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Molecular aspects of cutaneous T-cell lymphoma : genetic alterations underlying clinical behavior

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Marloes van Kester

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List of abbreviations

aCGH	array-based comparative genomic hybridization
ACP2	acid phosphatase 2, lysosomal
ARHB	synonym RHOB
ARPC4	actin related protein 2/3 complex, subunit 4, 20kDa
ATP5J2	ATP synthase, H ⁺ transporting, mitochondrial Fo complex, subunit F2
BAC	bacterial artificial chromosome
BCL2	B cell lymphoma 2
BCL7a	B-cell CLL/lymphoma 7A
BCL11a	B-cell CLL/lymphoma 11A (zinc finger protein)
C-ALCL	primary cutaneous anaplastic large cell lymphoma
CCL5	chemokine (C-C motif) ligand 5
CCR7	chemokine (C-C motif) receptor 7
CCR10	chemokine (C-C motif) receptor 10
CD30	synonym TNFRSF8
CDCA7	cell division cycle associated 7
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CGH	comparative genomic hybridization
CHN1	chimerin (chimaerin) 1
CLL	chronic lymphocytic leukemia
CNA	copy number alteration
C-PTCL-NOS	primary cutaneous peripheral T-cell lymphoma not otherwise specified
CRIP1	cysteine-rich protein 1 (intestinal)
CTCL	cutaneous T-cell lymphoma
DE	differentially expressed
DIABLO	diablo, IAP-binding mitochondrial protein
DUSP1	dual specificity phosphatase 1
EPHA4	EPH receptor A4
FAS	Fas (TNF receptor superfamily, member 6)
FASTK	Fas-activated serine/threonine kinase
FrAGL	Frequency of Amplicon, Gain, and Loss
GATA-3	synonym PRDM2
HDAC	histone deacetylase
IL32	interleukin 32

IRF4	interferon regulatory factor 4
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
JUNB	jun B proto-oncogene
KIR3DL2	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2
LyP	Lymphomatoid papulosis
MCR	minimal common region
MET	met proto-oncogene (hepatocyte growth factor receptor)
MF	Mycosis fungoides
miRNA	microRNA
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
MXI1	MAX interactor 1
NF- κ B	nuclear factor kappa B
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NKG7	natural killer cell group 7 sequence
PRDM2	PR domain containing 2, with ZNF domain
PRKCQ	protein kinase C, theta
PTPRG	protein tyrosine phosphatase, receptor type, G
PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2
PUVA	psoralen UVA
RANKL	synonym TNFSF11
RANTES	synonym CCL5
RHOF	ras homolog family member F (in filopodia)
RB1	retinoblastoma 1
SCYA5	synonym CCL5
SMAC	synonym DIABLO
STAT4	signal transducer and activator of transcription 4
Sz	Sézary syndrome
TBX21	T-Box 21
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)
TIA-1	TIA1 cytotoxic granule-associated RNA binding protein
T-MF	tumor-stage mycosis fungoides
TNFRSF8	tumor necrosis factor receptor superfamily, member 8
TNFSF11	tumor necrosis factor (ligand) superfamily, member 11
TRAF1	TNF receptor-associated factor 1
TWIST1	twist homolog 1 (Drosophila)
WHO-EORTC	World Health Organization-European Organization of Research and Treatment of Cancer classification



1

General introduction

Primary cutaneous T-cell lymphoma

Primary cutaneous lymphomas represent a group of lymphoproliferative disorders of neoplastic lymphocytes presenting in the skin with no evidence of extracutaneous disease at the time of diagnosis. Whereas most lymphomas arise in lymph nodes, a considerable proportion primarily involves extranodal sites. After the gastrointestinal tract, the skin is the second most common site of extranodal non-Hodgkin lymphoma, with an estimated annual incidence of 1:100,000.¹ Primary cutaneous lymphomas are for 75% of T-cell origin and for the remaining 25% of B-cell origin.² Primary cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of diseases. Several different classifications were used for CTCL before consensus was reached in the World Health Organization-European Organization of Research and Treatment of Cancer classification (WHO-EORTC) classification.³ Classification is an essential prerequisite for defining groups for molecular studies, as well as for determining prognosis and treatment. The WHO-EORTC classification is depicted in Table 1; CTCL that are studied in this thesis are described in more detail below.

Mycosis fungoides

Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma. MF generally has an indolent course with slow progression from patches to more infiltrated plaques and eventually development to tumors over a period of years or even decades.³ Preferred locations of the initial skin lesions are the buttocks and other sun protected areas. Patients with tumor-stage MF characteristically show a combination of patches, plaques, and tumors, which often show ulceration. MF typically affects older adults (median age at diagnosis is 55-60 years) and males more frequently than females (1.6-2.0:1).^{4,5} Early patch/plaque-stage MF is characterized by the presence of atypical T cells with hyperchromatic, cerebriform nuclei, which preferentially infiltrate the epidermis (epidermotropism). With progression to tumor stage, epidermotropism may be lost and dermal infiltrates become more diffuse with increasing number of blast cells. Transformation to large cell lymphoma may occur.⁶ The neoplastic T cells have a CD3⁺, CD4⁺, CD8⁻, CD45RO⁺ memory T-cell phenotype. The prognosis is dependent on the stage of disease: patients with limited plaque-stage disease have a 10-year disease specific survival of 98%, but in patients with tumor-stage disease the 10-year survival decreases to 42%.^{4,5} The first choice of treatment for disease limited to the skin are skin-directed therapies including topical steroids, PUVA photochemotherapy, UV-B phototherapy, topical nitrogen mustard and radiotherapy.⁷⁻⁹ Patients with limited patch-

stage disease can be treated with topical steroids. Skin tumors are commonly targeted by radiotherapy, and for wide-spread tumor stage refractory to skin-targeted therapies and extracutaneous disease multi-agent chemotherapy is indicated.¹⁰

Table 1 WHO-EORTC classification. Disease-specific survival of primary cutaneous lymphoma classified according to WHO-EORTC classification³

WHO-EORTC classification ³	Frequency %	Disease-specific 5-year survival %
Cutaneous T-cell lymphoma		
Mycosis fungoides	44	88
Variants of mycosis fungoides		
Folliculotropic MF	4	80
Pagetoid reticulosis	< 1	100
Granulomatous slack skin	< 1	100
Sézary syndrome	3	24
Primary cutaneous CD30 ⁺ lymphoproliferative disorders		
Primary cutaneous anaplastic large cell lymphoma	8	95
Lymphomatoid papulosis	12	100
Subcutaneous panniculitis-like T-cell lymphoma	1	82
Primary cutaneous NK/T-cell lymphoma, nasal-type	< 1	NR
Primary cutaneous peripheral T-cell lymphoma, unspecified	2	16
Primary cutaneous peripheral T-cell lymphoma, rare subtypes		
Primary cutaneous gamma/delta-T-cell lymphoma	< 1	NR
Primary cutaneous aggressive CD8 ⁺ T-cell lymphoma	< 1	18
Primary cutaneous CD4 ⁺ small/medium pleomorphic T-cell lymphoma	2	75
Cutaneous B-cell lymphoma		
Primary cutaneous marginal zone B-cell lymphoma	7	99
Primary cutaneous follicle center lymphoma	11	95
Primary cutaneous diffuse large B-cell lymphoma, leg type	4	55
NR indicates not reached.		

Sézary syndrome

Sézary syndrome (Sz) is a malignancy of skin-homing CD4⁺ T cells characterized by a triad of erythroderma, generalized lymphadenopathy and the presence of neoplastic T cells in the skin, lymph nodes and peripheral blood.^{2,3,11} Diagnostic criteria are a Sézary cell count > 1000 cells/mm³, a CD4/CD8 ratio of > 10 caused by an expanding population of CD4⁺ T cells, aberrant expression of T-cell antigens (loss of CD2, CD3, CD4, CD5 and/or CD7), and a T cell clone in the peripheral blood determined by molecular or cytogenetic tests.¹² Sézary patients have a poor prognosis with a disease specific 5-year survival of 24%.³ Historically, MF and Sz were regarded as part of the same spectrum of disease.^{13,14} Although different in clinical presentation and disease behavior, Sz is generally considered to be a leukemic phase or variant of MF. Therefore, these two CTCLs still share the same classification and staging system and are often included in the same clinical trials. Despite being considered earlier as a leukemic phase of MF, in the recent WHO-EORTC classification and WHO classification 2008, MF and Sz are included as separate disease entities based on their distinctive clinical features and disease behavior (see Table 1). However, controversy still remains whether Sz is a leukemic phase of MF, or whether these CTCLs should be regarded separately.¹⁵

In contrast to patients diagnosed with MF, who are most often treated with skin-directed therapies, patients diagnosed with Sézary syndrome require systemic therapy. First line treatment options are extracorporeal photophoresis (ECP), interferon alpha, bexarotene, low-dose methotrexate or denileukin diftitox. Most recent treatment guidelines recommend adding skin-directed therapy PUVA, topical nitrogen mustard or total skin electron beam radiation depending on the relative burden of disease, impact on quality of life, and rapidity with which disease progresses. Second line systemic treatment options are alemtuzumab, a combination of low-dose chlorambucil and prednisone, liposomal doxorubicin, HDAC inhibitors (vorinostat, romidepsin), gemcitabine, deoxycoformycin and high-dose methotrexate.¹⁶

Primary cutaneous anaplastic large cell lymphoma

Primary cutaneous anaplastic large cell lymphoma (C-ALCL), formerly designated as primary cutaneous CD30-positive large T-cell lymphoma, is a T-cell lymphoma composed of large cells with an anaplastic, pleomorphic or immunoblastic cytomorphology, that shows expression of the CD30 receptor in more than 75% of the neoplastic cells.³ Patients generally present with solitary or localized skin tumors that have a tendency to regress spontaneously. C-ALCL has an indolent clinical behavior and rarely shows extracutaneous dissemination. C-ALCL tumor cells have a characteristic morphology of anaplastic cells, with round, oval, or irregularly shaped nuclei, prominent eosinophilic nucleoli, and an abundant cytoplasm.

Reactive lymphocytes are often present at the periphery of the lesions. The neoplastic cells have an activated CD4⁺ T-cell phenotype with variable loss of CD2, CD5 and CD3, and frequent expression of the cytotoxic proteins granzyme B, TIA-1 and perforin.¹⁷⁻²⁰ Primary cutaneous ALCL lacks t(2,5) translocations seen in systemic ALCL.²¹ Patients have a relatively good prognosis with a 5-year survival exceeding 90%.^{20,22,23} C-ALCL treatment of choice is radiotherapy or surgical excision for solitary or lesions few in number; C-ALCL with multifocal skin lesions can be treated with low-dose methotrexate.^{20,24} C-ALCL together with lymphomatoid papulosis (LyP) constitutes a spectrum of primary cutaneous CD30⁺ lymphoproliferative disorders.²⁵ Lymphomatoid papulosis is a chronic, recurrent, self-healing papulonecrotic or papulonodular skin disease with histological features suggestive of C-ALCL.³

Primary cutaneous peripheral T-cell lymphoma not otherwise specified

Primary cutaneous peripheral T-cell lymphoma not otherwise specified (C-PTCL-NOS), formerly classified as primary cutaneous CD30-negative large T-cell lymphoma, is a heterogeneous group and diagnosis is made by exclusion of other (sub)types (including aggressive epidermotropic CD8⁺ CTCL; cutaneous gamma-delta T-cell lymphoma; CD4⁺ small/medium pleomorphic CTCL). Patients present with solitary or more often generalized skin tumors. C-PTCL-NOS displays aggressive clinical behavior and frequently disseminates to extracutaneous sites.³ Histologically, these tumors are nodular or diffuse infiltrates with variable numbers of medium-sized to large pleomorphic or immunoblast-like T cells. Large neoplastic cells represent at least 30% of the tumor cell infiltrate.²⁶ Epidermotropism is generally mild or absent. Most cases show an aberrant CD4⁺ T-cell phenotype with variable loss of pan-T-cell antigens. CD30 staining is negative or restricted to a few scattered tumor cells.²⁷ C-PTCL-NOS should be treated with multi-agent chemotherapy. However, results are often disappointing and the prognosis is generally poor with an estimated 5-year survival of less than 15%.^{26,28,29}

Oncogenesis

In normal homeostasis, biological processes in the cell such as differentiation, proliferation and cell death are tightly regulated. Dysregulation of these physiological processes can lead to malignant transformation of the cell. Malignant transformation is generally considered a multistep process in which several genetic changes need to be acquired.³⁰ Six acquired alterations in genes involved in cell physiology were proposed to be essential for tumor development: self-sufficiently in growth signals, insensitivity to anti-growth

signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.^{31,32} Genes involved in these cellular processes affected by genetic changes can broadly be categorized in 2 groups: oncogenes and tumor suppressor genes (Figure 1a).

Oncogenes are mutant, overactive forms of proto-oncogenes. Oncogenes undergo “gain-of-function” by amplification, activating point mutation, translocations creating chimeric genes or translocations to regions of transcriptionally active chromatin, leading to increased protein abundance or activity.³³ Oncogenes encode proteins classified into one the following functional classes: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers or apoptosis regulators.³³

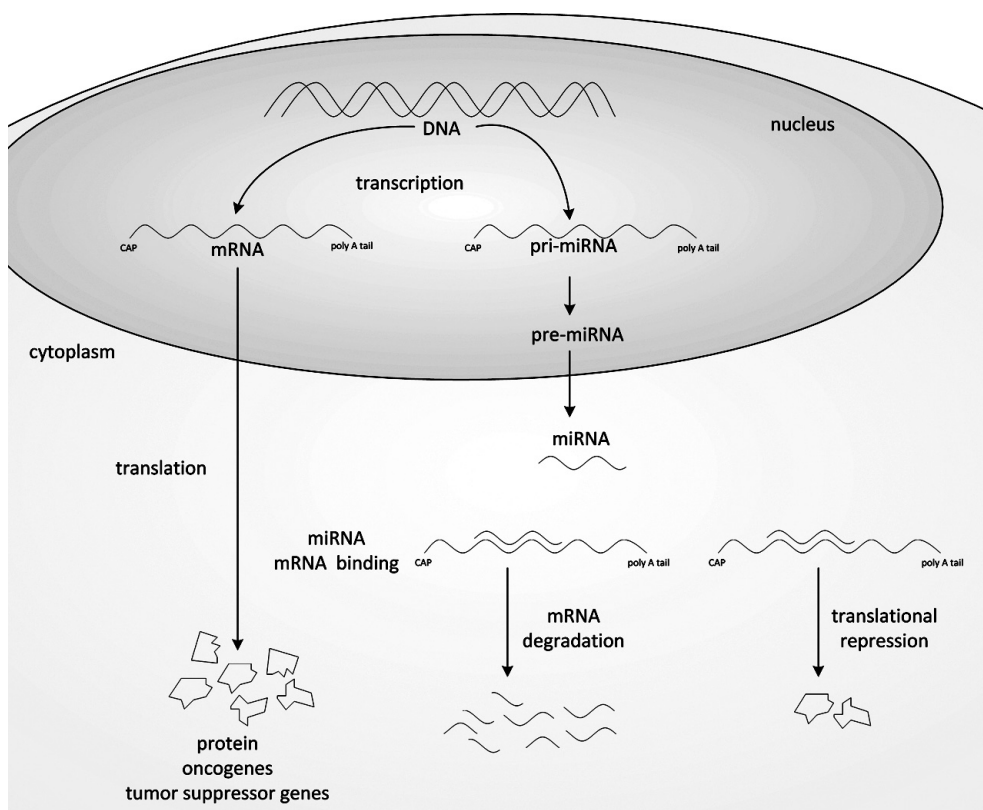


Figure 1 Interactions of DNA, mRNA and miRNA

Figure 1a during DNA transcription mRNA or pri-miRNA is synthesized of DNA Pri-miRNA is a precursor for miRNA. Following DNA transcription, protein is synthesized in the process of mRNA translation. Among other proteins, proto-oncogenes and tumor suppressor genes are synthesized by this means. miRNA can regulate protein expression by translational inhibition or induction of mRNA degradation leading to less available mRNA.

Tumor suppressor genes are gatekeepers of the cell, controlling crucial processes such as cell cycle and apoptosis. Both functional copies of a tumor suppressor gene have to be lost, as described by Knudson's two-hit hypothesis,^{34,35} to play a role in tumorigenesis. Tumor suppressor genes can be inactivated by inactivating mutations, deletions³⁶ or promoter hypermethylation.³⁷ In the typical tumor cell hundreds of mutations and chromosomal alterations are present, but only a part of these genetic defects are pathogenic and contribute to the malignant phenotype.

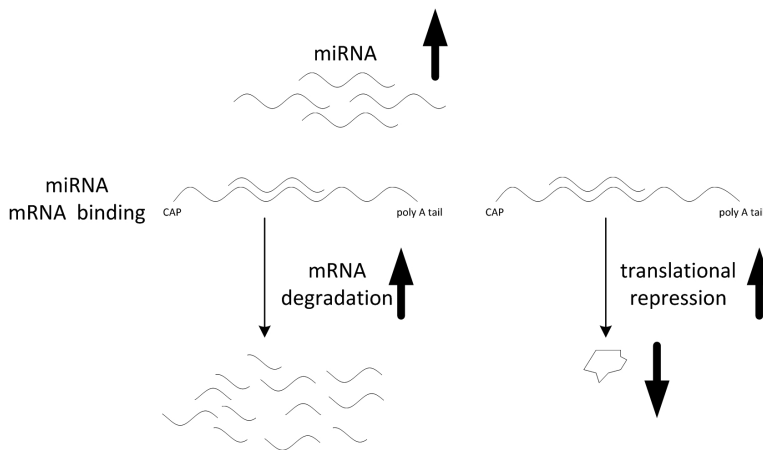


Figure 1b miRNAs can play a role in tumorigenesis by targeting proteins with a tumor suppressing function. Increased miRNA expression can lead to increased mRNA degradation and increased translational repression both leading to decreased protein synthesis

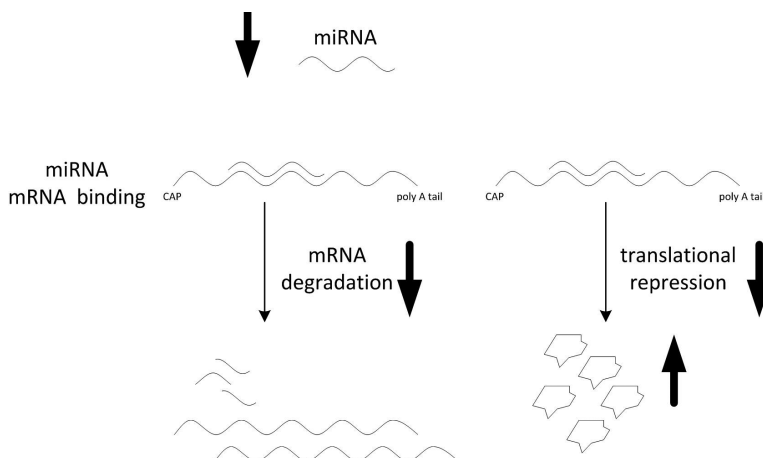


Figure 1c Likewise miRNAs can play a role by targeting oncogenes. Decreased miRNA expression can lead to decreased mRNA degradation and decreased translational repression both leading to increased protein synthesis

To acquire the necessary alterations in gene expression critical for tumor development, most tumors exhibit genomic instability.^{38,39} Two major types of genomic instability are chromosomal instability and microsatellite instability.^{40,41} The former stems from defects in DNA mismatch repair resulting in an increased mutation rate.^{40,42} Tumors show either microsatellite instability or chromosomal instability, but not both.⁴⁰ Most often tumors possess chromosomal instability, resulting in chromosomal alterations ultimately leading to translocations, gain of function amplifications of oncogenes and deletion of tumor suppressor genes.

Epigenetics is defined as heritable changes in gene expression that are not due to any alteration in the DNA sequence.⁴³ The field of epigenetics within oncogenesis covers DNA methylation, DNA hypomethylation and histone modification.⁴⁴ DNA hypermethylation of CpG islands in promoter regions of tumor-suppressor genes leads to inactivation of the tumor suppressor gene. The extent of DNA hypomethylation increases during the development of a tumor.⁴⁵ Three mechanisms have been proposed to explain the contribution of DNA hypomethylation to the evolution of a tumor cell: development of

Box 1**Array-based techniques used to decipher molecular alterations genome-wide in studies included in this thesis**

Array-based Comparative Genomic Hybridization (aCGH) is a technique to detect genome-wide DNA copy number alterations (gains and losses) at a higher resolution than conventional chromosome-based CGH. The microarrays used have a 1 Mb resolution (1 probe per 10^6 base pairs) containing 3500 probes (probe length: 10^5 base pairs) of mainly BAC clones and a set of subtelomeric sequences. Fluorescently labeled genomic DNA isolated from a tumor sample and reference DNA are hybridized to the probes on the array slides. Subsequently the fluorescent intensity ratio of tumor/reference is calculated and annotated to genomic location, hereby identifying gains and losses at specific chromosomal locations.

