS) of CTCL classified according to the WHO-EORTC classification, adapted from Willemze et al.⁷.

*, data are based on 1905 patients with a primary cutaneous lymphoma registered at the Dutch and Austrian Cutaneous Lymphoma Group between 1986 and 2002; †, primary cutaneous peripheral T-cell lymphoma, unspecified excluding the three provisional entities indicated with a double dagger (§); NR, not reached.

In 1806, Alibert introduced the name mycosis fungoides (MF) for a disease, which forms the prototype of CTCL at present day.² Since that time, various attempts have been taken to classify this heterogeneous group of malignant lymphoproliferations. In 1997, the EORTC task force on cutaneous lymphoma proposed the EORTC classification for primary cutaneous lymphomas³. The proposal was based on clinical, pathological, and immunohistochemical characteristics, and subgrouped CTCL into indolent (MF, MF-associated mucinosis follicularis, pagetoid reticulosis, large cell CD30+ CTCL, lymphomatoid papulosis), aggressive (Sézary syndrome, large cell CD30- CTCL), and some provisional entities. It has been clinically validated by several large studies, including follow-up data of more than 1300 patients suffering from primary cutaneous lymphoma^{3,4,5}. The scheme has been widely used in clinical and research settings until the WHO classification of tumors of hematopoietic and lymphoid tissues has been published in 2001⁶. In contrast to the EORTC proposal, the WHO classification primarily focuses on T-cell lineage and immunohistochemical characteristics. Clinical data, particularly clear prognostic and therapeutic implications, were mainly lacking. Furthermore, differences between both schemes with regard to the classification of cutaneous B-cell lymphomas

and of CTCL other than MF, Sézary syndrome, and primary cutaneous CD30+ T-cell lymphoproliferative disorders lead to considerable debates and confusion. As a result of consensus meetings, representatives of both classification systems developed the WHO-EORTC classification for cutaneous lymphomas, which was published in 2005⁷ (see table 1). In the meantime, this WHO-EORTC classification for cutaneous lymphomas has become the golden standard and most of its proposals have been adopted by the revised WHO classification of 2008⁸.

1.2 Mycosis fungoides

MF is characterized by a proliferation of epidermotropic small- to medium-sized T-lymphocytes with cerebriform nuclei. With a relative frequency of 44%, it represents the most common entity of primary cutaneous lymphomas. The term MF should be reserved to the classical entity, which is defined by the subsequent evolution of patches, plaques, and tumors, and to variants with similar clinical behavior as bullous and hyper- or hypopigmented MF. Folliculotropic MF (i.e. MF-associated mucinosis follicularis), pagetoid reticulosis, and granulomatous slack skin show distinct clinical and pathological features and, therefore, are separated from classical MF.

MF typically affects elderly males (median age at diagnosis: 55-60 years, male-to-female ratio: 1.6-2.0:1) and has an indolent clinical course^{9,10,11}. With a predilection for buttocks and other sun-protected areas, initial patches slowly progress into more infiltrated plaques and eventually into tumors within a period of years of sometimes decades. Typically, the individual patient shows a variegated pattern of variably infiltrated patches and plaques (and tumors). In cases with occurrence of tumors without concurrent patches and plaques, another type of CTCL must be considered. Only a minority of patients develops involvement of lymph nodes or visceral organs⁷.

Histopathologically, early patches are characterized by superficial band-like or lichenoid infiltrates, mainly consisting of lymphocytes and histiocytes. Only a few small- to medium-sized atypical cells can be identified by their cerebriform nuclei and their epidermotropism. In typical plaques, epidermotropism is more pronounced, and intraepidermal accumulations of atypical cells (Pautrier microabscesses) occur as highly characteristic, but infrequent feature¹². In tumor lesions, the dermal infiltrates become more diffuse and epidermotropism disappears. Atypical cells increase in number and size with variable proportions of cerebriform cells, blast cells with prominent nuclei, and intermediate forms.

Generally, the neoplastic cells express a mature memory T-cell phenotype (CD3+ CD4+ CD45RO+ CD8-). Demonstration of an aberrant phenotype (loss of pan-T-cell antigens as CD2, CD3, CD5) is often seen and represents an important adjunct in the diagnosis of MF¹³. In rare cases of otherwise classical MF, a CD4- CD8+ phenotype may be found.

The prognosis of patients with MF depends on type and extent of skin lesions and the presence of extracutaneous manifestations, as reflected by the revised TNM-classification of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization (EORTC, see table 2)¹⁴. In recent studies, 10-year disease-specific survival rate was 97%-98% for patients with limited patch/plaque disease (MF IA), 83% for generalized patch/plaque

disease (MF IB), 42% for tumor stage MF (IIB), and about 20% for histologically proven lymph node involvement (MF IVA)^{9,10,11}. Patients with lymph node or visceral involvement, and cases with transformation into a large T-cell lymphoma show an aggressive clinical course. Death usually occurs due to systemic involvement or infections.

TNMB Definitions	TNM Stage Groupings
Skin (T) T1 Limited patches, papules, and/or plaques, covering < 10% of the skin surface T2 Patches, papules and/or plaques covering ≥ 10% of the skin surface T3 One or more tumors, ≥1 cm diameter T4 Confluence of erythema, covering ≥80% body surface area Regional lymph nodes (N) N0 No clinically abnormal peripheral lymph nodes, biopsy not required N1 Clinically abnormal peripheral lymph nodes, histopathology Dutch grade 1 or NCI LN0-2, N1a = clone negative, N1b = clone positive N2 Clinically abnormal peripheral lymph nodes, histopathology Dutch grade 2 or NCI LN3, N2a = clone negative, N2b = clone positive N3 Clinically abnormal peripheral lymph nodes, histopathology Dutch grades 3-4 or NCI LN4, clone positive or negative Nx Clinically abnormal peripheral lymph nodes; no histologic confirmation Distant metastasis (M) M0 No visceral organ involvement M1 Visceral involvement, must have pathology confirmation, organ involved should be specified Blood (B) B0 Absence of significant blood involvement: ≤ 5% of peripheral blood lymphocytes are atypical (Sézary) cells, B0a = clone negative, B0b = clone positive B1 Low blood tumor burden: > 5% of peripheral blood lymphocytes are atypical (Sézary) cells, but not criterion B2, B1a = clone negative, B1b = clone positive B2 High blood tumor burden: ≥1000/μL Sézary cells with positive clone	Stage IA T1, N0, M0, B0-1 Stage IB T2, N0, M0, B0-1 Stage IIA T1-2, N1-2, M0, B0-1 Stage IIB T3, N0-2, M0, B0-1 Stage IIIA T4, N0-2, M0, B0 Stage IIIB T4, N0-2, M0, B1 Stage IVA ₁ T1–T4, N0-2, M0, B2 Stage IVA ₂ T1–T4, N3, M0, B0-2 Stage IVB T1–T4, N0-3, M1, B0-2

Table 2. ISCL/EORTC revision of the TNM-classification of mycosis fungoides and Sézary syndrome.¹⁴ For primary cutaneous lymphomas other than MF and Sézary syndrome, which show different clinical behavior, another TNM classification system should be applied, as published recently.¹⁵

In the present thesis, the TNM stages applied refer to the old classification of Bunn et al.¹⁶, which differs in the definition of the N stadia: In the revised ISCL/EORTC version, the old, clinically not relevant N2 stadium (no clinically abnormal lymph nodes, pathology positive for CTCL) is removed and all N stadia are more precisely defined. Furthermore, TCR clonality is added as suffix to the N and B stadia.