Gene expression microarray is a technology to determine the expression levels of tens of thousands of genes simultaneously. RNA isolated from fresh frozen tumor biopsies is reverse transcribed to synthesize cDNA. Biotin-labeled antisense RNA is synthesized from cDNA. Antisense RNA is hybridized to the array slides after fragmentation. After hybridization array slides are scanned to measure intensity values, while specialized software can calculate the expression values from the intensity values.

miRNA microarray is used to study miRNA expression levels genome-wide. Total RNA isolated from tumor samples and reference RNA is reverse transcribed, and tagged antisense cDNA of both sample and reference is hybridized to the sense array, containing probes with the sequences of interest spotted in quadruple. Following hybridization, tagged cDNA is fluorescently labeled and the array slides are scanned. Expression values can be calculated from the intensity values by specific software.

chromosomal instability, reactivation of transposable elements, and loss of imprinting.⁴⁴ Histone modification can regulate gene transcription, play a role in DNA repair and replication, and manage the organization of chromosomes.⁴⁴

MicroRNAs (miRNAs) are a recently discovered class of small RNA molecules regulating gene expression by translational inhibition or mRNA degradation of target genes (Figure 1a).⁴⁶ MicroRNAs targeting a tumor suppressor gene act as oncomirs and miRNAs targeting oncogenes have a tumor suppressing function (Figure 1b,c).^{47,48} MicroRNAs are more often encoded in fragile sites of the genome.⁴⁹ DNA copy number alterations can influence miRNA expression levels, implying that DNA copy number alterations can indirectly regulate gene expression through miRNAs.^{50,51} An example is the recurrent deletion of 13q14.3 in chronic lymphocytic leukemia (CLL). Pinpointing the minimal common region of overlap between tumors reveals deletion of the miR-15-16 cluster⁵² targeting BCL2.⁵³ BCL2 is often found to be up-regulated in CLL by previously unexplained mechanisms. Hence, decreased expression of miR-15-16 causes up-regulation of BCL2.^{52,53} Recently it was shown that increased expression of a single miRNA can act as a driver of cancer and initiate tumor formation.⁵⁴ There are several techniques to study DNA copy number alterations, gene expression and miRNA expression genome-wide, the techniques used in this thesis are described in Box 1.

Molecular pathogenesis of cutaneous T-cell lymphoma

In the previous section, the interactions between chromosomal alterations, oncogenes and tumor suppressor genes, epigenetics and miRNAs are explained, as well as the contribution of these different mechanisms to malignant transformation. In the following section results of studies investigating chromosomal alterations, gene expression, DNA methylation and miRNA expression contributing to lymphomagenesis in different types of CTCL will be reviewed.

Chromosomal aberrations

Sézary syndrome

Numerous cytogenetic studies investigating peripheral bloods samples of Sz patients for numerical and structural chromosomal alterations showed a heterogeneous pattern of alterations.⁵⁵⁻⁵⁸ Many studies describe only a single or a limited number of cases, in which MF and Sz cases are not well defined and often not studied as separate groups. Although use of comparative genomic hybridization (CGH) shows repeatedly losses on 10q and gains on 17q, no highly recurrent aberrations were found.^{55,59-61} The first study using aCGH

demonstrated highly recurrent alterations in Sz. Multiple regions of gains on 8q and 17q and losses on 10q were identified in which several oncogenes and tumor suppressor genes reside.⁶²

Mycosis fungoides

A limited number of cytogenetic studies describe chromosomal aberrations in karyograms of MF skin tumors.^{56,63-65} For karyotyping dividing cells are required. The proportion of dividing cells can be increased by stimulating cells with mitogens such as phytohemagglutinin during culturing, a technique that is easier to perform on blood samples. More often karyograms were made of peripheral blood samples of MF patients.^{57,58,66,67} However, it remains unclear whether the studied cells are representative of MF malignant cells. The use of comparative genome hybridization (CGH), not requiring dividing cells, provided a new opportunity to study chromosomal aberrations in skin lesions. Mao and colleagues were the first to use conventional CGH on skin lesions of MF. They described chromosomal aberrations in nine of the 16 skin lesions. However, no recurrent alterations were identified. The most recurrent alteration was loss of 1p36 in four of the 16 skin lesions.⁶¹ Subsequently, 19 cases representing various stages of MF and 11 cases of transformed MF were studied with CGH.^{59,68} Aberrations were mainly found in more advanced stages of disease.^{59,68} By studying DNA copy number alterations at a higher resolution with aCGH in early-stage MF, Carbone and colleagues identified recurrent loss of chromosome 19 (56% of the cases) and 12q24.31(44% of the cases) containing the genes *BCL7a*, *SMAC/DIABLO* and *RHOA*.⁶⁹ Hypermethylated CpG islands of the promotor regions of *BCL7a*, *PTPRG* and thrombospondin-4 were demonstrated.⁷⁰

CTCL, non MF/Sz

Studies describing chromosomal aberrations in C-ALCL and C-PTCL-NOS are few. CGH studies showed gains on 1p, 6p, 7q, 8p, 9 and 19, and losses on 6q and 18 in C-ALCL.⁷¹⁻⁷⁴ Mao and coworkers further studied chromosomal imbalances with aCGH in a subset of cases (n=5). Gains on 8p11 (3 cases), 1p13.2, 2p24.1, 3p25, 8p22, 15q26.1, and 21q22.3 (2 cases) were demonstrated.⁷²

Gene expression

Numerous studies describe the altered expression of one or more genes in CTCL. Gene expressions arrays provided the opportunity to study the expression of large sets of genes simultaneously (Box 1). Kari and colleagues compared 18 samples of 17 Sz patients with nine Th2-skewed controls identifying 385 differentially expressed genes,

including over expression of Th2 cell-specific transcription factors *Gata-3/PRDM2* and *JUNB*, as well as *ITGB1* (integrin $\beta 1$), *ARHB/RHOB* and *DUSP1*, and reduced expression of *CD26*, *STAT4* and *IL-1 receptors*.⁷⁵ Ten cases of Sz were compared with CD4⁺ T cells of healthy controls and benign erythroderma patients by van Doorn and colleagues. Using the Affymetrix U95Av2 arrays, they identified 176 differentially expressed genes.⁷⁶ High expression of *TWIST1*, *EPHA4* and *RANKL/TNFSF11* was demonstrated, while tumor suppressor genes *TGFB2*, *MXI1*, *PRDM2*, *BCL11a* and *STAT4* were down-regulated.⁷⁶ In the line of over expression of Th2-specific genes,⁷⁵ Hahtola and colleagues found down-regulation of Th1-specific genes (*TBX21*, *NKG7*, and *SCYA5 (RANTES/CCL5)*).⁷⁷ Although similarities exist between the studies, the array results are quite heterogeneous. This could be due to differences in experimental design using different array platforms and selection criteria of cases and controls. Tracey and colleagues studied genes associated with oncogenesis with CNIO Oncochips by comparing 29 cases of MF in various stages of disease with 11 cases of inflammatory dermatoses.⁷⁸ They identified 27 genes involved in tumorigenesis pathways. Studies investigating a comprehensive gene expression profile of C-ALCL and C-PTCL-NOS have not been performed yet.

miRNAs

MicroRNAs have thus far not been studied in cutaneous lymphoma, although studies in other types of lymphoma show altered expression of miRNAs and demonstrate a role in gene expression regulation of oncogenes and tumor suppressor genes.⁷⁹⁻⁸¹ In particular, miR-155 and miR-17-92 are frequently over expressed in various types of lymphoma and contribute to malignancy.^{82,83}

Aims and outline of this thesis

Although many molecular studies have been performed, much is still unknown regarding the tumor genetics underlying the differences in clinical presentation and prognosis of different types of CTCL. Within the scope of this thesis we performed several genome-wide inventory studies of copy number alterations, gene and miRNA expression with state-of-the-art techniques on well-defined CTCL cases to fill in gaps of knowledge and further elucidate the significance of specific molecular alterations in clinical behavior.

In search for differences in chromosomal alterations underlying the different clinical behavior and prognosis of patients with MF and Sz, in **Chapter 2** the DNA copy number

alterations of MF were investigated and compared with the previously published profile of Sz.⁶² We used the same aCGH platform to fine-map numerical chromosomal alterations at high resolution in the malignant T cells of tumor-stage MF, defined recurrent chromosomal alterations characteristic for tumor-stage MF and evaluated whether this pattern corresponds to the highly recurrent gains and losses observed in Sz. The second aim was to identify candidate oncogenes and tumor suppressor genes residing in chromosomal regions with recurrent copy number alteration (CNA) in tumor-stage MF contributing to tumorigenesis. To this end, chromosomal alteration and gene-expression patterns of MF tumor samples were integrated to determine which genes located in minimal common regions (MCRs) with CNA demonstrated dysregulated expression associated with chromosomal alteration. A third line of enquiry was aimed at finding chromosomal alterations with prognostic significance.

To identify altered gene expression contributing to lymphomagenesis not due to copy number effect we re-analyzed in **Chapter 3** the gene expression profiles of 22 cases of tumor-stage MF previously performed on commercial available arrays comprehending the entire human genome. A bioinformatic approach was used to identify a gene expression profile characteristic for tumor-stage MF comparing MF tumors simultaneously with normal T cells, normal skin and inflamed skin to circumvent the issue of admixing cells in the tumor biopsies. This profile could provide more insight in the pathogenesis of this type of lymphoma, describing altered expression of genes involved in oncogenesis, the (immuno)phenotype and the discovery of novel putative diagnostic markers and therapeutic targets.

The contribution to malignancy of miRNAs regulating gene expression is studied in **Chapter 4** and **5**. In **Chapter 4**, the first study of miRNA expression in cutaneous lymphoma, investigating the miRNA profile of Sézary syndrome, is described. To explore the effects of DNA copy number alteration on gene expression, altered miRNA expression was correlated to chromosomal alterations. To study the contribution of altered miRNA expression to lymphomagenesis the effect of altered miRNA expression on proliferation and apoptosis was investigated by functional assays.

In **Chapter 5**, miRNA expression was investigated in tumor-stage MF. Firstly, to identify aberrantly expressed miRNAs specific for tumor-stage MF, the miRNA profiles of tumor-stage MF were compared with benign inflammatory dermatoses. Secondly, to correlate miRNA expression with chromosomal aberrations locations of up- and down-regulated miRNAs were inspected for previously described DNA copy number alterations

characteristic for tumor-stage MF. Thirdly, the correlation between miRNA expression and gene expression was studied to elucidate the role of miRNAs as gene regulators in tumor-stage MF.

C-ALCL and C-PTCL-NOS are two types of primary cutaneous lymphomas with a very different clinical behavior and prognosis. **Chapter 6** discusses the results of aCGH analysis and gene expression profiling performed on C-ALCL and C-PTCL-NOS tumor samples. For both these types of lymphoma, the chromosomal aberrations were determined and compared in an effort to elucidate differences and similarities, and the minimal common regions were examined for oncogenes and tumor suppressor genes. Furthermore to better understand the molecular mechanisms underlying these two clinically different types of CTCL the gene expression profiles were compared.

Chapter 7 summarizes the main findings of the previous chapters

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Oncogenomic analysis of mycosis fungoides reveals major differences with Sézary syndrome

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Abstract

Mycosis fungoides (MF), the most common cutaneous T-cell lymphoma (CTCL), is a malignancy of mature, skin-homing T cells. Sézary syndrome (Sz) is often considered to represent a leukemic phase of MF. In this study, the pattern of numerical chromosomal alterations in MF tumor samples was defined using array-based CGH; simultaneously, gene expression was analyzed using microarrays. Highly recurrent chromosomal alterations in MF include gain of 7q36, 7q21-7q22 and loss of 5q13 and 9p21. This pattern characteristic of MF differs markedly from chromosomal alterations observed in Sz. Integration of data from array-based CGH and gene expression analysis yielded several candidate genes with potential relevance in the pathogenesis of MF. We confirmed that the *FASTK* and *SKAP1* genes, residing in loci with recurrent gain, demonstrated increased expression. The *RB1* and *DLEU1* tumor suppressor genes showed diminished expression associated with loss. In addition, it was found that presence of chromosomal alterations on 9p21, 8q24 and 1q21-1q22 was associated with poor prognosis in patients with MF. This study provides novel insight into genetic alterations underlying MF. Furthermore, our analysis uncovered genomic differences between MF and Sz, which suggest that the molecular pathogenesis and therefore therapeutic requirements of these CTCLs may be distinct.

Introduction

Mycosis fungoides (MF), the most common type of primary cutaneous T-cell lymphoma (CTCL), is a malignancy of mature, skin-homing T cells. MF commonly presents with erythematous patches and plaques and generally behaves as a low-grade lymphoma with an indolent disease course.^{1,2} A subset of patients with MF experiences disease progression, which is characterized by the formation of skin tumors, the appearance of blast-like cells in the tumoral infiltrate and extracutaneous dissemination of malignant T cells. Progressive MF is often refractory to treatment and has an unfavorable prognosis.³ In recent years progress has been made in defining cytogenetic alterations in MF using conventional comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) methods.^{4,5} In addition, mutations affecting the *CDKN2A*, *FAS* and *JUNB* genes and alterations of JAK/STAT and death receptor signalling have been identified in subgroups of patients with MF.⁶⁻¹¹ However, the molecular genetic alterations underlying this T-cell lymphoma remain poorly understood.¹²⁻¹³

A CTCL that is closely related to MF is Sézary syndrome (Sz). Sz is characterized by the triad of erythroderma, generalized lymphadenopathy and presence of malignant T cells in peripheral blood. Patients with Sz have a considerable leukemic T-cell burden and a dismal prognosis with an estimated 5-year survival rate of 24%.¹⁴ Recently, we identified several highly recurrent copy number alterations (CNAs) in Sz, including gain of loci on chromosome 17q24 and 8q24 and loss of regions on 17p13 and 10q25, occurring in up to 85% of patients.¹⁵ Additional evaluation of candidate oncogenes and tumor suppressor genes residing in loci with chromosomal alteration pointed to dysregulation of the *MYC* oncogene, several of its regulators and IL-2 receptor signalling pathway components in Sz.

MF and Sz are both clonal proliferations of T cells with cerebriform nuclei and a CD4⁺, CD45RO⁺, CLA⁺ immunophenotype.¹³ Despite differences in clinical presentation and disease behavior of these two disease entities, Sz is often designated as a leukemic phase or variant of MF and it has been suggested that differences between both conditions are a matter of stage of disease.¹⁶⁻¹⁸ Therefore these two CTCLs, sometimes collectively termed MF/Sz, share the same classification and staging system and are managed using similar treatment regimens.^{19,20} Although previously classified as a variant of MF, the current World Health Organization-European Organization of Research and Treatment of Cancer classification (WHO-EORTC) classification lists Sz as a separate disease entity based on its distinctive clinical features and disease behavior.¹⁶ Because of the existence of both shared and dissimilar immunophenotypical and genetic properties, controversy has remained as to whether MF and Sz should be regarded as distinct disorders with a

different pathogenesis and therapeutic requirements or whether differences reflect distinct stages of a similar disease process.

In this study, numerical chromosomal alterations in malignant T cells from tumor-stage MF samples were mapped using array-based CGH. The first purpose was to define the pattern of recurrent chromosomal alterations characteristic of MF. We then evaluated whether this pattern corresponds to the highly recurrent gains and losses observed in Sz. The second purpose was to identify candidate oncogenes and tumor suppressor genes residing in chromosomal regions with recurrent copy number alteration in MF. To this end, chromosomal alteration and gene expression patterns of MF tumor samples were integrated to determine which genes located in minimal common regions (MCRs) with CNA demonstrated dysregulated expression associated with chromosomal alteration. A third line of enquiry we pursued was aimed at finding chromosomal alterations with prognostic significance.

Material and Methods

Selection of Patients

Lesional skin tumor biopsy samples containing at least 70% malignant T cells from 22 patients with tumor-stage MF (TNM stage $T_3N_0M_0B_0$ in 21 patients and $T_3N_3M_0B_0$ in 1 patient) were included in this study. They included 18 male and 4 female patients with a mean age at time of biopsy of 66 years. All biopsy samples were obtained before treatment, except in patients diagnosed with plaque-stage disease ($T_1N_0M_0B_0$ or $T_2N_0M_0B_0$) previously who had been treated with local corticosteroids or phototherapy. The malignant phenotype of T cells in tumoral infiltrates was assessed on the basis of cytonuclear atypia and immunophenotypical characteristics by an expert panel of pathologists. Lymphoid cells were $CD4^+$ and $CD8^-$ in all cases. Histopathologically, all included tumor samples showed large cell transformation, indicated by the presence of at least 25% large cells in the tumoral infiltrate. After a mean follow-up period of 23 months, 12 patients had died because of MF, 3 had died of other causes and 7 patients were alive. Results of array-based CGH analysis were compared to those previously obtained from peripheral blood mononuclear cells of 20 patients with Sz using identical methods.¹⁵ In that study Sz was defined according to criteria of the WHO-EORTC classification. Clinical characteristics of MF and Sz patients are summarized in Supplementary Table S1. Approval was obtained from the Leiden University Medical Center institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Extraction of DNA and RNA

DNA and RNA were isolated from the same tumor biopsy for array-based CGH and gene expression analysis, using oligonucleotide arrays and quantitative real-time PCR (qPCR), respectively of all 22 included patients. DNA was isolated from 25 x 20 μ M frozen sections using the Genomic-tips 20/G kit (Qiagen, Hilden, Germany), yielding 10-60 μ g genomic DNA. RNA was extracted from 25 x 50 μ M frozen sections using the RNeasy kit (Qiagen), yielding 25 to 60 μ g total RNA. RNA used for gene expression analysis and for confirmatory qPCR analysis was isolated from the same tumor biopsy sample.

Array-based CGH analysis

Genome-wide analysis of CNAs was performed using array-based CGH containing approximately 3500 bacterial artificial chromosomes (BACs) produced at the Leiden University Medical Center. The particular BAC set used to produce the arrays was distributed by the Wellcome Trust Sanger Institute (Hinxton, United Kingdom) and contains large insert clones spaced at approximately 1 Mb density over the full genome, a set of subtelomeric sequences for each chromosome arm, and a few hundred probes selected for their involvement in oncogenesis. Fabrication and validation of the array, hybridization methods and analytical procedures have been described in detail elsewhere, whereas the clone content is available in the Cytoview window of the Sanger Center mapping database site Ensembl (<http://www.ensembl.org>).²¹ Data were analyzed using CAPWeb and visualized using VAMP.²² Log² ratios were classified as copy number gain (> 0.25) or genomic loss (< -0.25). Identified CNA of regions with copy number variations described in the Database of Genomic Variants (<http://projects.tcag.ca/variation>) were excluded from analysis.

Gene expression profiling

Samples and microarrays (Human Genome U133plus2.0 array, Affymetrix Santa Clara, CA, USA), interrogating over 47000 human transcripts and variants, were processed according to the manufacturer's protocol as described previously.²³ The array images were quantified utilizing the Genechip operating system (GCOS) v1.2 software (Affymetrix). The average fluorescence intensity was determined for each microarray, and then the output of each experiment was globally scaled to a target value of 200. Further normalization and variance stabilization was performed using variance-stabilizing normalization in the R statistical software package.²⁴ All microarray data have been deposited with Gene Expression Omnibus under accession number GSE12902.²⁵

Data analysis

BAC clone and oligonucleotide probe positions were established based on Ensembl release 44 (April 2007). Recurrent MCRs with CNA affecting at least 35% of analyzed samples were computed in CAPWeb using the algorithm proposed by Rouveirol *et al.*²⁶ Only CNAs characterized by gain or loss of at least 2 clones were taken into consideration. The nearby borders of adjacent clones were chosen to delineate MCRs. Copy number was divided into the categories gain, normal, and loss. To determine whether MCRs with recurrent CNA contained a statistically significantly higher number of genes showing increased expression in case of gain, or diminished expression in case of loss the sign test was performed. The normalized expression levels of genes residing in these MCRs as measured by oligonucleotide microarray analysis were then compared between tumor samples with and without the particular CNA. Independent-samples *t*-tests were performed (equality of variances not assumed) using the SPSS 14.0 statistical software package. Genes demonstrating a statistically significant increased expression in MF samples with gain or decreased expression in case of loss were considered of primary interest ($p < 0.05$). From this collection of genes with CNA-associated expression, candidate genes with pathobiological relevance were selected by focusing on genes listed as oncogene or tumor suppressor gene in the European Bioinformatics Institute cancer gene prediction database (<http://cgg.ebi.ac.uk/services/cgp>) with a probability exceeding 30%. Disease-specific actuarial survival rates of patients were calculated from the date of tumor biopsy for array-based CGH analysis and compared using the log-rank test.