1.3 Clonal T-cell receptor rearrangement

In early stages, an unspecific clinical and histopathological picture often hampers definitive diagnosis of MF. To overcome this problem, different approaches have been introduced: Calculation of a nucleus-contour-index of the cerebriform nuclei of the atypical cells by electron microscopy¹⁷ showed high sensitivity and specificity in the differentiation between reactive and neoplastic T-cell infiltrates¹⁸ but is time and labor consuming. Flow-cytometric estimation of the ratio of diploid and aneuploid cells¹⁹ could increase diagnostic specificity²⁰, but is not suited for daily practice. Cytogenetic studies identified many structural and numerical chromosomal abnormalities, in particular in the advanced stages of MF, but recurrent, MF-specific chromosomal translocations have not been found^{21,22}. By immunophenotyping, the neoplastic cells show a (non-specific) mature memory T-cell phenotype (CD3+ CD4+ CD45RO+ CD8-). However, loss of pan-T-cell antigens as CD2, CD3, CD5 represents an important adjunct in the diagnosis of MF¹³.

It is generally accepted that carcinogenesis is initiated by a mutation event in a single cell, which leads to uncontrolled growth (or defects in cell death) of that cell. By subsequent cell division, genetically identical cells arise and form a neoplastic cell clone. During their maturation, T-lymphocytes rearrange the genes encoding the T-cell receptor (TCR)²³. This rearrangement leads to a unique gene sequence, which defines every single T-cell (see figure 1A). Consequently, every single CTCL can be defined by the unique sequence of its TCR genes, and demonstration of the predominance of a single TCR rearrangement (i.e. clonal TCR rearrangement) identifies the presence of a T-cell clone.

Two different techniques have been developed to detect clonal TCR rearrangements:

1) Southern blotting (SB): Genomic DNA is digested by endonucleases, restriction fragments are separated by electrophoresis and blotted on nylon membranes. Hybridization of the blots with gene probes for the constant region of the TCR genes results in germ line bands (derived from cells without TCR rearrangement), a smear (derived from the multiple different TCR rearrangements of the T-cell background), and clonal (non-germ line) bands derived from the predominant TCR gene. Clonal bands become visible if clonal cells comprise 2-5% of all analyzed cells^{24,25}. The technique is costly in labor and requires relatively high amounts of intact DNA. SB analysis of early stage MF often fails to detect a clonal TCR rearrangement²⁶.

2) PCR techniques: TCR genes of a given sample are amplified by PCR. Due to its limited combinatorial diversity, the TCR γ locus allows for simplified PCR primers and is often preferred for this analysis. PCR amplicons are subsequently separated on high-resolution gels. Predominant TCR rearrangements appear as sharp bands or high peaks (see figure 1 B-D). The diagnostic sensitivity of this approach has been estimated at 1-5%²⁷. Both steps of the technique are prone to give pseudoclonal results: Low T-cell numbers in the sample or the use of (semi-) nested PCR assays may result in imbalanced amplification of the TCR genes. Differentiation between sharp (clonal) band and non-clonal smears after electrophoresis remains very subjective, especially when low-resolution techniques are applied. False-positive PCR results can be reduced by application of standardized primer sets (as the Biomed-2 approach²⁸), by the use of more objective separation techniques (fluorescence fragment analysis³⁰) and by analysis of several skin samples per patient²⁹. PCR analysis

detects clonal TCR rearrangements in most MF cases²¹. In our hands, detection of TCR clonality succeeds in three-fourths of MF stage I patients (see table 3).