Quantitative real-time PCR

cDNA synthesis was performed on 1 µg of total RNA, after treatment with RQ1 DNase I (Promega, Madison, WI, USA), using IScript reverse transcriptase (Bio-Rad, Veenendaal, the Netherlands), oligo(dT)₁₂₋₁₈ and random hexamer priming (Bio-Rad) in a final volume of 20 µl. Real-time PCR was performed with the MyIQ instrument and the SYBR Green Supermix (Bio-Rad). The cycle parameters for transcripts of interest and for the reference genes *U1A* and *RPS11* used for normalization were as follows: denaturing for 15 s at 97 °C; annealing and extension for 20 s at 60 °C, for 40 cycles. Primer sequences (Invitrogen, Breda, The Netherlands) for selected transcripts are given in Supplementary Table S2. Data were evaluated using MyIQ software (Bio-Rad) and the second derivative maximum algorithm, whereas confirmation of the specificity of the PCR product and standard curves were performed as previously described.²⁷

Immunohistochemistry

Immunostaining on formalin-fixed, paraffin-embedded skin sections with antibodies against RB1 (dilution 1:400; phosphorylation-nonspecific, 14001A, BD PharMingen, San Diego, CA USA) and SKAP1 (dilution 1:400; HPA002969, Sigma-Aldrich, St Louis, MO USA) was performed using a standard 3-step streptavidin-biotin-peroxidase-based technique after antigen retrieval with microwave heating as described previously.²⁸

Results

Pattern of copy number alterations of mycosis fungoides

Clinical characteristics and follow-up data of the 22 patients with tumor-stage MF included in the study are presented in Supplementary Table S1. Array-based CGH methodology was used to catalog CNAs in the genomes of malignant T cells present in skin tumor biopsy samples. All MF tumor samples showed extensive losses and gains of both large and smaller chromosomal regions. Copy number gains were more frequent than losses. The frequency and cumulative pattern of gains and losses in the tumor samples is depicted in Figure 1a. As a first step towards determining biologically significant patterns of genomic alterations in MF, we computed MCRs with CNA. MCRs represent the smallest recurrent chromosomal region with altered probes common to the set of aCGH profiles and are considered to harbor genes with biological relevance in tumor progression.^{26,29} We identified 24 MCRs present in at least 35 % of the 22 MF patients, ranging in size from 1.2 to 41 Mb. These MCRs are presented in Table 1 and are indicated by vertical lines in a visual representation of averaged CGH data in Figure 1b. Fifteen of these recurrent MCRs with CNA represent gains of chromosomal regions and 9 correspond to losses. Among the most frequently observed alterations were gain of regions on the long arm of chromosome 7 with a MCR on 7q36, observed in 59% of samples, and gains of several other regions on 7q32-7q35, 7q21-7q22 and 7q11.2. The chromosomal regions second most frequently affected with gain were 7p13-7p14, 7p21-7p22, 1q31-1q32 and 1p36.2, occurring in 45% of the patients. Losses were most frequently observed on 5q13, 9p21 and 13q14-13q31.

Comparison of genomic profiles of mycosis fungoides and Sézary syndrome

We then evaluated the similarity of chromosomal alterations observed in MF with those present in Sz. Recently, we have studied chromosomal alterations in malignant T cells from peripheral blood of 20 Sz patients using an identical array-based CGH platform and bioinformatic analysis.¹⁵ Malignant T cells from patients with Sz are characterized by

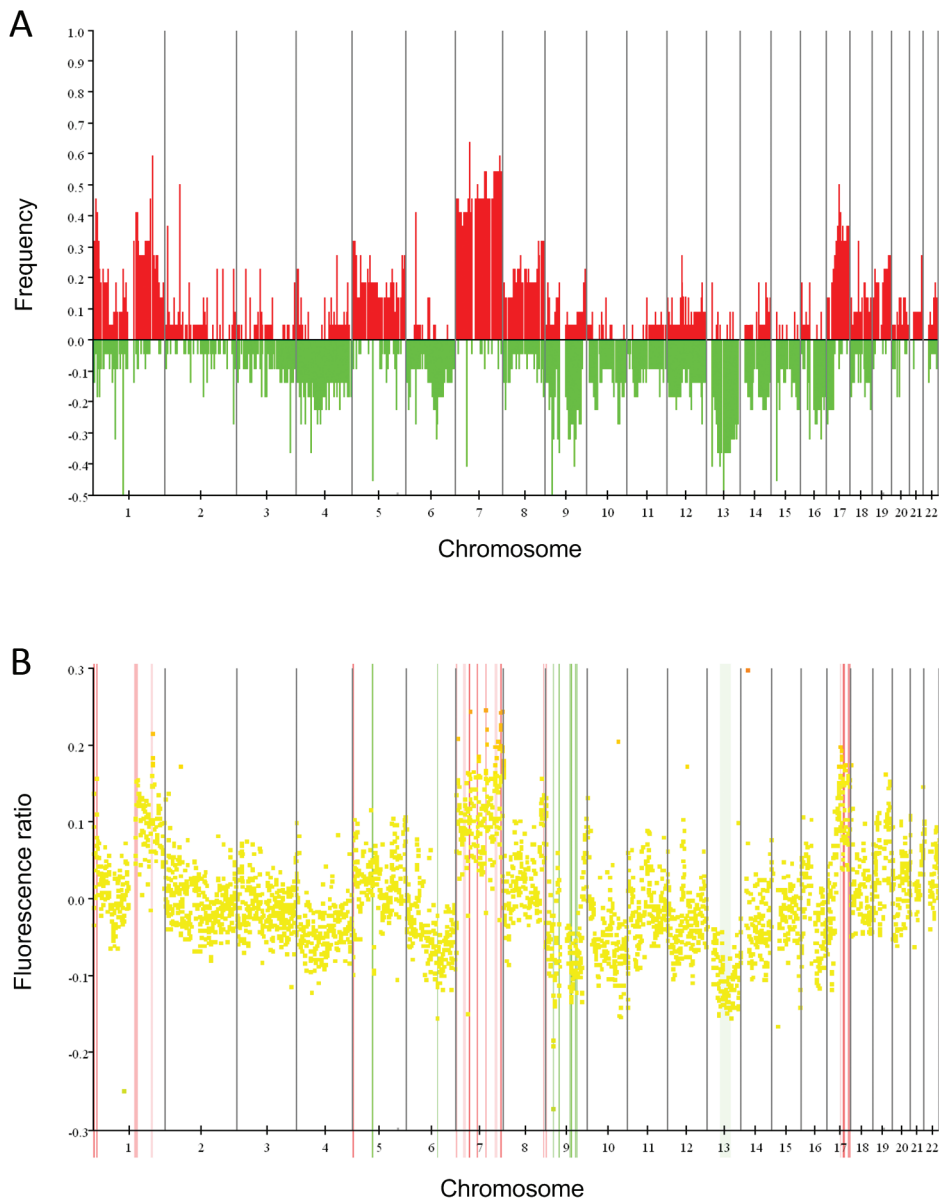


Figure 1 Visualization of the array-based CGH results by VAMP

(A) Overall frequency of CNAs in MF patient tumor biopsies, calculated using the FrAGL (Frequency of Amplifications, Gains and Losses) option of VAMP. Losses are represented on the negative scale as green bars; gains are presented on the positive scale as red bars. (B) Averaged CGH pattern of all 22 MF tumor samples. MCRs with loss occurring in at least 35% of patients are indicated as green vertical lines and MCRs with gain as red vertical lines. All data are presented ordered by chromosomal map position of the clones, excluding sex chromosomes.

several highly recurrent alterations, including gain of 17q23-25 (in 80% of patients), gain of 8q24 (in 75%, harboring the *MYC* oncogene) and loss of 17p13 (in 75%, harboring the *TP53* tumor suppressor gene). These specific chromosomal alterations are present much less frequently in MF tumor samples, with frequencies of 32%, 23% and 9% respectively. Conversely many highly recurrent alterations in MF, including gain on 7q36, only rarely occur in Sz. Whereas the overall pattern of chromosomal alterations of MF is characterized by gains on chromosome 1 and 7 and losses on chromosome 9, Sz demonstrates gains of regions on chromosome 8 and 17 and loss on chromosome 10.

Table 1 Minimal common regions with copy number alteration in mycosis fungoides

Cytogenetic band	Copy number alteration	Adjacent clones		Clone position (Mb)		Affected patients (%)
		start	stop	start	stop	
1p36.2	gain	RP4-539L13	RP11-196P5	11098993	12351219	45
1q21-1q22	gain	RP4-790G17	RP11-172I6	146342686	156056126	41
1q31-1q32	gain	RP11-572A16	RP11-534L20	198714422	205087972	45
5q13	loss	RP11-551B22	RP11-497H16	67677068	70179512	45
7p22-7p21	gain	RP11-510K8	RP4-733B9	1081263	7947777	45
7p15-7p14	gain	RP11-99O17	RP11-302L6	24659178	37825117	41
7p14-7p13	gain	RP11-36H20	RP11-52M17	43272694	45048103	45
7q11.2	gain	RP11-313P13	RP11-107L23	71274704	76190020	50
7q21-7q22	gain	RP4-550A13	RP11-333G13	97314794	102514284	55
7q32-7q35	gain	RP11-329I5	RP11-298A10	130270796	143852574	55
7q36	gain	RP11-24N19	RP4-548D19	148089302	151558264	59
8q24.2	gain	RP11-71N3	RP11-343P9	132799581	137773461	32
8q24.3	gain	RP5-1118A7	RP5-1056B24	142790550	telomere	36
9p21	loss	RP11-113D19	RP11-149I2	20351121	22479496	41
9p21	loss	RP11-495L19	RP11-33K8	22579721	24877888	32
9p13-9p11.1	loss	RP11-211N8	RP11-475I24	39990599	42614658	32
9q21	loss	RP11-490H9	RP11-336N8	78213759	80495074	32
9q21	loss	RP11-174K23	RP11-432M2	79930787	84622895	32
9q21	loss	RP11-439A18	RP1-292F10	84783002	86180561	32
9q22-9q31	loss	RP11-463M14	RP11-75J9	101410218	105214273	32
13q14-13q31	loss	RP11-168P13	RP11-464I4	42301191	83766576	36
17q21	gain	RP5-905N1	RP11-361M10	39091531	44639847	41
17q22-17q23	gain	RP11-312B18	RP11-156L14	48664511	59626448	32
17q25	gain	RP11-478P5	GS-362-K4	69639765	telomere	36

More detailed comparison of MCRs with recurrent CNAs in MF and Sz revealed clear differences, including many gains and losses that were present at a high frequency in MF but not in Sz. In Table 2 the 10 most frequent MCRs with CNA in MF and Sz are highlighted and frequencies in both entities are indicated. These findings argue against the notion that differences between these CTCLs are a matter of stage and strongly suggest that the molecular pathogenesis of MF and Sz follows distinct pathways.

Table 2 Comparison of most highly recurrent CNAs in MF and Sz

MYCOSIS FUNGOIDES			
Cytogenetic band	CNA	Affected MF patients (%)	Affected Sz patients (%)
7q36	gain	59%	15%
7q21-7q22	gain	55%	20%
7q32-7q35	gain	55%	10%
7q11.2	gain	50%	15%
1p36.2	gain	45%	15%
1q31-1q32	gain	45%	0%
5q13	loss	45%	40%
7p22-7p21	gain	45%	20%
7p14-7p13	gain	45%	15%
1q21-1q22	gain	41%	5%
SÉZARY SYNDROME			
Cytogenetic band	CNA	Affected Sz patients (%)	Affected MF patients (%)
17q23	gain	85%	32%
17q22-17q23	gain	80%	32%
17q24-17q25	gain	80%	27%
8q24.1-8q24.2	gain	75%	23%
8q24.2-8q24.3	gain	75%	27%
8q22-8q23	gain	70%	18%
17p13	loss	70%	9%
17q25	gain	70%	32%
8q12-8q21.1	gain	65%	18%
8q11.2-8q12	gain	60%	18%

Identification of genes relevant in the pathobiology of MF through integrated genomic analysis

Chromosomal gains and losses can contribute to the development and progression of lymphoma by altering the expression levels of genes residing in loci with CNA. We sought to identify such biologically relevant genes in MF by evaluating the expression levels of genes located in MCRs with recurrent CNA. A schematic representation of the strategy used for identifying these genes is depicted in Figure 2. First we asked which genes, residing in any of the 24 MCRs affecting at least 35% of patients, showed increased expression associated with gain or decreased expression associated with loss. A total of 1504 annotated genes interrogated by the Affymetrix oligonucleotide arrays are located in the 24 MCRs. In tumor samples affected by gain of any of the 15 identified highly recurrent MCRs, significantly more genes residing in these chromosomal regions showed increased expression.

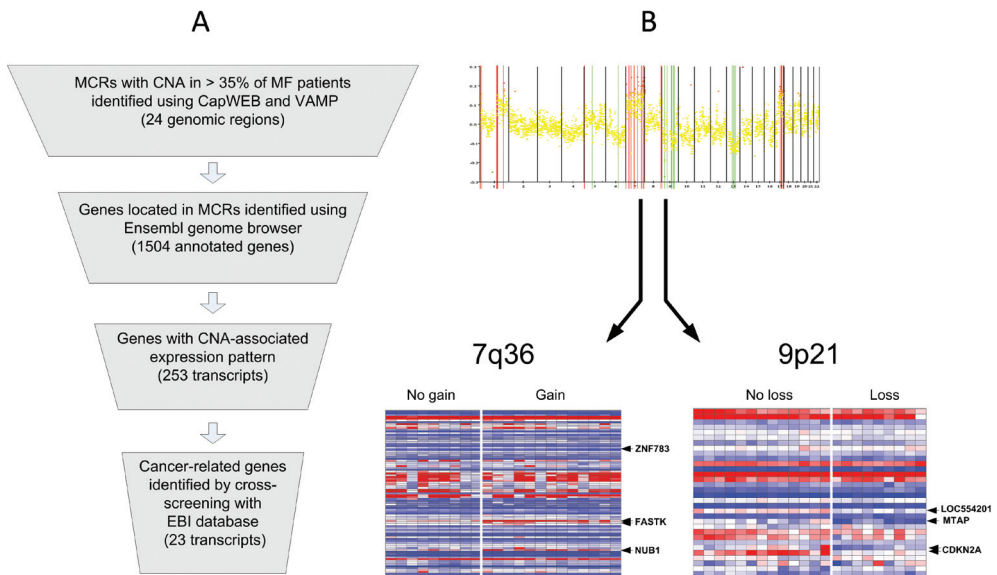


Figure 2 Integration of array-based CGH and gene expression data

(A) Stepwise approach to identification of genes potentially relevant in the development or progression of MF residing in loci with frequent CNA. Transcripts corresponding to genes localized in MCRs (see Figure 1b) were identified and extracted using the Ensembl genome browser and cross-referenced with microarray probes. Gene doses effects on expression levels were then statistically evaluated. Genes demonstrating significantly higher expression associated with gain or lower expression associated with loss are summarized in Table 3. (B) Visual illustration of the integration method applied for aCGH and gene expression data. For 2 exemplary MCRs, with gain on 7q36 and loss on 9p21, heatmaps of resident gene expression patterns were generated. Genes with a CNA-associated expression pattern are indicated with arrows.

In addition, in 8 of the 9 MCRs with loss there was a significant excess of genes displaying decreased expression in the tumor samples affected by loss of these MCRs according to the sign test (Supplementary Table S3). To examine the effect of gene dosage on mRNA abundance we tested whether gene expression correlated with CNA for each individual gene residing within these MCRs by comparing the gene expression levels in samples harboring chromosomal gain or loss to the samples not affected by CNA using Student's *t*-test. A total of 223 annotated genes showed increased expression associated with gain and 30 genes decreased expression associated with loss ($p < 0.05$). Genes demonstrating such CNA-associated expression pattern, i.e. significantly increased expression in samples with gain or decreased expression in samples with loss of a certain chromosomal region, are listed for each of the 24 MCRs in order of frequency of occurrence in Table 3. For each chromosomal region we then prioritized these genes for potential biological relevance by triangulating with genes listed as cancer-related in the EBI cancer gene database, indicated in bold in Table 3. The resulting list of candidate oncogenes and tumor suppressor genes includes *MDMX*, *MCL1* and *RB1*. In addition, the *CDKN2A* gene with an established role in MF progression is among this refined list of candidate genes.⁶ The 2 *CDKN2A* probesets emerging from integrated genomic analysis indicated in Figure 2b both target a region common to the p16 and p14 transcripts. The chromosomal region most frequently affected by gain is 7q36, amplified in 59% of MF patients. Only 3 of the 56 genes residing at the 7q36 locus demonstrate increased expression in the tumor samples with gain (*FASTK*, *NUB1* and *LOC791120*). The *FASTK* gene encodes FAS-activated serine/threonine kinase, an anti-apoptotic protein expressed in T cells.^{30,31}

Confirmation of gene expression data by quantitative real-time PCR and immunohistochemistry

To validate the results of microarray analysis, we selected several candidate oncogenes and tumor suppressor genes, located in MCRs affecting at least 35% of patients and predicted to show CNA-associated dysregulation (Table 3). Expression levels of these genes were analyzed using qPCR and compared between MF samples with and without CNA of the chromosomal region harboring these genes (Figure 3). Expression levels of the *FASTK*, *SKAP1* and *MCL1* genes, located in MCRs with gain on 7q36, 17q21 and 1q21-22 respectively, were analyzed. In addition, expression of the tumor suppressor genes *RB1* and *DLEU1*, located in a MCR on 13q14-13q31 lost in 36% of patients, was investigated. The *FASTK* gene and *SKAP1* gene, also known as *SKAP55*, were selected because of their essential role in T-cell apoptosis and T-cell activation, respectively.^{30,32} *MCL1*, *RB1* are reported to be cancer-related according to the EBI cancer gene database. The putative tumor suppressor gene *Deleted in Lymphocytic Leukemia 1 (DLEU1)* was selected for

confirmatory PCR analysis because it has also been found to be affected by promoter hypermethylation in MF.³³ The mean expression intensity of *FASTK* was significantly higher for patients with a corresponding gain of DNA content than for those without gain (fold difference 1.7, *t*-test $P=0.03$). Similarly, the *SKAP1* gene was thus confirmed to demonstrate a CNA-associated expression pattern (fold difference 2.6, $P=0.01$). Expression of the *MCL1* gene was higher in samples with gain, but the difference did not reach statistical significance. Expression of *RB1* and *DLEU1* was significantly diminished in patients demonstrating loss of the chromosomal region on 13q14 (fold difference -1.8 and -2.4; $P=0.02$ and 0.01 respectively). These results indicate that gene dosage influences transcript abundance of these tumor-related genes. The relatively large standard error apparent in the data in Figure 3 on the one hand reflects heterogeneity in expression levels within the group of samples, but may also suggest that gene expression levels are influenced by other factors such as multiple copy gain and promoter hypermethylation. In addition, protein expression of RB1 and SKAP1 was evaluated by immunohistochemical staining of tissue sections of 10 MF tumor samples. We found that RB1 was expressed by tumor cells of samples without loss of the locus harboring this gene. However, expression of RB1 was absent in the majority of tumor cells in 2 of the 5 tumor samples demonstrating loss of the locus harboring this gene, indicative of loss or epigenetic silencing of the other allele. Loss of RB1 protein expression in CTCL has been reported previously.^{34,35} SKAP1 showed strong cytoplasmic staining in lymphoid cells in all MF samples. Although tumor samples in which gain of the SKAP1 locus had been detected appeared to display slightly more intense staining, no significant difference in staining intensity between samples with and without gain of the locus harboring the gene could be discerned. Results of exemplary stainings are shown in Supplementary Figure S4.

Chromosomal alterations with prognostic significance

Next, we determined possible relationships between the occurrence of specific chromosomal alterations and the clinical behavior of these MF patients. For each of the 24 MCRs with CNA affecting at least 35% of patients, we compared the disease-specific survival rate in the group of patients harboring this CNA to survival in the group of patients not affected by the particular CNA. Patients whose tumor cells showed loss of 9p21 (Mb position 20351121-22479496), gain of 8q24.3 or gain of 1q21-1q22 had a statistically significantly lower survival rate (log-rank test, $P=0.011$, 0.013 and 0.031 respectively). Figure 4 shows survival curves of patients with and without these 3 CNAs with prognostic significance. These loci may contain genes that modify the biological behavior or treatment response of MF. Loss of the 9p21 locus, harboring the *CDKN2A* tumor suppressor gene, has been reported to predict more aggressive disease behavior in cutaneous B-cell lymphoma and nodal lymphomas previously.^{36,37}

Table 3 Results from integration of expression and aCGH results: candidate oncogenes and tumor suppressor in MF

Cytogenetic band	Copy number alteration	Clone position (Mb)		Affected patients (%)	Candidate genes
		start	stop		
7q36	gain	148089302	151558264	59	<u>FASTK</u> , NUB1, LOC791120
7q21-7q22	gain	97314794	102514284	55	AP1S1 , SMURF1, ZKSCAN1, C7orf38, CLDN15, ZNF789, RASA4, ZNF498, ZNF789, ARMC10, POLR2J2, ZNHIT1, ZCWPW1, MGC40499
7q32-7q35	gain	130270796	143852574	55	TRIM24, CNOT4, PTN, C7orf49, KIAA0738, LUC7L2
7q11.2	gain	71274704	76190020	50	GTF2IRD1 , ABHD11, NSUN5, NSUN5B, NSUN5C, ELN, WBSCR22, TRIM73
1p36.2	gain	11098993	12351219	45	MFN2
1q31-1q32	gain	198714422	205087972	45	MDM4 , NAV1 , RBBP5, IPO9, CSRP1, KIF21B, PPP1R15B, NUCKS1, TIMM17A, SNRPE, KIF14
5q13	loss	67677068	70179512	45	TAF9 , SERF1A, SERF1B, SMN1, TAF9, GUSBP1
7p14-7p13	gain	43272694	45048103	45	CAMK2B , POLR2J4
7p22-7p21	gain	1081263	7947777	45	WIPI2, LOC222967, FTSJ2, MICALL2
1q21-1q22	gain	146342686	156056126	41	<u>MCL1</u> , CLK2 , PRCC , ARHGEF11 , HDGF, GPATCH4, JTB, MSTO1, FLAD1, CRTC2, SMG5, ADAR, MRPL24, KRTCAP2, SETDB1, C1orf2, SF3B4, PRPF3, SEMA4A, MTX1, ISG20L2, SNAPAP, ENSA, PLEKHO1, ISG20L2, DAP3, GON4L, C1orf85, APOA1BP, C1orf43, RUSC1, UBAP2L, CDC42SE1, MAPBPIP, SCAMP3, C1orf77, PYGO2, PSMD4, GATAD2B, PEAR1, FDPS, VPS72, MRPL9, IQGAP3, DENND4B, TNFAIP8L2, UBQLN4, SLC39A1, TPM3, PRUNE
7p15-7p14	gain	24659178	37825117	41	TAX1BP1 , HOXA10 , CREB5 , HERPUD2, JAZF1, LOC441212, HNRPA2B1, C7orf41, LOC401320, KBTBD2
9p21	loss	20351121	22479496	41	CDKN2A , MTAP, LOC554202
17q21	gain	39091531	44639847	41	FMNL1 , NMT1, NPEPPS, <u>SKAP1</u> , DBF4B, LOC641522, KPNB1, NFE2L1, ARL17P1, GPATCH8, LRRC37A2, TMUB2, ARL17, CCDC43, MAPT, EFTUD2, OSBPL7, ACBD4

Cytogenetic band	Copy number alteration	Clone position (Mb)		Affected patients (%)	Candidate genes
		start	stop		
8q24.3	gain	142790550	telomere	36	HSF1, RECQL4, PLEC1 , PPP1R16A, NFK-BIL2, LRRC14, SCRIB, CPSF1, SIAHBP1, CPSF1, RPL8, GPAA1, MGC70857, GPR172A, ZNF7, GPR172A, C8orf33, FBXL6, BOP1, GPAA1, PYCRL, EXOSC4, C8orf30A, CYHR1, SHARPIN, ZNF707, JRK, CYC1, EEF1D, KIFC2, MAF1, COMMD5
13q14-13q31	loss	42301191	83766576	36	RB1, KLF12 , TPT1, LMO7, HUWE1, RBM26, UTP14C, FNDC3A, DNAJC15, RNASEH2B, NDFIP2, INTS6, RPL13A, PTMA, COG3, <u>DLEU1</u>
17q25	gain	69639765	telomere	36	CBX4 , RECQL5, HGS, SPHK1, MIF4GD, B3GNTL1, UBE2O, NT5C, LOC124512, FLJ21865, SAP30BP, NUP85, C17orf56, NPLOC4, ACTG1, RAB40B, TRIM65, C17orf70, H3F3B, MIF4GD, FLJ30594, KIAA0195, PRPSAP1, MXRA7, FLJ35220, EXOC7, MFSD11, WDR45L, RHBDLF2, TSEN54, TIMP2, TNRC6C
8q24.2	gain	132799581	137773461	32	ST3GAL1, PHF20L1, KIAA0143
9p13-9p11.1	loss	39990599	42614658	32	(no genes with CNA-associated expression)
9p21	loss	22579721	24877888	32	(no genes with CNA-associated expression)
9q21	loss	78213759	80495074	32	CEP78, VPS13A
9q21	loss	79930787	84622895	32	(no genes with CNA-associated expression)
9q21	loss	84783002	86180561	32	UBQLN1 , C9orf103, GKAP1, LOC389765
9q22-9q31	loss	101410218	105214273	32	TEX10, MRPL50, TXNDC4, RNF20, ZNF189
17q22-17q23	gain	48664511	59626448	32	SUPT4H1, DHX40 , PTRH2, AKAP1, FLJ44342, TLK2, RPS6KB1, TUBD1, HEATR6, C17orf71, INTS2, MRPS23, COIL, GPD1, METTL2A, DDX42, FTSJ3, LOC51136, ICAM2, MKS1, MSI2

Genes reported as cancer-related according to the EBI cancer gene database are shown in bold; the remaining genes are ordered according to statistical significance of differential expression. mRNA expression levels of genes underlined are determined by qPCR

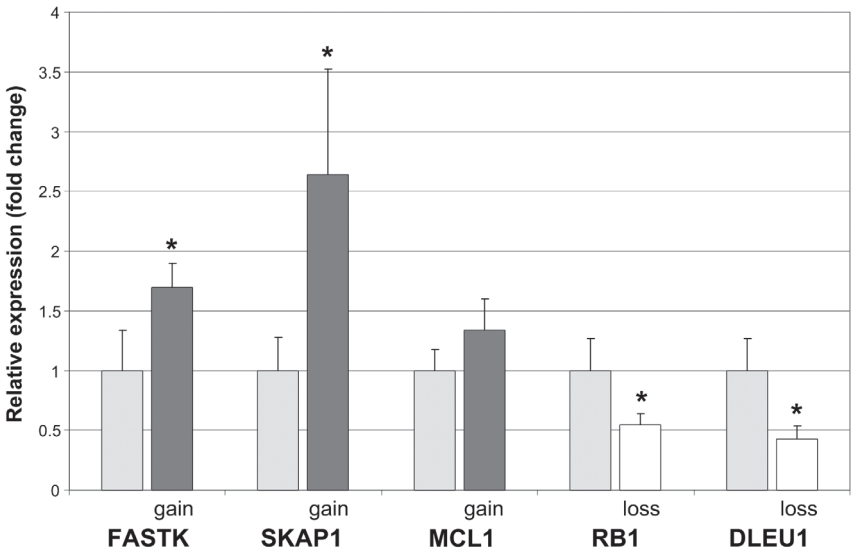


Figure 3 Relative mRNA expression in MF tumor samples as measured by qPCR. Data (mean $\pm$ S.E.M) of 3 independent qPCR experiments are depicted relative to the reference genes *RPS11* and *U1A*. Grey bars: qPCR results using cDNA synthesized from RNA isolated from samples with no CNAs. Black bars: qPCR results from samples with copy number gains. White bars: qPCR results from samples with copy number loss. Asterisks indicate statistically significant differential expression ($P<0.05$).

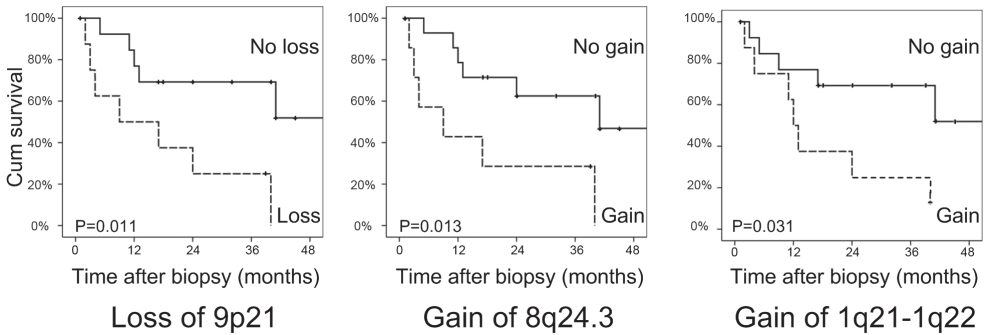


Figure 4 Chromosomal alterations associated with lower disease-specific survival rates in MF. Patients were divided based on the loss of a MCR on 9p21, or gain of MCRs on 8q24.3 or gain of 1q21-22. Actuarial survival rates were calculated from the date of biopsy using the Kaplan-Meier technique. The log-rank test was used to analyze differences between survival rates.

Discussion

Our study provides a genome-wide analysis of recurrent chromosomal alterations in a panel of 22 well-defined tumor-stage MF cases. A primary goal of this investigation was to compare the patterns of chromosomal alterations observed in MF with those recently identified in Sz. Both conditions are malignancies originating from activated, skin-homing, memory T cells with cerebriform nuclei. In 1975, based on the morphological and immunophenotypical similarities between MF and Sz and related lymphoid malignancies, Lutzner and colleagues proposed the encompassing term CTCL for this group of diseases.³⁸ In many subsequent studies on CTCL no distinction has been made between MF and Sz. In reviews and textbooks, Sz is often designated as a leukemic phase or variant of MF, suggesting that differences between both conditions are mainly a matter of stage of disease.^{13,16-20} However, Sz presents with erythroderma, lymph node and blood involvement and has a poor prognosis, whereas MF generally behaves as a low-grade lymphoma with limited, skin-confined disease for years or decades.^{1,2} There are also histopathological differences between both conditions. Whereas infiltration of the epidermal basal layers is the hallmark of early MF, in Sz the atypical cells are predominantly found around the dermal blood vessels, although a variable degree of epidermotropism may be present as well.³⁹ Consistent with its leukemic nature, involved lymph nodes in Sz are typically overrun by a monotonous infiltrate of Sézary cells, whereas dermatopathic lymphadenopathy as seen in early involvement by MF tumor cells is often absent.⁴⁰ MF and Sz have been reported to share several chromosomal alterations, analyzed using conventional CGH, such as loss of chromosomal regions on 1p, 10q and 17p.^{4,5} However, in line with our findings also differences in CNA patterns of MF and Sz, including a higher frequency of gain of 17q in Sz have been recognized.¹⁸ More recently, expression of *CDO1* and *DNM3*, genes specifically expressed in Sz, could not be demonstrated in MF.⁴¹

The mapping resolution of the array-based CGH method applied in this study allows a more detailed definition of chromosomal alterations than obtained by FISH and conventional CGH, used in previous studies of CTCL. The detailed genomic profiles of chromosomal imbalances of MF tumor cells displayed marked differences with those previously identified in Sz cells using identical methods. Numerical chromosomal alterations most frequently observed in MF include gain of 7q21-36, 1p36.2 and loss of 5q13 and 9p21, whereas Sz is characterized by gain of 17q22-25, 8q22-24 and loss of 17p13 and 10q25. Amplification of the locus containing the *MYC* gene on 8q24, observed in 75% of patients with Sz and associated with increased expression of this oncogenic transcription factor, was detected in only a minority of patients with MF.¹⁵ Notably, several aberrations commonly observed in MF are not or infrequently seen in Sz, arguing against

the notion that Sz represents an advanced stage of MF. Gain or loss of chromosomal regions may be associated with altered expression of resident oncogenes or tumor suppressor genes and thereby have a causative role in the development and progression of lymphoma. The pattern of chromosomal alterations, in particular highly recurrent focal gains and losses, is therefore often characteristic of a certain type of malignancy and can be informative of its pathogenesis. Although the chromosomal alterations in MF and Sz show heterogeneity within the group, the overall patterns clearly differ. This strongly suggests that the molecular pathogenesis of these CTCLs follows distinct pathways. By implication, patients with these two CTCL subtypes may respond differently to treatment regimens. In current clinical trials patients with MF and Sz are often included collectively as CTCL or MF/Sz.⁴² It is conceivable that the efficacy of experimental therapeutics, such as inhibitors of STAT or MYC transcription factors, would differ considerably between MF and Sz. Therefore patients with MF and Sz should be entered in clinical trials separately or results of such trials should at least be stratified according to CTCL type.

The molecular genetic alterations underlying the development and progression of MF are largely unresolved. The second goal of this study was to identify pathobiologically relevant genes in MF by evaluating the expression of genes residing in smallest overlapping chromosomal regions (MCRs) with highly recurrent CNA. A subset of the 253 genes that demonstrated CNA-associated dysregulated expression is known to be cancer-related, and several other genes have been reported to have essential roles in T-cell activation and proliferation. By integrating array-based genetic maps with gene expression signatures derived from the same MF tumor biopsy samples, we thus identified several oncogenes and tumor suppressor genes, including *RB1*, *CDKN2A*, *MCL1* and *MDMX* as targets of gain and loss in MF. Interestingly, the most frequently observed CNA, gain of a MCR on chromosome 7q36 affecting 59% of MF patients, was associated with increased expression of the *FASTK* gene. The protein encoded by this gene is a member of the serine/threonine protein kinase family and is normally expressed in human T cells.³¹ Although some earlier reports suggested that FASTK may be involved in the induction of Fas-induced apoptosis, most evidence indicates that this protein has anti-apoptotic properties.³⁰ FASTK attenuates apoptosis induced by UV-radiation and FAS ligation, in part by increasing expression of XIAP and cIAP1. Short interfering RNA(siRNA)-mediated interference with *FASTK* expression increases apoptosis in human cells.³⁰ Moreover, FASTK regulates splicing of several genes, including *FGFR2* and *FAS*.⁴³ Previously, our group has noted aberrant splicing of the *FAS* gene in MF.⁸ It is conceivable that *FASTK* dysregulation may be related to *FAS* splicing alterations in MF tumor cells, and this possible relationship may be a subject of further study. Consistent with previous reports, we found recurrent loss of 9p21 and 13q14 and diminished expression of the *CDKN2A*

and *RB1* tumor suppressor genes residing in these loci.^{6,34,35} In a subset of MF patients with loss of the *RB1* locus protein, expression of this essential cell cycle regulator was diminished. In a study by Zhang and colleagues, the RB1 protein was found to be functionally inactivated in a subset of patients with advanced MF through hyperphosphorylation.³⁵ In addition, the *DLEU1* gene is located on 13q14.3 and shows reduced expression. The promoter of *DLEU1* displays frequent hypermethylation in MF, suggesting that genetic and epigenetic mechanisms collectively act to silence this gene.³³ In addition to *RB1* and *DLEU1*, the 13q14 region lost in 36% of MF patients, also contains the *miR-15a* and *miR-16-1* gene cluster. These microRNA genes have tumor suppressive properties as their expression inhibits translation of the anti-apoptotic protein BCL2. Loss of 13q14.3 and concomitant reduced expression of these tumor suppressive microRNA genes, resulting in elevated protein levels of BCL2, is a frequent event in chronic lymphocytic leukemia.⁴⁴ Consistently, malignant T cells in MF skin lesions have been reported to demonstrate high expression of BCL2.⁴⁵

Finally, we attempted to evaluate the prognostic relevance of registered recurrent CNAs. Patients with MF who demonstrated loss of the MCR on 9p21, gain 8q24.3 or gain of 1q21-1q22 appeared to have significantly lower survival rates than patients whose tumor cells were not affected by these CNAs. The chromosomal region on 9p21 harbors the *CDKN2A* tumor suppressor gene, which showed reduced expression in patients with loss of this region. Loss of 9p21 and reduced expression of p16 encoded by *CDKN2A* have been found to predict an unfavorable prognosis in various haematopoietic malignancies.^{36,37} Consistent with clinical observations, inactivating mutation in *CDKN2A* promote tumorigenesis and resistance to chemotherapy in experimental lymphoma in murine model systems. As in experimental lymphoma, treatment resistance in MF patients whose tumor cells are affected by loss of *CDKN2A* may be explained by defects in the induction of apoptosis and senescence in response to therapy. The locus with prognostic significance on 8q24.3 contains 28 genes with gain-associated increased expression, including the *HSF1* gene. This heat shock response regulator has been found to be a determinant of chemotherapeutic efficacy in malignancy.⁴⁶ Gain of chromosome 8q was previously identified as a hallmark of progressive MF associated with shorter survival.^{47,48} Gain of the chromosomal region on 1q21-22 is associated with significantly higher expression of a number of genes including the *MCL1* gene. This anti-apoptotic gene was recently observed to be part of a gene cluster up-regulated in patients with advanced CTCL.⁴⁹ Protein levels of MCL1 have been demonstrated to be elevated in advanced skin lesions of patients with CTCL.⁵⁰ It is tempting to speculate that dysregulated expression of this gene influences the disease course of patients with MF, since MCL1 has been shown to modulate glucocorticoid resistance in lymphoid malignant cells.⁵¹

Interestingly, it was reported in that study that the mTOR-inhibitor rapamycin can modulate MCL1 activity and thereby restore glucocorticoid sensitivity, suggesting that addition of rapamycin to chemotherapy of patients with treatment-refractory MF, especially in case of 1q21-22 gain, could potentially enhance therapeutic efficacy. Whereas our study and those of others^{5,47,48} have focused on genomic alterations associated with an adverse prognosis, Shin and colleagues aimed to identify gene expression patterns marking patients with aggressive disease.⁴⁹ Apart from MCL1, no other candidate genes detected in their study as being associated with aggressive CTCL, were found to reside in the 3 loci with prognostic significance we identified. The observed associations of specific chromosomal alterations and gene expression patterns with prognosis require further investigation in independent prospective studies.

In conclusion, we have attempted to provide a comprehensive characterization of recurrent chromosomal alterations of MF, a thus far poorly understood malignancy. The application of array-based CGH has revealed important molecular distinctions between MF and Sz not previously appreciated. These findings may have consequences not only for our understanding of the pathogenesis of these CTCLs, but also clinically for the design of trials to evaluate the efficacy of novel treatments. The integration of high-resolution copy number and gene expression data has afforded relevant novel insights into molecular genetic alterations underlying MF. Over expression of *FASTK*, *MCL1*, *SKAP1* associated with chromosomal gain and reduced expression of *CDKN2A*, *RB1* and *DLEU1* related to loss are important candidate oncogenic events in MF. Elucidation of the biological role of the identified candidate oncogenes and tumor suppressor genes in the development and progression of MF should be the focus of further studies.

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3

A meta-analysis of gene expression data identifies a molecular signature characteristic for tumor-stage mycosis fungoides

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Abstract

Mycosis fungoides (MF) is the most common type of primary cutaneous T-cell lymphoma (CTCL). To identify a molecular signature characteristic of MF tumor stage, we used a bioinformatic approach involving meta-analysis of publically available gene expression datasets combined with previously generated gene expression data. Results for a selection of genes were further refined and validated by quantitative PCR and inclusion of additional controls. With this approach, we identified a profile specific for MF tumor stage consisting of 989 aberrantly expressed genes, the majority (718 genes) statistically significantly more expressed in MF compared to normal skin, inflamed skin and normal T cells. As expected, the signature contains genes reflecting the highly proliferative character of this T-cell malignancy, including altered expression of cell cycle and kinetochore regulators. We uncovered details of the immunophenotype suggesting that MF originates from IL-32 producing cells and identified previously unreported therapeutic targets and/or diagnostic markers, for example, *GTSF1* and *TRIP13*. Loss of expression of the NF- κ B inhibitor, *NFKBIZ*, may in part explain the enhanced activity of NF- κ B, which is a hallmark of MF and other CTCLs.