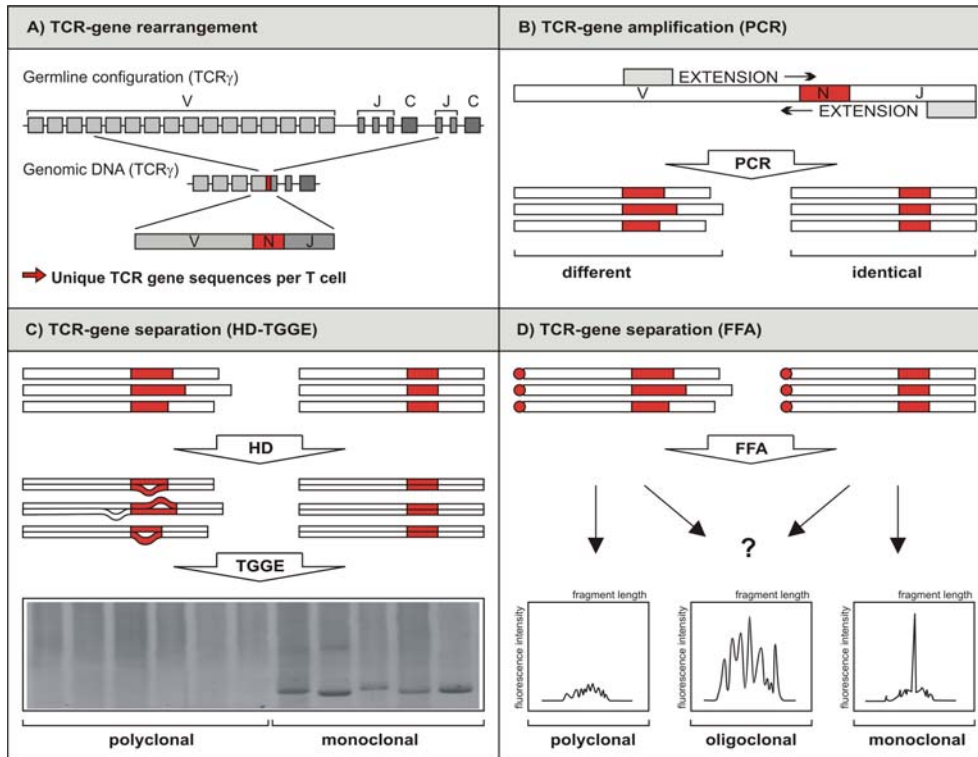


Figure 1. Detection of clonal TCR rearrangements. A) TCR rearrangement. During T-cell maturation distinct variable (V) and joining (J) segments are rearranged from germ line DNA to form a functional TCR gen (genomic DNA). Due to modifications during the recombination, the N region occurs. Combinatorial (chosen V and J gene segment) and junctional (N region) diversity ensure uniqueness of the TCR gene sequence. B) TCR amplification. All TCR genes of a sample are amplified by PCR. In case of predominant rearrangement, multiple identical amplicons occur. C) Hetero-duplex temperature gradient gel electrophoresis. Amplicons are denatured and slowly re-natured. Predominant amplicons more often form homoduplexes (right), whereas the remaining amplicons form heteroduplexes (left). After electrophoresis in a temperature gradient, homoduplexes appear as sharp bands (right), whereas heteroduplexes form a broad smear (left). D) Fluorescence fragment analysis. Fluorescence labeled amplicons are separated on an automated sequencer. Fluorescence intensity is depicted dependent on fragment length. Predominant fragments form high peaks. In this approach, estimation of TCR clonality can be objectified by calculation of a peak-height-ratio³⁰.

Currently, PCR approaches represent the preferred technique for the estimation of TCR clonality. However, detection of TCR clonality does not demonstrate malignancy. Immune responses to human immunodeficiency virus or Epstein-Barr virus as well as to vaccines can result in T-cell clones representing up to 40% of all peripheral T-lymphocytes. Moreover, TCR clonality has been demonstrated in skin samples of various benign dermatoses (see table 3). Consequently, TCR clonality studies represent only a complementary tool in the diagnostics of CTCL. Since every single CTCL can be defined by the unique sequence of its TCR genes, the technique offers advantages in disease monitoring as staging procedures and therapy control. Moreover, it can be applied to identify the neoplastic cells in various issues of lymphoma research, as microanatomical studies or clone-

specific in-situ hybridization^{31,32}.