Introduction

Mycosis fungoides (MF) is the most common type of primary cutaneous T-cell lymphoma (CTCL), consisting of skin-homing CD45RO⁺ effector memory T cells. MF patients present with an evolution of patches, plaques and tumors. Stages are related to life expectancy; tumor-stage MF has an unfavorable prognosis with a 10-year survival of approximately 40%.^{1,2} Although for MF numerous genetic and genomic studies are described, ranging from investigating individual gene(mutation)s^{3,4} to genome-wide (array-based) analyses,⁵⁻⁸ the molecular (patho)biology of the disease is still poorly understood.

Reconstruction of (aberrant) gene expression patterns by comparing gene expression profiles from MF tumor biopsies with normal counterparts offers the possibility to identify pathobiologically relevant genes in MF tumor cells. However a genuine comparison of MF tumor cells with normal (skin-homing) T cells is difficult to achieve, because skin biopsies of MF contain tumor T cells, but also an admixed infiltrate of immune cells and resident cells (keratinocytes, fibroblasts, endothelial cells, etc). Previous gene expression studies on MF and other cutaneous lymphoma tried to circumvent this drawback by either comparing different types of lymphoma,^{9,10} different stages of the disease,¹¹ or analyzing copy number effect on mRNA expression.⁸ One previous study that directly compared MF with benign counterparts identified 27 genes implicated in tumorigenesis, but in this study the expression of only a limited number of genes was analyzed.¹²

In this study, we performed a meta-analysis on raw gene expression data available in public repositories selecting high quality datasets from normal T-cell subsets, skin, inflamed skin, and tumor-stage MF (T-MF), generated with commercially available Genechips. Subsequently these datasets were corrected for inaccurate Gene annotations.¹³ We took advantage of recent developments in bioinformatics and subjected the data sets to a robust statistical analysis comparing expression data of MF tumor samples with normal T-cell subsets and normal skin, as well as inflamed skin from experimentally induced allergic contact dermatitis simultaneously. Finally, we confirmed altered expression of selected genes by reverse transcriptase-coupled quantitative (RT-q)PCR in a series of controls including benign T-cell dermatoses and early-stage MF. Using this approach, we identified a gene expression pattern characteristic for MF tumor stage providing more insight in the pathogenesis of this lymphoma, a description of its (immuno)phenotype and the discovery of previously unreported putative diagnostic markers and therapeutic targets.

Material & Methods

In silico analysis

We designed a strategy to identify the molecular signature of MF tumor stage (the workflow is shown in Figure 1). All data analyses were performed in R using packages present in Bioconductor (www.bioconductor.org). The Gene expression omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and ArrayExpress (<http://www.ebi.ac.uk/microarray-as/ae/>) databases were screened for datasets, generated with Affymetrix U133 version 2 Gene chips (GPL570) in combination with keywords skin and/or (activated) T cells or T-cell lymphoma, allowing comparison with gene expression profiles of T-MF previously generated.⁸ In addition, gene expression data of two additional MF tumor biopsies from our lab (both processed in parallel with previous MF samples) and the CTCL cell lines Seax, HuT-78 and MyLa (all cultured under standard conditions) were included in the analysis.

The quality of all datasets were checked using a series of QC metrics recommended by Affymetrix (using the R-script described by Heber and Sick¹⁴) in order to confirm that arrays were hybridized correctly, that sample quality was acceptable and batches of datasets could reliable be compared in a meta analysis. Data sets in which more than 30% of the individual samples required exclusion solely based on RNA quality, were discarded entirely. Next, raw gene expression data from Affymetrix CEL files passing all QC controls were reannotated according to the Entrez genome annotation using CDF-files¹³ followed by GC-RMA (robust multiarray averaging) normalization. Samples passing QC which were included in the final normalization are summarized in Supplementary Table S1. For subsequent analysis, all T-cell expression data from healthy volunteers were grouped to create a reference of normal T cells. Gene expression profiles of skin biopsies were clustered and labeled according to the supplementary information given in GEO or ArrayExpress or accompanying papers^{15,16} (Table 1), resulting in groups labeled as normal skin or inflamed skin (Pedersen data) or normal skin, uninvolved skin psoriasis and lesional skin psoriasis (Yao data). Differentially expressed (DE) genes between groups and MF tumor stage were identified with LIMMA¹⁷ using a log 2 fold change ≥ 1 as threshold and were considered statistically significant at an adjusted *P*-value of < 0.01 (using Benjamini-Hochberg multiple testing correction). Subsequently, the “AND” operator¹⁸ was used to perform a comparison between the lists of DE genes from the individual comparisons to identify consistently up- or down-regulated transcripts in MF. This comparison resulting in a list of genes enriched or depleted in MF. Gene enrichment analyses of DE genes were performed with PPI spider, DAVID, Panther and Webgestalt.¹⁹⁻²³

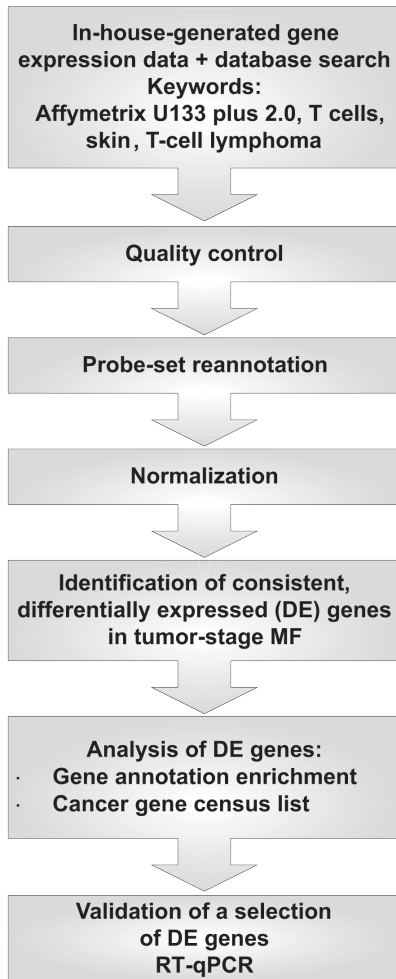


Figure 1a Workflow of the strategy used to identify the molecular signature of MF tumor stage

1) Data sets were obtained by searching for Affymetrix U133 vs 2 Gene chip (GPL570). 2) The quality of the data-sets was checked using a series of QC metrics. 3) Gene expression data were reannotated according to the Entrez genome annotation using CDF-files. 4) GC-RMA normalization was applied. 5) Differentially expressed (DE) genes were identified with LIMMA using a log 2 fold change ≥ 1 as a threshold, an adjusted P -value of < 0.01 and the "AND" operator to identify consistently up- or down-regulated genes. 6) Analysis of DE genes. 7) RT-qPCR was used to validate differential expression in additional samples.

Reverse transcription-coupled quantitative PCR (RT-qPCR)

Array results were validated by RT-qPCR on RNA isolated from fresh-frozen skin biopsies of 21 MF tumors, 6 chronic discoid lupus erythematosus (CDLE) lesions, 8 early-stage MF (IA/B) patients, 4 patients with chronic eczematous dermatitis (CED), normal skin ($n=6$), freshly isolated CD4⁺ T cells of 6 healthy donors as described previously²⁴ and MyLa cells. cDNA was synthesized of 1 μ g total RNA, after treatment with RQ1 DNase I (Promega, Madison, WI), using IScript reverse transcriptase (Bio-Rad, Veenendaal, the Netherlands), oligo(dT)₁₂₋₁₈ and random hexamer priming (Bio-Rad) in a final volume of 20 μ l. RT-qPCR was performed with the MyIQ Detection System and the SYBR Green Supremix (Bio-Rad).

Table 1 Samples included in the comparative analyses

group	subtype	reference	#samples (passing QC)
MF	MF tumor skin biopsy	van Doorn et al.	20
Normal T cells	CD3 ⁺ T cells	Mosig et al.	12
	CD4 ⁺ T cells	Piccaluga et al.	2
	CD8 ⁺ T cells	Piccaluga et al.	4
	Resting CD3 ⁺ T cells	Piccaluga et al.	5
	Activated CD3 ⁺ T cells	Piccaluga et al.	5
	CD4 ⁺ T cells	Ledieu et al.	7
	CD8 ⁺ T cells	Ledieu et al.	7
Skin	control skin biopsies + ACD with no clinical signs	Pedersen et al.	16
Inflamed skin	ACD skin biopsy (clinical signs)	Pedersen et al.	9
Skin	normal skin biopsy	Yao et al.	17
Psoriasis	uninvolved skin psoriasis biopsy	Yao et al.	27
Psoriasis	lesional skin psoriasis biopsy	Yao et al.	31

Abbreviations: ACD, allergic contact dermatitis; MF, mycosis fungoides; QC, quality control. Database numbers for these (and other) studies are provided in Supplementary Tabel S1 online.

Primers were intron-spanning designed with Primer3 (<http://frodo.wi.mit.edu/primer3/>), and tested *in silico* with Beacon Designer (Premier Biosoft, Palo Alto, CA). Before use all primers were tested experimentally, assessing the slope, Efficiency and R^2 value of dilution series using cDNA synthesized from human reference RNA (Stratagene Europe, Amsterdam, The Netherlands) as a template. Primer sequences are listed in Supplementary Table S5. The reference gene set was identified by testing several optimized primers on all samples included in the validation experiment and using GeNorm as earlier described.²⁵ The set of *ARF5*, *EIF2C4*, *TMEM87a* and *ERCC3* was identified as the best option and further used as the reference gene set. The cycle parameters for transcripts of interest and for the reference genes used for normalization were as followed: denaturing for 15 s at 97 °C; annealing and extension for 20 s at 60 °C, for 40 cycles. The nonparametric Mann-Whitney *U*-test (one tailed; Graphpad Prism 5, GraphPad Software Inc., La Jolla, CA) was used for statistical evaluation of the RT-qPCR results.

Methylation-specific Melting-Curve Analysis (MS-MCA) PCR

For the bisulfite conversion by the EZ DNA methylation kit (Zymo Research, Orange, CA, USA), 1 µg genomic DNA (isolated from T-MF skin biopsies⁸) was used. Primers (Supplementary Table S5) were designed to anneal to the bisulfite-sensitive, unmethylated strand and the bisulfite-resistant, methylated strand. Under these conditions, both

methyated and non-methyated DNA will be amplified. MS-MCA PCR reactions were performed as described earlier²⁶ with the MyIQ Detection System and the SYBR Green Supermix (Bio-Rad) in a 25 µl reaction volume. Cycle parameters for all analyzed CpG islands were as followed: denaturing at 96 °C for 30 s, annealing at temperatures varying from 65 °C to 58 °C depending on the primer set used for 40 s and extension at 72 °C for 40 s for eight cycles; followed by denaturing for 30 s, annealing at 60 °C for 40 s and extension at 72 °C for 40 s for 35 cycles. DNA melting curves were acquired directly after amplification by measuring the fluorescence of SYBR Green Supermix (Bio-Rad) during a linear temperature transition from 65 °C to 94.8 °C at 0.2 °C /s.

Sensitivity and specificity of the MS-MCA was validated for all primer sets using (mixtures of) methylated human DNA (Chemicon, Hampshire, UK) or unmethylated human semen DNA as input. Approval for these studies was obtained from the institutional review board of the Leiden University Medical Center. Informed consent was provided according to the Declaration of Helsinki Principles.

Results

***In silico* analysis: identification of differentially expressed genes**

Screening and filtering strategy for the identification of MF tumor-specific genes

To identify MF tumor genes we designed a strategy (the workflow is shown in Figure 1a) eventually generating a list of DE genes characteristic for MF tumor stage. First, gene expression data sets were obtained by searching for Affymetrix U133 plus 2.0 Gene chip (designated as GPL570 in the Gene Expression Omnibus, GEO) in combination with the keywords skin and/or (activated) T cells and/or T-cell lymphoma, revealing approximately 15 suitable hits. Downloaded CEL files and previously generated expression profiles (amongst which 22 T-MF⁸) were subjected to the Affymetrix QC metrics and individual samples passing the control (Table 1 and Supplementary Table S1) were included for further analyses. Next, we compared gene expression profiles of T-MF one-to-one with skin or (reference) T cells. Not surprisingly, comparing MF tumor samples with skin (or inflamed skin only) revealed a large number of typical T-cell (related) genes, while comparison between MF tumor samples and T cells produced a long list of DE genes indicative for skin (results not shown). To identify genes that are specifically enriched or depleted in T-MF ("unique genes") we decided to perform a comparison between the lists of DE genes, identified by the pairwise comparisons, using an "AND" operator¹⁸ and to look for genes that were consistently up- or down-regulated. To that end the T-MF

samples (n=20) were compared with the Pedersen data of skin (clinically normal skin, n=16) AND inflamed skin (n=9) AND all reference T cells (n=42). Because of the limited group sizes, we restricted the number of false positives by applying multiple testing correction (Benjamini-Hochberg) at stringent settings (false discovery rate < 0.01). This overlap analysis resulted in a list of 989 genes, of which 271 were down-regulated and 718 were over expressed in T-MF (The top lists are given in Table 2a and b, while a full searchable table is available in the supplementary information Table S2). This list was used for detailed analysis described below. To further validate our approach we also carried out a similar comparison for T-MF, T cells and the data set from Yao *et al.*,¹⁶ containing normal skin, uninvolved and lesional skin from psoriasis patients. This analysis showed that the majority of genes (but not all) found to be characteristic for T-MF are also consistently and differentially expressed compared to these datasets (596 up and 195 down; results are provided as supplementary information Table S3).

Systematic and integrative analysis of differentially expressed genes

Gene-annotation enrichment analysis

Next, Internet-based gene-annotation category enrichment analysis programs (PPI spider, DAVID, Panther, Webgestalt)¹⁹⁻²³ were applied to gain further insight into the (clinical) relevance of the MF DE genes and gave all similar results. E.g. using PPI spider classification (Supplementary Table S4a-c), we observed that genes associated with “mitosis”, “cell division”, “cell cycle”, “spindle” and “spindle organization” are

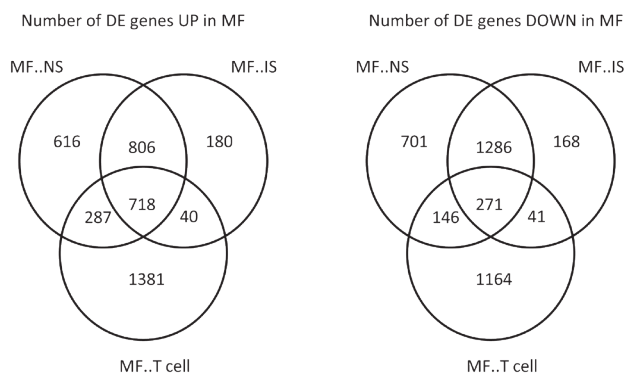


Figure 1b Venn diagram illustrating the number of genes that show altered expression in MF tumor stage compared to normal skin, (NS) inflamed skin (IS) and T cells. Values indicate the number of genes significantly up-regulated (left) or down-regulated (right). The intersecting regions represent number of genes that are common to the specific comparisons.

overrepresented in the list of DE MF genes, which is in line with the malignant (proliferative) phenotype of tumor-stage MF. The enrichment for genes involved in “immune response” is consistent with the T-cell origin of MF. Closer inspection of the “immunity genes” in Supplementary Table S4 revealed over expression of several interferon-responsive genes (*IFI27*, *IFI6*, *IFI30*, *IFI35*) interleukin/chemokines genes (*IL10*, *IL15*, *IL26*, *IL32*, *CCL18*, *CXCL9*, *-10*, *-11* and *-13*) and receptors (*IL13RA2*, *IL15RA*, *CCR1*, *-8* and *-10*), and down-regulation of *IL11RA*. Functional annotation analysis for down-regulated genes identified a single category: RNA processing (Supplementary Table S4c).

Comparison of molecular signatures

Comparison of the “T-MF signature” with the most recent Cancer Gene census list (updated Nov 15 2011; first described by Futreal *et al.*²⁷) identified 32 *bona fide* cancer genes (see Supplementary Table S5). We also determined whether differential expression of genes could be related to known genomic alterations. However, none of the “top 30” differentially expressed genes (Table 2a and b) resides within previously described minimal common regions of genetic imbalances.⁸

Promoter hypermethylation

We investigated the promoter sites of down-regulated genes for CpG islands using the UCSC genome browser (<http://genome.ucsc.edu/>) and found CpG islands present in the promoter start sites in more than 70% of the down-regulated genes, suggesting a possible role of promoter hypermethylation. Subsequently, bisulfite conversion followed by PCR and melt curve analysis was used to test CpG islands for methylation.^{26,28} Bisulfite-treated DNA isolated from tumor biopsies of MF patients (n=22) and CD4⁺ T-cell controls (n=6) were used as input and CpG islands in the promoter regions of *NFKBIZ*, *ATXN7* and *MXI1* were amplified. Primers were developed in such a way that both methylated and unmethylated sequences are amplified using the same bisulfite-treated DNA as PCR template. In none of the resulting melting curves analyses of PCR products (see Supplementary Figure S1) could methylation be demonstrated, although all contained PCR products representing unmethylated DNA. We therefore concluded that promoter hypermethylation does not play a role in down-regulation of these genes in MF tumor stage.

Verification of MF DE genes using qPCR

Differential expression for a selection of genes (since material is limited) was verified using RT-qPCR (results summarized in Figure 2; *P*-values from statistical evaluation (Mann-Whitney) can be found in Supplementary Table S6). We included mRNA isolated from skin biopsies of patients with chronic discoid lupus erythematosus (CDLE), early-stage

MF (IA/B), chronic eczematous dermatitis, normal skin and freshly isolated CD4⁺ T cells of healthy donors as additional samples. Instead of solely confirming the highest differences, we focused on genes that represent different classes, which might give further insights into the disease and/or previously unreported putative targets for diagnosis/therapy. We noticed that the array comparison indicated dysregulation of multiple genes involved in the miRNA biogenesis/machinery and aberrant expression of proteins involved in miRNA processing has been observed in T-cell lymphoma including Sézary syndrome.²⁹ We therefore included also *RNASEN*, *DICER*, *EIF2C1*, *EIF2C2*, *EIF2C3* and *EIF2C4* in the RT-qPCR validation. GeNorm analysis²⁵ revealed that *EIF2C4* was stably expressed in all samples and therefore in addition to *ARF5*, *TMEM87a* and *ERCC3* (See Materials and Methods) used as a reference gene. Initial experiments could not detect any significant differences in *DICER* and *EIF2C3* expression between samples and controls; therefore, these genes were excluded from subsequent analysis. With RT-qPCR down-regulation of *ATXN7*, *ZBTB20*, *NFKBIZ*, in T-MF in comparison to CD4⁺ T cells and normal skin could be affirmed. *ZBTB20* was also significantly less expressed in T-MF compared to CLE and early-stage MF, whereas *NFKBIZ* is lower in T-MF compared to all controls. Expression of *ATXN7*, however, was higher in T-MF versus CED and early-stage MF.

We observed that the expression of *RNASEN* and *EIF2C1* in T-MF is not different from control CD4⁺ T cells, but *RNASEN* is higher in T-MF compared to early-stage MF and CED, whereas that of *EIF2C1* is lower in T-MF compared to CLE. *EIF2C2* expression in T-MF was lower in CD4⁺ T cells and CED, but not different from early-stage MF, CED and normal skin. With RT-qPCR the over expression of *CXCL13*, *TRIP13*, *GTSF1* and *IL32*, in T-MF compared to freshly isolated CD4⁺ T cells and normal skin was confirmed (Figure 2). *CXCL13*, a marker for follicular helper T cells, showed variable expression among all biopsies but is over expressed in T-MF versus early-stage MF. *TRIP13*, a gene encoding a key protein for chromosome development, is highly up-regulated in T-MF versus control biopsies, whereas *IL32*, a gene belonging to the immune cluster, and the gene encoding the gametocyte specific protein, *GTSF1* (gametocyte specific factor 1), are nearly exclusively expressed in T-MF patients.

Figure 2 Relative mRNA expression levels for a selection of genes in normal skin (NS), CD4⁺ T cells, cutaneous discoid lupus erythematosus (CDLE), chronic eczematous dermatitis (CED), early-stage MF (MF, IA/B) MF tumor (MF, T) samples and the cell line MyLa. The mRNA expression levels were measured by RT-qPCR and calculated relative to *ARF5*, *EIF2C4*, *TMEM87a* and *ERCC3*, used as a reference gene set and depicted for individual samples as dots. The median and Interquartile range for each sample and gene under study are given. Summary of statistical evaluation (Mann Whitney *U*-test) denotes relative expression of gene in MF tumor versus sample group. *P*-values: * = <0.05, ** = < 0.01, *** = <0.001, full data are provided as a Supplementary Table (S6). NC = No change, NA = Mann-Whitney not applicable since gene is not expressed in this group resulting in *ex aequo* values.

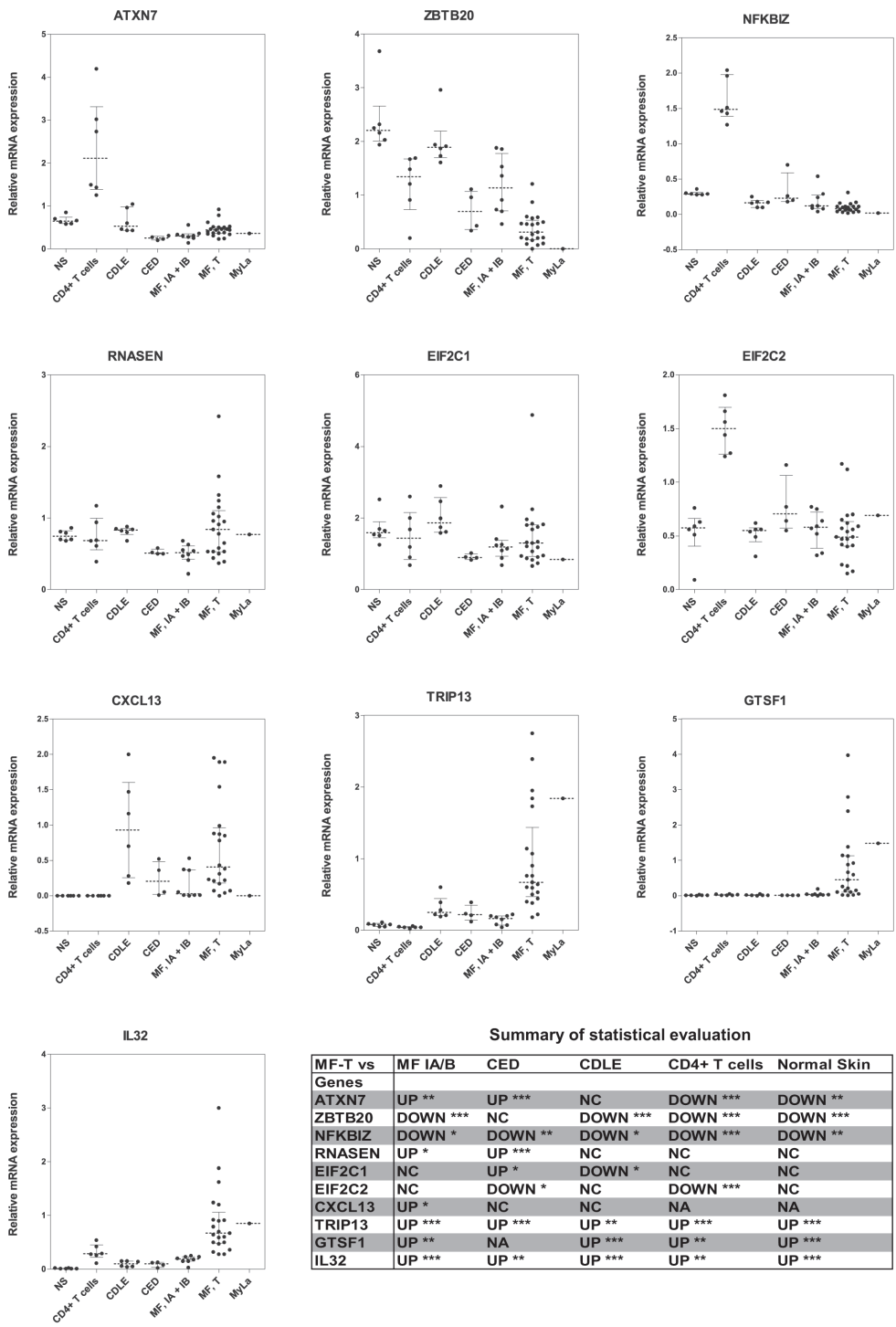


Table 2 Top lists (30) of genes differentially expressed in MF versus NS and IS and T cells: ranked on (a) log fold change MF versus IS (in bold) and (b) log fold change MF versus T cells (in bold)

A									
Down in MF		Log ² Fold change			Up in MF		Log ² Fold change		
HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cells	HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cells
TAOK1	57551	-5,30	-5,20	-4,26	ADAMDEC1	27299	6,93	7,20	6,72
PSAPL1	768239	-4,56	-4,77	-1,00	IL32	9235	7,97	7,12	3,20
NR1D2	9975	-5,31	-4,55	-5,46	GTSF1	121355	6,64	6,65	4,98
ZBTB20	26137	-4,72	-4,25	-3,66	CXCL13	10563	6,44	6,50	4,58
ATXN7	6314	-3,24	-4,13	-5,45	RRM2	6241	6,92	6,21	4,95
UGCG	7357	-3,70	-3,82	-3,28	PTPRCAP	5790	6,86	6,04	1,09
EPHA4	2043	-3,68	-3,68	-2,66	CXCL9	4283	5,83	5,81	8,22
KIAA0754	643314	-2,99	-3,65	-3,02	SPP1	6696	5,81	5,69	6,22
SLC16A7	9194	-3,26	-3,61	-3,07	PTPN7	5778	6,61	5,43	1,15
MAST4	375449	-2,98	-3,59	-1,83	DLGAP5	9787	4,72	5,30	4,95
ABCA5	23461	-4,05	-3,50	-3,06	UBD	10537	5,77	5,22	7,99
ZBTB16	7704	-3,64	-3,47	-1,95	CKS2	1164	4,08	5,15	3,33
NKTR	4820	-3,26	-3,46	-3,86	CCL18	6362	7,36	5,04	8,57
NFKBIZ	64332	-2,75	-3,42	-4,07	MMP1	4312	5,79	4,81	5,74
RPS27P19	100129905	-2,62	-3,39	-5,51	CEP55	55165	4,73	4,77	4,52
DICER1	23405	-3,42	-3,36	-3,25	TMEM163	81615	4,68	4,68	4,17
ZBTB43	23099	-2,77	-3,26	-2,70	UBE2C	11065	4,96	4,66	5,79
MALAT1	378938	-2,48	-3,26	-2,75	IDO1	3620	4,17	4,57	3,99
IL6ST	3572	-3,18	-3,22	-2,64	HMMR	3161	4,17	4,55	4,27
ATP7A	538	-3,81	-3,17	-2,63	MAD2L1	4085	4,21	4,54	2,53
EIF2C2	27161	-2,83	-3,16	-3,82	PLA2G2D	26279	4,54	4,54	4,50
NSUN6	221078	-2,83	-3,12	-3,53	IQCG	84223	4,74	4,45	4,44
PLEKHA1	59338	-3,37	-3,11	-2,10	AURKB	9212	4,49	4,41	4,36
EIF2C3	192669	-2,78	-3,03	-2,74	TRIP13	9319	4,84	4,39	4,75
TCF7L2	6934	-3,93	-2,94	-1,35	ASF1B	55723	4,58	4,38	4,16
GSK3B	2932	-2,36	-2,92	-1,61	CDC20	991	4,56	4,38	5,20
DKFZP586I1420	222161	-2,57	-2,92	-2,71	CCNA2	890	4,50	4,37	4,34
PIK3R1	5295	-3,27	-2,91	-3,46	CXCL10	3627	4,95	4,31	6,79
C2orf40	84417	-3,79	-2,91	-1,34	CENPA	1058	3,97	4,30	4,22
LOC654340	654340	-2,70	-2,89	-2,89	APOBEC3B	9582	3,87	4,27	3,59

Abbreviations: HGNC, HUGO Gene Nomenclature Committee; IS, inflamed skin; MF, mycosis fungoides; NS, normal skin. Left, downregulated genes; right, upregulated genes.

B

Down in MF		Log ² Fold change		
HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cells
FGFBP2	83888	-3,62	-2,45	-5,93
RPS27P19	100129905	-2,62	-3,39	-5,51
NR1D2	9975	-5,31	-4,55	-5,46
ATXN7	6314	-3,24	-4,13	-5,45
TAOK1	57551	-5,30	-5,20	-4,26
NFKBIZ	64332	-2,75	-3,42	-4,07
GPRASP1	9737	-2,87	-1,76	-4,06
LOC100132279	100132279	-1,61	-1,50	-4,00
NKTR	4820	-3,26	-3,46	-3,86
EIF2C2	27161	-2,83	-3,16	-3,82
ZNF331	55422	-1,38	-1,36	-3,82
UHMK1	127933	-2,32	-2,42	-3,76
ZBTB20	26137	-4,72	-4,25	-3,66
RASA2	5922	-1,28	-1,50	-3,61
BEX2	84707	-1,70	-1,07	-3,57
NSUN6	221078	-2,83	-3,12	-3,53
ZNF44	51710	-1,78	-1,99	-3,52
PIK3R1	5295	-3,27	-2,91	-3,46
NLRP1	22861	-1,19	-1,53	-3,45
C2orf82	389084	-1,82	-1,32	-3,44
ADRB2	154	-2,84	-2,50	-3,36
NR3C2	4306	-3,65	-2,61	-3,35
UGCG	7357	-3,70	-3,82	-3,28
FLJ10213	55096	-1,80	-2,37	-3,27
DICER1	23405	-3,42	-3,36	-3,25
C5orf41	153222	-2,24	-1,37	-3,22
LOC731484	731484	-1,44	-1,21	-3,20
ZCCHC2	54877	-1,96	-2,23	-3,13
LCOR	84458	-1,97	-1,91	-3,11
SLC16A7	9194	-3,26	-3,61	-3,07

Up in MF		Log ² Fold change		
HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cells
CCL18	6362	7,36	5,04	8,57
KRT6C	286887	3,92	4,19	8,46
IFI27	3429	1,66	1,19	8,28
CXCL9	4283	5,83	5,81	8,22
UBD	10537	5,77	5,22	7,99
C1QC	714	2,70	2,61	7,59
C1QA	712	2,54	2,91	7,54
C1QB	713	3,15	3,41	7,17
CXCL10	3627	4,95	4,31	6,79
ADAMDEC1	27299	6,93	7,20	6,72
APOE	348	1,01	1,00	6,64
VCAM1	7412	2,88	2,40	6,23
SPP1	6696	5,81	5,69	6,22
UBE2C	11065	4,96	4,66	5,79
TMEM176B	28959	2,62	2,94	5,77
MMP1	4312	5,79	4,81	5,74
CTS2	1522	4,59	4,18	5,22
CDC20	991	4,56	4,38	5,20
MMP9	4318	5,29	3,74	5,11
GTSF1	121355	6,64	6,65	4,98
CDC2	983	3,88	4,08	4,95
DLGAP5	9787	4,72	5,30	4,95
RRM2	6241	6,92	6,21	4,95
TOP2A	7153	3,61	3,94	4,84
TYMP	1890	4,84	3,01	4,79
TRIP13	9319	4,84	4,39	4,75
MS4A4A	51338	1,60	2,37	4,67
BUB1B	701	3,68	3,96	4,61
TYMS	7298	4,48	3,98	4,61
SLAMF8	56833	3,78	3,12	4,60

3

Discussion

The purpose of this study was to distill the gene expression profile of tumor-stage mycosis fungoides, aiming to gain more insight in the pathogenesis and the molecular basis of this disease. We performed a meta-analysis using high quality datasets generated with commercially available Genechips, a robust statistical analysis, and compared/studied expression data of T-MF with normal T-cell (subset)s, normal skin and inflamed skin (from experimentally induced allergic contact dermatitis or psoriasis). With this approach, we identified 989 genes significantly differentially expressed in T-MF, the majority of which (718) are higher expressed and 271 genes are lower expressed in T-MF. After submitting the extracted gene lists to Internet-based gene set enrichment tools, various classes of genes could be distinguished. The most apparent classes contained genes which can be considered as “usual suspects”, being hallmarks of proliferating cells such as genes involved in mitosis, cell division, cell cycle, including kinetochore formation, and DNA replication. From these clusters several genes and corresponding proteins were previously described in MF e.g. the over expression of MCM7 protein, a member of the minichromosome maintenance complex, in T-MF.³⁰ Our results, however, suggest that not only MCM7, but also MCM2-6, ORCL1, CDC6 and CDC7 belonging to the MCM7 complex are up-regulated. These results are in line with the notion that (collective) up-regulation of kinetochore and proliferation genes can lead to aberrant chromosome separation, hence contribute to genomic instability in tumors including lymphoma.³¹ On the basis of our results, in particular the high expression of UBE2C, one of the key regulators of cell cycle completion and marker of grade of malignancy in lymphoma,³² might play a central role in chromosomal instability as observed in MF.^{7,8} In analogy, the over expression of *TRIP13* in T-MF (confirmed by RT-qPCR) is of interest. *TRIP13* has a prominent role in chromosome recombination and chromosome structure development and mRNA over expression was recently correlated with prostate cancer progression.³³ We noticed that a large proportion of down-regulated genes in T-MF contained CpG islands in their promoter region. As DNA methylation of tumor suppressor genes has been found in MF,³⁴ we screened several of these genes for DNA promoter hypermethylation. For none of the genes tested however, promoter DNA hypermethylation could be confirmed, indicating that other mechanisms are responsible for down-regulation of these genes (e.g. aberrant expressed transcription factors or miRNA induced mRNA degradation).³⁵ Among the down-regulated genes, in comparison with healthy CD4⁺ T cells and benign dermatoses, we also detected (and confirmed by RT-qPCR) decreased expression of Argonaute 2 (*EIF2C2*), a protein belonging to the RISC complex and an essential component of the miRNA machinery.³⁶ As aberrant expression of other proteins

involved in miRNA processing has been observed in T-cell lymphoma including Sézary syndrome,²⁹ we also determined expression of Argonaute 1, 3 and 4 (encoded by *EIF2C1*, *EIF2C3* and *EIF2C4*), *DICER* and *Drosha* (*RNASEN*) using RT-qPCR. We could only demonstrate up-regulation of *RNASEN* in MF tumor stage compared to early-stage MF and down-regulation of *EIF2C1* in T-MF in comparison with CDLE. When comparing the T-MF “signature” with signatures for CTCL^{11,37} we identified 9 genes in the so-called “cluster 1” that are shared with our study: *IL26*, *PTPN7*, *TNFSF14*, *TNFSF4*, *CCR8*, *FUT7*, *CXCL13*, *LILRB4* and *ST8SIA4* (all up-regulated in cluster 1). Comparison of the T-MF DE genes with the Cancer Gene census database²⁷ revealed differential expression of 32 *bona fide* cancer genes in T-MF.

Immunophenotype

Our results show up-regulation of both interferon responsive genes (e.g. STAT1³⁸) and several interleukin/chemokines genes previously demonstrated to be up-regulated in MF tumor cells (*IL10*³⁹, *IL15*⁴⁰) or surrounding cells (*CXCL9*, *10* in keratinocytes⁴¹; *CCL18* in macrophages⁴²). Our analysis could not confirm elevated expression of *CCL17*.⁴³ With regard to the cytokine receptors, high expression of *IL15RA* is in line with the reported sensitivity of MF cells for *IL15*,⁴⁴ whereas expression of *IL13RA2* is not yet described for (cutaneous) lymphoma. We noticed increased expression of the chemokine receptors *CCR1*, *CCR8* and the skin-homing receptor *CCR10*. A role for CCR8 in localization of cutaneous memory T cells to the skin was proposed earlier⁴⁵ though no data on CCR8 (protein) expression in MF are not available yet. In contrast to FACS-based data of Campbell *et al.*,⁴⁶ and Clark *et al.*,⁴⁷ our gene expression data do not show up-regulation of CCR4 in T-MF. This might be explained by a high variable expression in either group (T-MF or controls) and consequently do not reach statistical significance. The gene expression data provide some evidence for the suggestion that MF is derived from Th17 cells⁴⁸: increased IL-26 mRNA levels though increased expression of IL-17 is not detected, whereas genesis from Th22 (no increase in IL-22), Treg (no FoxP3 over expression) or T follicular helper cells (no up-regulation of ICOS, or PD1) is unlikely. Instead, we observed a large degree of heterogeneity in expression of another putative T-follicular helper marker, *CXCL13*, as well as increased expression of programmed cell death 1 ligand 2 (*PD1L2*).

We did find high and consistent expression of IL-32 mRNA in all patients which was confirmed by RT-qPCR data showing expression of IL-32 in T-MF and MyLa, but not in early-stage MF, benign controls and normal skin. Although these findings suggest that MF might originate from “Th32” cells, it remains to be proven that the tumor T cell is the source of IL-32 in particular since recent studies described IL-32 production by fibroblasts (in rheumatoid synovium,⁴⁹ keratinocytes⁵⁰ and mast cells.⁵¹

Therapeutic targets and diagnostic markers

Our data are in full agreement with the previously described aberrant expression of B-Lymphoid kinase (*BLK*) in MF, which enhances proliferation induced by constitutive activation of NF- κ B⁵² and the described over expression of Pololike kinase 1 (*PLK1*).⁵³ Here we also demonstrate that *NFKBIZ*, a gene encoding a NF- κ B signaling inhibitor, is down-regulated in MF (supported by RT-qPCR data) which might be an explanation for enhanced NF- κ B activity, a hallmark of MF.⁵⁴ In this respect, targeting of RMM2 (among the DE genes up-regulated in T-MF), which induces NF- κ B -dependent MMP9 activation (up-regulated in T-MF and in agreement with published protein data⁵⁵) and thereby enhances cellular invasiveness⁵⁶ warrants further studies; several potent inhibitors for RRM2 protein were recently described which leads to growth suppression of tumors.⁵⁷ *CD74* over expression, could be targeted by milatuzumab, a humanized antibody currently tested on lymphoma and multiple myeloma patients in phase I trials.⁵⁸ Neither *CD52* nor *NOTCH-1* or-3 over expression could be reproduced in our analysis. *CD52* is a target of alemtuzumab (also known as Campath), and although previously described as being up-regulated on the mRNA level in CTCL⁵⁹ its use in the treatment of mycosis fungoides and Sézary syndrome is with varying results.⁶⁰ *NOTCH-1* is over expressed on the protein level in advanced MF,⁶¹ but data on mRNA expression are solely obtained from CTCL cell lines. Over expression of *TOP2A*, also identified in nodal peripheral T-cell lymphoma,⁶² might serve as a target of anthracyclines, such as doxorubicin and etoposide. We observed that indoleamine 2,3-dioxygenase 2, *IDO2*, playing a role in immunomodulation and tumor escape is over expressed in T- MF. A recent study demonstrated that the small molecule inhibitor INCB024360 is able to inhibit IDO2 protein and can act as an effective immunotherapeutic agent.⁶³

Finally, we identified a limited number of genes for which expression appears to be restricted to MF tumor stage and which might also serve as diagnostic (bio)markers. Amongst these is *GTSF1* (gametocyte specific factor 1). Expression thus far is only described for gametocytes⁶⁴ and according to our mRNA expression analysis (RT-qPCR) is limited to MF tumor samples. As male *GTSF1* knockout mice are sterile owing to massive apoptotic death of their germ cells, aberrant (over)expression of *GTSF1* might play a role in apoptosis resistance in MF.

In summary, we determined a molecular signature characteristic for mycosis fungoides tumor stage offering more insight in the pathogenesis of this disease. Moreover we uncovered more details of its immunophenotype: over expression of interferon-responsive genes (*IFI27*, *IFI6*, *IFI30*, *IFI35*) interleukin/chemokine genes (*IL10*, *IL15*, *IL26*, *IL32*, *CCL18*, *CXCL9*, *10*, *11* and *13*) and receptors (*IL13RA2*, *IL15RA*, *CCR1*, *8* and *10*) and down-regulation of *IL11RA*. Finally, our data suggests previously unreported therapeutic

targets and/or diagnostic markers: *IDO2*, *RRM2*, *CD74*, *TOP2A*, *GTSF1*, *TRIP13*, and *NFKBIZ*, which warrant further research.

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MicroRNA expression in Sézary syndrome: Identification, Function and Diagnostic potential

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Abstract

MicroRNAs are commonly aberrantly expressed in many cancers. Very little is known of their role in T-cell lymphoma, however. We therefore elucidated the complete miRNome of purified T cells from 21 patients diagnosed with Sézary syndrome (SzS), a rare aggressive primary cutaneous T-cell (CD4⁺) lymphoma. Unsupervised cluster analysis of microarray data revealed that the miRNA expression profile was distinct from CD4⁺ T-cell controls and B-cell lymphomas. The majority (104 of 114) of SzS-associated miRNAs ($P < 0.05$) were down-regulated and their expression pattern was largely consistent with previously reported genomic copy number abnormalities and were found to be highly enriched ($P < 0.001$) for aberrantly expressed target genes. Levels of *miR-223* distinguished SzS samples ($n = 32$) from healthy controls ($n = 19$) and patients with mycosis fungoides ($n = 11$) in >90% of samples. Furthermore, we demonstrate that the down-regulation of intronically encoded *miR-342* plays a role in the pathogenesis of SzS by inhibiting apoptosis and describe a novel mechanism of regulation for this miRNA via binding of *miR-199a** to its host gene. We also provide the first *in vivo* evidence for the down-regulation of members of the *miR-17-92* cluster in malignancy and demonstrate that ectopic miR-17-5p expression increases apoptosis and decreases cell proliferation in SzS cells.