Entity	Detection of a T-cell clone		Technique
	n	%	
CTCL ³³	46/58	80	TCR γ PCR/ HD-TGGE
MF IA	9/12	75	
MF IB	17/24	71	
MF II-IV	3/4	(75)	
Sézary syndrome	3/4	(75)	
Pleomorph small/medium-sized CTCL	12/12	100	
CTCL, not otherwise specified	2/2	(100)	
MF ³⁴	47/64	74	TCR γ PCR/ FFA
MF IA	14/18	78	
MF IB	28/39	72	
MF II-IV	5/7	(71)	
Benign dermatoses ³²	11/100	11	TCR γ PCR/ HD-TGGE
Drug eruption	0/7	(0)	
Lupus erythematosus	5/23	22	
Morphea	4/27	15	
Lichen rubber	0/20	0	
Prurigo simplex	2/23	9	
Lichenoid dermatoses	56/144	39	TCR γ PCR/HD-PAGE
Pityriasis lichenoides et varioliformis acuta ³⁵	13/20	65	
Lichen sclerosus et atrophicus ³⁶	18/39	46	
Lichen sclerosus et atrophicus ³⁶	16/29	55	
Lichen ruber ³⁷	9/36	25	
Lichen ruber ³²	0/20	0	
Chronic dermatitis vs. MF ³⁸			TCR γ PCR/ DGGE
MF	16/27	59	
Suggestive for MF, subsequently developing MF	11/22	50	
Suggestive for MF, subsequently MF excluded	6/32	19	
Chronic dermatitis	3/31	10	
Small plaque parapsoriasis (SPP)	3/25	12	TCR γ PCR/ DGGE
SPP ³⁹	2/3	(67)	
SPP ⁴⁰	1/8	(13)	
SPP ⁴¹	0/14	0	

Table 3. Detection of clonal T-cells by TCR γ PCR assays in different dermatoses and CTCL. HD, heteroduplex; PAGE, polyacrylamide gel electrophoresis; TGGE, temperature gradient gel electrophoresis; DGGE denaturing gradient gel electrophoresis; FFA, fluorescence fragment analysis.

1.4 Outline of the thesis

In the early 1990-ties, several groups applied SB techniques to evaluate an extracutaneous spread of malignant T-cells in MF and other CTCL. The majority of these studies detected circulating clonal T-cells only in Sézary syndrome and some cases of advanced MF or pleomorphic CTCL. Therefore, it was postulated that blood involvement is restricted to advanced cutaneous lymphoma and is associated with poorer prognosis, lymph node involvement as well as an enlarged total body tumor burden.^{42,43,44,45} In contrast, frequent occurrence of multifocal or diffuse cutaneous MF lesions as well as the recirculatory behavior of T-cells^{46,47} support the hypothesis of an early occurrence of

malignant T-cells in the peripheral blood. Consequently, a few groups have supposed early hematogeneous involvement in MF^{20,40,48,49}.

The overall outline of the research described in this thesis was to obtain more insight into T-cell clonality in the peripheral blood of patients suffering from MF. Four main topics were addressed. The first concerned the occurrence of circulating clonal T-cells (chapter 2). Frequency of peripheral blood T-cell clonality with regard to MF stage was analyzed in detail, and the identity of the T-cell clones detected in skin and blood of a given patient was determined. Topics 2 and 3 focused on the significance of the peripheral blood T-cell clonality in CTCL. The prognostic relevance of simultaneous detection of clonal T-cells in peripheral blood and skin of MF patients was analyzed in extended studies with special emphasis for early stages (chapter 3). The linkage between clonal chromosomal aberrations indicating qualitative and functional alteration of the affected cells, and TCR clonality was addressed. In addition, the relation between genomic aberration and survival in CTCL was investigated (chapter 4). Main topic four concerned the confirmation of our TCR clonality data by a golden standard. After analyzing a large series of paraffin-embedded CTCL samples by our in-house PCR and the Biomed-2 protocol, the results of both approaches were compared with special regard to sensitivity, specificity and match of the outcomes (chapter 5). Finally, the main results of the studies presented in this thesis are summarized and the diagnostic and prognostic significance of T-cell receptor gene rearrangement analysis in CTCL is discussed (chapter 6).

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