Introduction

Sézary syndrome (SzS) is a rare aggressive form of primary cutaneous T-cell lymphoma (CTCL) characterized by erythroderma, generalized lymphadenopathy, and the presence of neoplastic cerebriform nucleated CD4⁺ T cells (Sézary cells) in peripheral blood.¹ Patients with SzS typically have a high leukemic burden and a poor prognostic outcome with an estimated 5-year survival of only 24%.¹ The molecular pathogenesis of this devastating disease, however, remains poorly understood.

There is emerging evidence that miRNAs are involved in the pathogenesis of many cancers including B-cell lymphomas.² There is, however, very little published data to date on the involvement of miRNAs in human T-cell lymphomas. Therefore, we undertook a comprehensive study to elucidate the miRNome of tumor cells from 21 SzS patients and normal CD4⁺ T cells using microarrays containing probes against 655 human miRNAs (miRBase 10.1).

Materials and Methods

Patients samples

Peripheral blood were obtained from 21 patients attending either the Skin Tumour Unit, St John's Institute of Dermatology, St Thomas' Hospital, London, UK (patients SzS1-SzS17) or the Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands (patients SzS18-SzS21). All patients had a T-cell clone detected in the peripheral blood as determined by T-cell receptor (TCR) gene rearrangement studies and fulfilled the World Health Organization-European Organization for the Research and Treatment of Cancer (WHO-EORTC) diagnostic criteria for Sézary Syndrome.³ Individual patient characteristics are shown in Supplementary Table S1. With the exception of patients SzS18, SzS19 and SzS21 which were Ficoll-purified PMBCs, CD4⁺ cells were purified from SzS patient peripheral blood using the RosetteSep™ CD4⁺ T-cell enrichment kit (Stem Cell Technologies, London, UK). Immunomagnetic separation (Miltenyi Biotec, Bisley, UK) was used to purify CD4⁺ T cells/ CD3⁺ T cells or CD19⁺ B cells from peripheral blood of healthy control donors as indicated in Figures 1 through 3.

A further cohort of SzS patient samples ($n = 15$), samples of patients with mycosis fungoides (MF) with no peripheral blood involvement ($n = 11$), and healthy donor controls ($n = 12$) were used as a validation set for testing the diagnostic ability of miRNA levels by qRT-PCR. CD4⁺ T cells from these additional patients were also purified by RosetteSep™ CD4⁺ T-cell enrichment kit; patient characteristics are given in Supplementary Table S2.

All patients gave informed consent to be included in this study in accordance with the Declaration of Helsinki 1975 as revised in 2005. The samples from patients SzS1 to SzS17, SzS22 to SzS36 and MF1 to MF11 were obtained from an ethically approved research tissue bank (St Mary National Research Ethics Committee: 07/H10712/106).

RNA purification and microarray analysis

MicroRNA (and total RNA) was isolated from samples SzS1 to SzS17 using Trizol (Invitrogen, Paisley, UK) and RNeasy columns as described by the manufacturer (Qiagen, Crawley, UK), whereas only miRNA was purified from samples SzS18 to SzS21 using the mirVana kit (Ambion, Warrington, UK). MicroRNA (approximately 500 ng) were labeled and hybridized to μ RNA microarrays as previously described⁴ using tonsillar material (pooled from 12 healthy individuals) as a common reference in a dye-balanced design. The arrays contained 655 human probes (miRBase v.10.1). Probe details can be found at MicroRNA world.⁵

Image analysis was carried out with BlueFuse v3 software (BlueGnome, Cambridge, UK). Raw image data were global median-normalized within arrays and normalized between arrays using the LIMMA package.⁶ The normalized log ratios (average of 4 replicates per probe) were used for subsequent analysis in Genespring 7.2 (Agilent Technologies, CA, US). MicroRNAs were filtered prior to ANOVA analysis to remove those that had a median intensity less than 1.5x background (200 fluorescence units). ANOVA *P*-values were adjusted using the Benjamini-Hochberg correction method. Differentially expressed genes were tested for their ability to predict sample class using the leave-one-out cross-validation support vector machine (SVM) function in Genespring. All microarray data are available in the GEO public database under accession number GSE21697.⁷

Quantitative RT-PCR (qRT-PCR)

Due to insufficient material, only patient samples which had been CD4⁺ purified (i.e. patients SzS1 to SzS17) were used for qRT-PCR and subsequent analysis. CD4⁺ (*n* = 7) and CD3⁺ (*n* = 6) T cells purified from a total of 13 healthy individuals were used as controls. MicroRNA and gene-expression qRT-PCR was carried out using Taqman probes as described by the manufacturer (Applied Biosystems, Warrington, UK) using 20 ng of miRNA or total RNA per reaction in a Roche LightCycler 480 machine. Triplicate samples were used throughout. Levels of β -2-microglobulin (*B2M*) and *U6* were used as control genes for gene expression and miRNA expression assays respectively. The mean *C_t* value of each triplicate was used for analysis, by the ΔC_t method (ΔC_t = mean *C_t* of control – mean *C_t* of gene of interest). Expression levels were compared using Mann-Whitney independent *t*-test (Graphpad Prism v.4.0, La Jolla, CA).

To assess the ability of miRNA expression levels to discriminate between SzS and control samples we used the k-fold cross-validation algorithm based on logistic regression to calculate receiver operator curve (ROC) statistics for these data. Analysis was carried out by 2-class logistic regression modeling with a ridge estimator using the Logistic function in WEKA version 3.6.1 in the 10-fold cross validation test mode.⁸

Methylation specific-PCR (MS-PCR)

Genomic DNA was bi-sulphite treated using the EpiTect kit from Qiagen. *EVL* methylation status was measured using a CpG Wiz kit from Millipore (Watford, UK) and *EVL*-specific primers as previously described.⁹ Supplied universally methylated and unmethylated control DNA were used as positive and negative controls. Colorectal cancer cell lines RKO and VACO400 which were previously demonstrated to be methylated and unmethylated, respectively, at the *EVL* gene were used as additional controls.⁹

Luciferase assay

The complete 3'UTR sequence of the *EVL* gene was amplified from IMAGE clone no. 5222624 by PCR to include *XhoI* and *NotI* restriction sites. This was cloned immediately downstream of *Renilla* luciferase (RL) in the psiCHECK2 vector (Promega, Southampton, UK) that also encodes for firefly luciferase (FL) which acts as an internal transfection control for experiments. The resulting plasmid (psiCHECK-EVL-3'UTR) was sequence verified. HeLa cells were cotransfected with 2.5 µg of psiCHECK-EVL-3'UTR and 20nM of synthetic RNA oligo (MWG Biotech, Ebersberg, Germany) encoding either *miR-199a** (ACAGUAGUCUGCACAUGGUUA) or a scrambled version of this sequence (*Scramble-miR-199a** (ACAGUAGUCUGCACAUGGUUA)) with no known homology against any known human miRNA or gene sequence. FL and RL activity was measured 48 hours after transfection in triplicate using a Glomax luminometer (Promega) according to the manufacturer's recommendations. The RL/FL ratio of cells transfected with *miR-199a** or *Scramble-miR-199a** were compared with cells transfected only with psiCHECK-EVL-3'UTR. Experiments were carried out in triplicate.

Cell transfection, cell-proliferation and apoptosis assays

Jurkat cells were transfected with *miR-199a**, *Scramble-miR-199a** or mock transfected (no sequence). SeAx cells were transfected with miRIDIAN® *miR-342*, *miR-17-5p* or *anti-miR-199a** from Dharmacon (Lafayette, CO), *Scramble-miR-199a** or were mock transfected. All cells were transfected by electroporation using the Amaxa nucleofactor machine as described by the manufacturer (Lonza, Cologne, Germany) and transfection efficiency was measured using a green fluorescent protein (GFP)-containing plasmid and by qRT-PCR.

Apoptosis was quantified in triplicate 72hrs post-transfection in SeAx cells using the Cell Death Detection ELISA Kit (Roche Applied Science), while cell proliferation was measured using CellTiter 96® Aq_{ueous} One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions. Experiments were carried out in triplicate.

Results

Most aberrantly expressed miRNAs in Sézary syndrome are down-regulated

We elucidated the complete (miRBase v.10.1) miRNA profile of CD4⁺ T cells from 21 SzS patients and 6 healthy controls. In order to see how the miRNA profiles of SzS samples related to other hematological malignancies, we carried out meta-analysis on data generated previously.^{4,10} Unsupervised cluster analysis of these data revealed that all the clinical lymphoma samples clustered distinctly from cell lines and normal lymphocyte subsets, and that T-cell lymphoma (SzS) samples were distinct from B-cell lymphoma samples (Figure 1A). Using an extended human probe set (miRBase v.10.1; $n = 655$), unsupervised cluster analysis again placed the SzS samples distinctly from control CD4⁺ T cells (Figure 1B).

To identify miRNAs that are aberrantly expressed in SzS we compared expression levels with controls by ANOVA. This resulted in the identification of 114 miRNAs (adjusted $P < 0.05$), only 10 of which were up-regulated in SzS samples (Table 1-2). To validate the microarray data, 9 of the most up- and down-regulated miRNAs and 6 miRNAs previously associated with malignancy (ie, *miR-15*, *miR-16*, *miR-24*, *miR-17-5p*, *miR-106a*, *miR-19a*) were measured by qRT-PCR in SzS patient samples ($n = 17$), 7 CD4⁺ and 6 CD3⁺ T-cell control samples. These data were consistent with the microarray results (Figure 1-3).

Many miRNAs are encoded in clusters, and members of these clusters often exhibit the same pattern of expression.¹¹ A higher proportion of SzS-associated miRNAs (70 of 114 [61%]) were encoded in clusters than generally observed (215 of 474 [45%]; source:MiRGen Clusters database¹²). In 23 (74%) of the 31 clusters encoded by SzS-associated miRNAs, all members of the cluster were significantly differentially expressed (Table 1-2, Supplementary Table S3).

MicroRNA expression levels have diagnostic potential for Sézary syndrome

Using the 10 most discriminatory (based on P -value) up- and down-regulated miRNAs (Table 1) for cluster analysis distinguished SzS and control samples (Supplementary Figure S1) and correctly predicted diagnosis in 26 of 27 of samples (96%) by leave-one-out cross-validation SVM analysis.

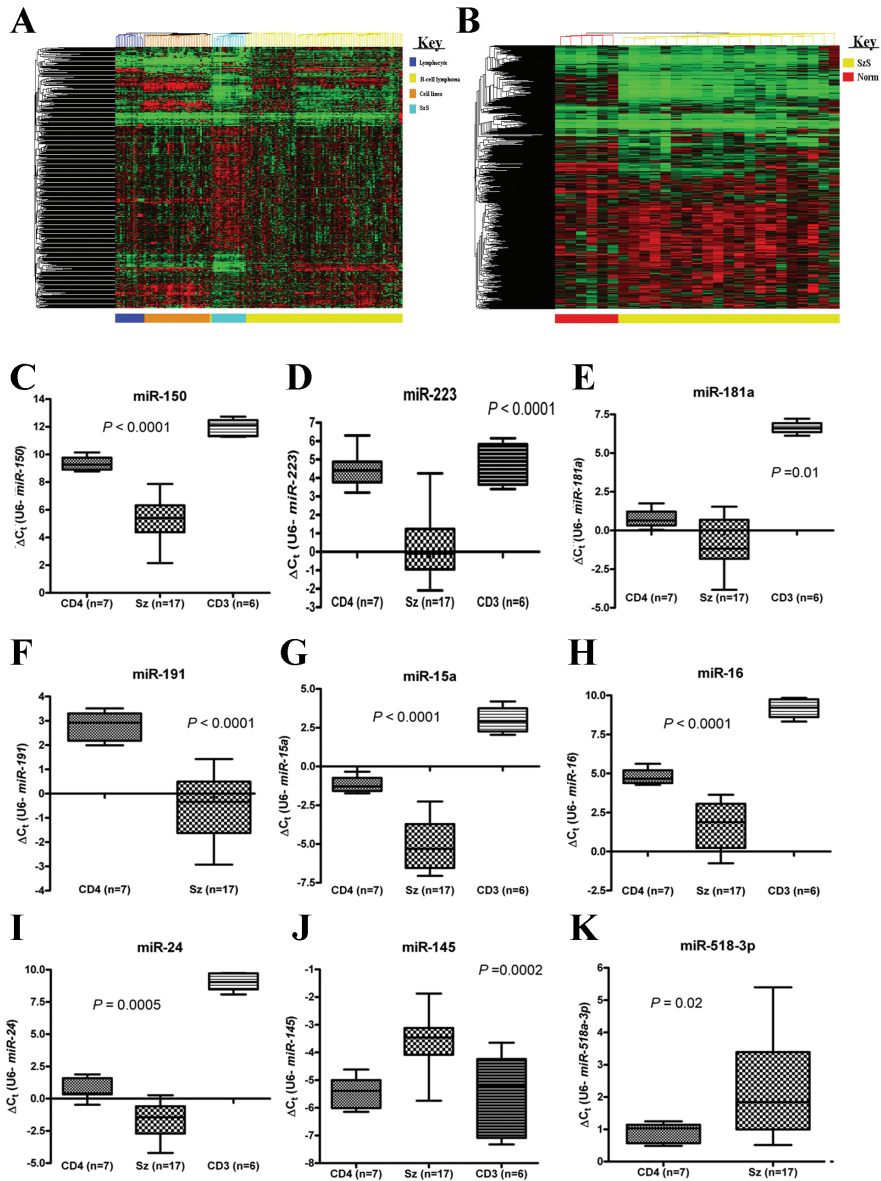


Figure 1 MicroRNAs are aberrantly expressed in SzS

(A) Unsupervised cluster analysis of miRNA expression data (miRBase v. 9.0) for purified lymphocyte subsets (n = 18), B-cell lymphoma samples (n = 98), hematological cell lines (n = 42) and Sézary syndrome (SzS) samples (n = 21). (B) Unsupervised cluster analysis of control CD4⁺ T cells (n = 6) and SzS samples (n = 21) using extended human probe set (miRBase v. 10.1; n = 655). Expression levels of (C) *miR-150*, (D) *miR-223*, (E) *miR-181a*, (F) *miR-191*, (G) *miR-15a*, (H) *miR-16*, (I) *miR-24*, (J) *miR-145* and (K) *miR-518a-3p* in SzS samples (n = 17), CD4⁺ T cells (n = 7) and CD3⁺ T cells (n = 6) controls measured by qRT-PCR. P-values relate to SzS versus control CD4⁺ T cells (Mann-Whitney independent t-test).

To see if miRNA levels measured by qRT-PCR yielded similar results we analyzed training set data (i.e. 17 SzS and 7 CD4⁺ samples; Figure 1-3) using k-fold cross-validation based on logistic regression analysis. A similar statistical approach was previously used to identify genes with diagnostic potential in SzS.¹³ The individual expression levels of 4 miRNAs (*miR-150*, *miR-191*, *miR-15a* and *miR-16*) correctly predicted diagnosis with 100% accuracy, whereas *miR-223* and *miR-17-5p* were 96% accurate (Supplementary Table S4).

To extend these analyses, we measured levels of the 6 miRNAs in an independent validation set consisting of an additional 15 SzS samples, a further 12 healthy controls and 11 cases of non-erythrodermic MF patients (patient characteristics are given in Supplementary Table S2). Taking the validation set data in isolation, levels of *miR-223* correctly discriminated between SzS and control (i.e. MF and healthy controls) samples in 34 of 38 of cases (90%) with a specificity of 87% and sensitivity of 92% corresponding

Table 1 Ten most discriminatory up- and down-regulated microRNAs differentially expressed ($P < .05$) between SzS ($n = 21$) and CD4⁺ controls ($n = 6$)

Order	microRNA	P	Fold change	Chromosome	Cluster
1	<i>miR-145</i>	5.68×10^{-4}	24.38	5q33.1	143-145
2	<i>miR-574-5p</i>	2.65×10^{-2}	12.82	4p14	-
3	<i>miR-200c</i>	9.67×10^{-3}	11.12	12p13.31	141-200c
4	<i>miR-199a*</i>	2.60×10^{-2}	8.58	19p13.2/1q24.3	199a-214
5	<i>miR-143</i>	3.84×10^{-3}	7.38	5q33.1	143-145
6	<i>miR-214</i>	3.43×10^{-3}	4.94	1q24.3	199a-214
7	<i>miR-98</i>	2.48×10^{-3}	4.91	Xp11.22	98-let-7f
8	<i>miR-518a-3p</i>	5.08×10^{-3}	3.18	19q13.41	-
9	<i>miR-7</i>	5.23×10^{-3}	2.46	9q21.32/15q26.1/19p13.3	-
10	<i>miR-152</i>	2.66×10^{-2}	2.05	17q21.32	-
-1	<i>miR-342</i>	2.31×10^{-9}	-9.94	14q32.2	-
-2	<i>miR-223</i>	2.41×10^{-9}	-13.99	Xq12	-
-3	<i>miR-150</i>	3.63×10^{-9}	-6.72	19q13.33	-
-4	<i>miR-189(24*)</i>	2.82×10^{-8}	-3.97	9q22.32/19p13.12	24-23a/23b-24
-5	<i>miR-186</i>	5.04×10^{-8}	-6.4	1p31.1	-
-6	<i>miR-423-3p</i>	5.04×10^{-8}	-2.24	17q11.2	-
-7	<i>miR-92</i>	5.22×10^{-8}	-5.6	13q31.3/Xq26.2	17-92/106a-363
-8	<i>miR-181a</i>	5.22×10^{-8}	-4.4	1q32.1/9q33.3	181a-181b
-9	<i>miR-191</i>	6.72×10^{-8}	-4.59	3p21.31	425-191
-10	<i>miR-376a</i>	6.72×10^{-8}	-3.91	14q32.31	-

Positive fold changes are up-regulated in SzS samples and negative values down-regulated compared to controls. MicroRNAs also associated ($P < .05$) with B-cell lymphoma¹⁰ are indicated in bold.

to an area under the curve (AUC) value of 0.977. Values of the other 5 miRNAs however did not discriminate well (Supplementary Table S5). Taking all data into account (i.e. training and validation set equals 32 SzS samples plus 30 controls), *miR-223* levels were 90% accurate with a specificity of 91% and sensitivity of 90% corresponding to an AUC value of 0.938 (Supplementary Table S6). Combining data from multiple miRNAs did not improve the performance with this data.

***miR-342* expression in Sézary syndrome is negatively regulated by *miR-199a** expression**

Because the intronically encoded *miR-342* is epigenetically silenced in colorectal cancer due to CpG-island methylation in the promoter region of host *EVL* gene,⁹ we wanted to see whether the same mechanism could explain the down-regulation observed in SzS. Both *miR-342* and *EVL* were down-regulated ($P < 0.001$ and 0.04, respectively) in SzS (Figure 2A-B). Using the same *EVL*-specific primers and colorectal cancer cell line controls as previously described,⁹ we did not find any evidence for *EVL* CpG-island promoter hypermethylation in SzS patient samples or control CD4⁺ T cells (Figure 2C). In addition, we sequenced areas of approximately 1kbp in SzS patients representing the promoter regions of both *EVL-201/2* and *EVL-203* transcripts that included the CpG-island and other identified regulatory sequences¹⁴ but found no mutations (data not shown). In case the intron encoding *miR-342* had been deleted or mutated, we also sequenced this region in the SzS samples, but found no mutations or deletions (data not shown).

We next investigated whether any of the SzS-associated miRNAs potentially targeted the *EVL* gene. Using the miRGen suite of predictive algorithms,¹² we identified *miR-199a** (*miR-199a-3p*), which is up-regulated in SzS (Figure 2D; $P = 0.01$), as potentially targeting *EVL* (Figure 2E). To test this hypothesis we cloned the 3'UTR sequence of *EVL* into a luciferase-reporter vector. As can be seen from Figure 2F transfection with *miR-199a** reduced luciferase activity significantly ($P = 0.004$), compared to either vector alone or transfection with a scrambled sequence.

In order to see whether *miR-199a** could also directly regulate *miR-342* expression in SzS we transfected the SeAx cell line with a *miR-199a** inhibitor. This resulted in greater than 26-fold decrease in the levels of *miR-199a** by 72 hours after transfection (data not shown) and an approximately 5-fold increase in endogenous levels of *miR-342* (Figure 2G). In contrast transfection of the SeAx cell line with *miR-342* did not significantly affect *miR-199a** levels (data not shown). The converse experiment was also carried out by transfecting the Jurkat T-cell line that has high endogenous levels of *miR-342*⁴ with *miR-199a** which resulted in an approximately 4-fold decrease in *miR-342* levels by 48 hours after transfection (Figure 2H).

Table 2 Down-regulated microRNAs differentially expressed ($P < .05$) between SzS ($n = 21$) and CD4+ controls ($n = 6$) showing chromosomal location, inclusion in microRNA clusters, and number of predicted target genes up-regulated in SzS

Order	microRNA	Adjusted P	Fold change	Chromosome	Cluster	No. of target genes
1	<i>miR-342</i>	2.31×10^{-9}	-9.94	14q32.2	-	2
2	<i>miR-223</i>	2.41×10^{-9}	-13.99	Xq12	-	8
3	<i>miR-150</i>	3.63×10^{-9}	-6.72	19q13.33	-	2
4	<i>miR-189</i>	2.82×10^{-8}	-3.97	9q22.32/19p13.12	24-23a/23b-24	6
5	<i>miR-186</i>	5.04×10^{-8}	-6.40	1p31.1	-	6
6	<i>miR-423-3p</i>	5.04×10^{-8}	-2.24	17q11.2	-	0
7	<i>miR-92</i>	5.22×10^{-8}	-5.60	13q31.3/Xq26.2	17-92/106a-363	6
8	<i>miR-181a</i>	5.22×10^{-8}	-4.40	1q32.1/9q33.3	181a-181b	10
9	<i>miR-191</i>	6.72×10^{-8}	-4.59	3p21.31	425-191	5
10	<i>miR-376a</i>	6.72×10^{-8}	-3.91	14q32.31	-	9
11	<i>miR-425-5p</i>	1.01×10^{-7}	-4.31	3p21.31	425-191	5
12	<i>miR-15b</i>	1.11×10^{-7}	-4.16	3q26.1	15b-16	11
13	<i>miR-181c</i>	1.11×10^{-7}	-3.87	19p13.12	181c-181d	10
14	<i>miR-93</i>	1.15×10^{-7}	-4.07	7q22.1	106b-25	6
15	<i>miR-423</i>	1.33×10^{-7}	-1.47	17q11.2	-	0
16	<i>miR-582</i>	1.55×10^{-7}	-5.13	5q12.1	-	0
17	<i>miR-363</i>	1.90×10^{-7}	-3.89	Xq26.2	106a-363	6
18	<i>miR-128b</i>	2.07×10^{-7}	-3.83	2q21.3/3p22.3	-	4
19	<i>miR-30c</i>	2.24×10^{-7}	-8.79	1p34.2	30e-30c	6
20	<i>miR-25</i>	2.24×10^{-7}	-5.05	7q22.1	106b-25	4
21	<i>miR-181b</i>	2.24×10^{-7}	-3.42	1q32.1/9q33.3	181a-181b	9
22	<i>miR-194</i>	2.87×10^{-7}	-4.28	1q41/11q13.1	215-194/192-194	3
23	<i>miR-652</i>	5.02×10^{-7}	-3.14	Xq22.3	-	3
24	<i>miR-505</i>	5.05×10^{-7}	-3.12	Xq27.1	-	4
25	<i>miR-30b</i>	6.54×10^{-7}	-8.76	8q24.22	30b-30d	4
26	<i>miR-128a</i>	7.84×10^{-7}	-3.09	2q21.3/3p22.3	-	4
27	<i>miR-142-3p</i>	7.93×10^{-7}	-12.33	17q22	-	4
28	<i>miR-532</i>	9.93×10^{-7}	-5.65	19q13.41	-	1
29	<i>miR-140-5p</i>	1.27×10^{-6}	-3.99	16q22.1	-	3
30	<i>miR-361</i>	1.43×10^{-6}	-5.91	Xq21.2	-	6
31	<i>let-7e</i>	1.54×10^{-6}	-3.17	19q13.33	99b-125a	5
32	<i>miR-140-3p</i>	1.65×10^{-6}	-4.43	16q22.1	-	3
33	<i>miR-532-3p</i>	2.32×10^{-6}	-3.49	Xp11.23	532-502	0

MicroRNAs also associated ($P < .05$) with B-cell lymphoma¹⁰ are indicated in bold

follow up table 2

Order	microRNA	Adjusted <i>P</i>	Fold change	Chromosome	Cluster	No. of target genes
34	<i>miR-345</i>	2.32×10^{-6}	-3.06	14q32.2	-	0
35	<i>let-7a</i>	2.61×10^{-6}	-3.02	11q24.1/9q22.32/22q13.31	100-let7a/let-7a-7d/let-7a-7b	5
36	<i>miR-500</i>	2.96×10^{-6}	-3.39	Xp11.23	532-502	2
37	<i>miR-16</i>	3.45×10^{-6}	-3.56	13q14.3/3q26.1	15a-16/15b-16	12
38	<i>miR-29a</i>	3.52×10^{-6}	-10.79	7q32.3	29a-29b	5
39	<i>miR-18b</i>	5.47×10^{-6}	-3.64	Xq26.2	106a-363	3
40	<i>miR-142-5p</i>	5.52×10^{-6}	-6.01	17q22	-	5
41	<i>miR-31</i>	8.73×10^{-6}	-5.57	9p21.3	-	6
42	<i>miR-27a</i>	9.50×10^{-6}	-6.37	19p13.12	24-23a	9
43	<i>miR-146b</i>	1.01×10^{-5}	-5.45	10q24.32	-	5
44	<i>miR-30a-3p</i>	1.21×10^{-5}	-5.47	6q13	-	6
45	<i>miR-30e-5p</i>	1.43×10^{-5}	-9.81	1p34.2	30e-30c	11
46	<i>miR-107</i>	1.50×10^{-5}	-7.52	10q23.31	-	9
47	<i>miR-17-5p</i>	2.00×10^{-5}	-6.66	13q31.3	17-92	11
48	<i>miR-338</i>	3.33×10^{-5}	-3.99	17q25.3	338-657	4
49	<i>miR-422b (378)</i>	5.21×10^{-5}	-3.10	5q33.1	-	4
50	<i>miR-106a</i>	5.37×10^{-5}	-6.22	Xq26.2	106a-363	3
51	<i>miR-484</i>	5.96×10^{-5}	-5.40	16p13.11	-	2
52	<i>miR-30a-5p</i>	1.09×10^{-4}	-7.11	6q13	-	9
53	<i>miR-30d</i>	1.14×10^{-4}	-4.31	8q24.22	30b-30d	5
54	<i>miR-455</i>	1.14×10^{-4}	-3.14	9q32	-	1
55	<i>miR-20b</i>	1.17×10^{-4}	-6.34	13q31.3	17-92	2
56	<i>miR-28-3p</i>	1.40×10^{-4}	-4.20	3q28	-	1
57	<i>miR-103</i>	1.44×10^{-4}	-6.51	5q35.1/20p13	-	10
58	<i>miR-590</i>	1.53×10^{-4}	-5.04	7q11.23	-	2
59	<i>miR-338-3p</i>	1.60×10^{-4}	-4.73	17q25.3	338-657	4
60	<i>miR-378</i>	1.82×10^{-4}	-3.13	5q33.1	-	4
61	<i>miR-18a</i>	2.15×10^{-4}	-3.85	13q31.3	17-92	6
62	<i>miR-30e</i>	2.29×10^{-4}	-10.19	1p34.2	30e-30c	11
63	<i>miR-365</i>	2.92×10^{-4}	-5.90	16p13.12/17q11.2	193b-365	2

MicroRNAs also associated ($P < .05$) with B-cell lymphoma¹⁰ are indicated in bold

follow up table 2

Order	microRNA	Adjusted <i>P</i>	Fold change	Chromosome	Cluster	No. of target genes
64	<i>miR-95</i>	3.25×10^{-4}	-4.58	4p16.1	-	6
65	<i>miR-374b</i>	3.25×10^{-4}	-4.13	Xq13.2	545-374	4
66	<i>miR-215</i>	3.46×10^{-4}	-3.12	1q41	215-194	4
67	<i>miR-23a</i>	3.49×10^{-4}	-7.30	19p13.12	24-23a	5
68	<i>miR-362-3p</i>	3.60×10^{-4}	-2.94	Xp11.23	532-502	4
69	<i>miR-20a</i>	3.62×10^{-4}	-7.85	13q31.3	17-92	2
70	<i>miR-22</i>	4.04×10^{-4}	-4.81	17p13.3	-	8
71	<i>miR-660</i>	4.27×10^{-4}	-4.95	Xp11.23	532-502	0
72	<i>miR-26a</i>	4.48×10^{-4}	-11.13	3p22.2/12q14	-	8
73	<i>miR-29c</i>	4.62×10^{-4}	-14.25	1q32.2	29b-29c	7
74	<i>miR-23b</i>	5.65×10^{-4}	-7.35	9q22.32	23b-24	7
75	<i>miR-19a</i>	6.17×10^{-4}	-9.72	13q31.3	17-92	7
76	<i>miR-125a-5p</i>	9.14×10^{-4}	-7.00	19q13.33	99b-125a	7
77	<i>miR-24</i>	1.17×10^{-3}	-8.02	9q22.32/19p13.12	24-23a/23b-24	3
78	<i>miR-27b</i>	1.40×10^{-3}	-6.29	9q22.32	23b-24	8
79	<i>miR-19b</i>	1.83×10^{-3}	-11.82	13q31.3/Xq26.2	17-92/106a-363	9
80	<i>miR-340</i>	1.93×10^{-3}	-4.61	5q35.3	-	0
81	<i>miR-374</i>	2.78×10^{-3}	-12.45	Xq13.2	545-374	4
82	<i>miR-15a</i>	2.92×10^{-3}	-8.09	13q14.3	15a-16	9
83	<i>miR-28</i>	3.78×10^{-3}	-3.88	3q28	-	1
84	<i>miR-148b</i>	4.04×10^{-3}	-3.71	12q13.13	-	5
85	<i>miR-106b</i>	4.10×10^{-3}	-4.31	7q22.1	106b-25	6
86	<i>miR-192</i>	4.59×10^{-3}	-2.29	11q13.1	192-194	3
87	<i>miR-29b</i>	4.74×10^{-3}	-15.39	1q32.2	29b-29c/29a-29b	6
88	<i>miR-32</i>	4.78×10^{-3}	-1.63	9q31.3	-	6
89	<i>miR-185</i>	9.45×10^{-3}	-4.75	22q11.2	-	7
90	<i>miR-146a</i>	1.05×10^{-2}	-2.19	5q33.3	-	5
91	<i>miR-133b</i>	1.17×10^{-2}	-3.34	6p12.2	133b-206	3
92	<i>miR-320</i>	1.38×10^{-2}	-3.32	8p21.3	-	9
93	<i>miR-151-5p</i>	1.42×10^{-2}	-4.96	8q24.3	-	5
94	<i>miR-26b</i>	1.60×10^{-2}	-13.43	2q35	-	9
95	<i>miR-590-3p</i>	1.60×10^{-2}	-2.69	7q11.23	-	2

MicroRNAs also associated ($P < .05$) with B-cell lymphoma¹⁰ are indicated in bold

follow up table 2

Order	microRNA	Adjusted <i>P</i>	Fold change	Chromosome	Cluster	No. of target genes
96	<i>miR-429</i>	1.69×10^{-2}	-2.39	1p36.33	200a-429	5
97	<i>miR-141</i>	2.21×10^{-2}	-3.38	12p13.31	141-200c	4
98	<i>miR-99b</i>	2.47×10^{-2}	-4.84	19q13.33	99b-125a	2
99	<i>miR-335</i>	2.49×10^{-2}	-4.11	7q32.2	-	3
100	<i>miR-100</i>	2.75×10^{-2}	-4.79	11q24.1	100-let7a	2
101	<i>miR-361-3p</i>	2.84×10^{-2}	-13.00	Xq21.2	-	6
102	<i>miR-10a</i>	2.84×10^{-2}	-6.85	17q21.32	-	6
103	<i>miR-148a</i>	3.40×10^{-2}	-13.52	7p15.2	-	4
104	<i>miR-125b</i>	3.51×10^{-2}	-6.85	11q24.1/21q21	-	5

MicroRNAs also associated ($P < .05$) with B-cell lymphoma¹⁰ are indicated in bold

Reconstitution of down-regulated miRNAs *miR-342* or *miR-17-5p* in Sézary cells inhibits apoptosis

Transfection with either *miR-342* or *miR-199a** inhibitor resulted in a significant increase in levels of apoptosis (Figure 2I; $P \leq 0.01$) but had no effect on cell proliferation levels (data not shown). In contrast, transfection of SeAx cells with *miR-17-5p* caused both an increase in apoptosis levels (Figure 3F; $P = 0.01$) and also a decrease in levels of cell proliferation (Figure 3G; $P = 0.01$).

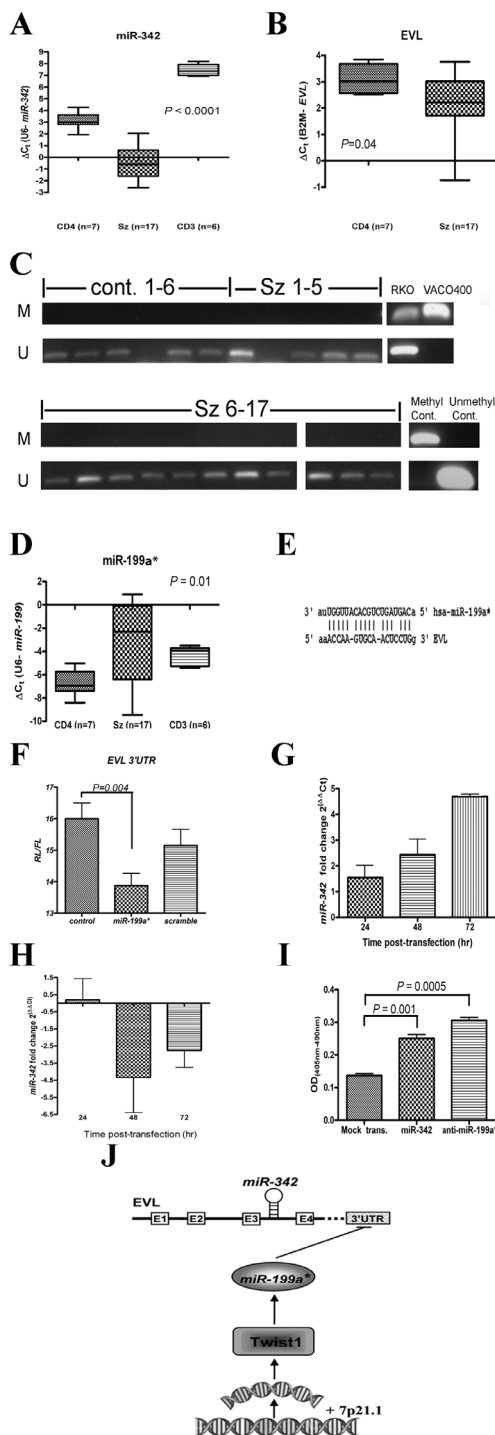


Figure 2 Down-regulation of pro-apoptotic *miR-342* is mediated by *miR-199a** which targets the *miR-342*-encoding gene *EVL*

Levels of (A) *miR-342* and (B) *EVL* in SzS samples ($n = 17$), CD4⁺ T cells ($n = 7$) and CD3⁺ T cells ($n = 6$) controls measured by qRT-PCR. Data shown as box-whisker plots. (C) CpG-island methylation status of *EVL* in SzS samples ($n = 17$) and CD4⁺ T cell controls ($n = 6$) measured by MS-PCR. Universally methylated and unmethylated DNA were used as controls. Colorectal cell lines RKO and VACO400 were methylated and unmethylated respectively as previously reported.⁷ (D) Levels of *miR-199a** in SzS samples ($n = 17$), CD4⁺ T cells ($n = 7$) and CD3⁺ T cells ($n = 6$) controls measured by qRT-PCR. (E) Predicted binding site for *miR-199a** within the 3'UTR sequence of *EVL* gene. (F) Transfection of *miR-199a** in HeLa cell line suppressed *EVL* 3'-UTR luciferase reporter activity compared with vector only control or Scramble-*miR-199a** sequence. (G) Inhibition of *miR-199a** in SeAx cells resulted in increased levels of *miR-342* measured by qRT-PCR. Fold change levels shown are relative to Scramble-*miR-199a** transfected control (i.e. $\Delta\Delta C_t$). (H) Transfection of Jurkat cell line with *miR-199a** resulted in decreased levels of *miR-342* measured by qRT-PCR. Fold change levels shown are relative to Scramble-*miR-199a** transfected control (i.e. $\Delta\Delta C_t$). (I) Expression of *miR-342* or inhibition of *miR-199a** in SeAx cell line resulted in an increase in apoptosis levels compared to mock-transfected control. Values shown are mean values from 3 experiments. (J) Schematic diagram of proposed pathway of *miR-342* regulation in SzS.

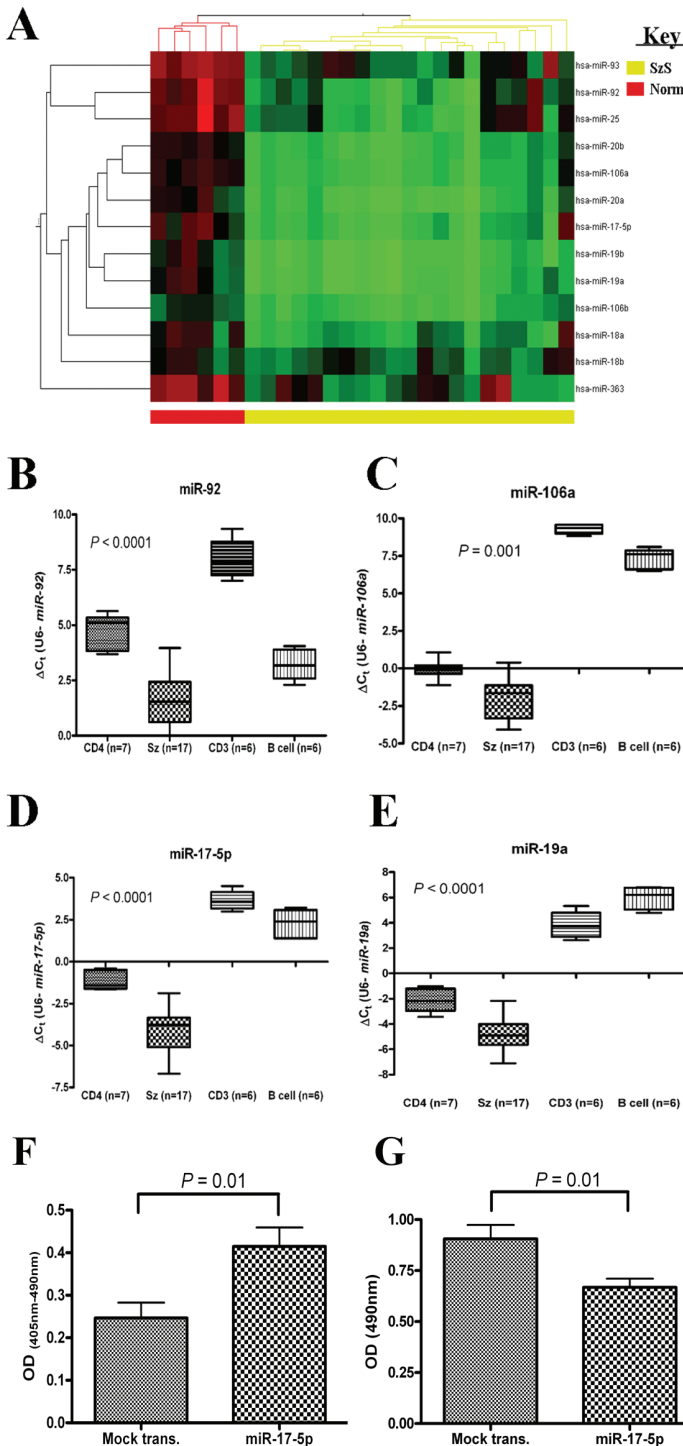


Figure 3 Members of the *miR-17-92* (and homologous) clusters are down-regulated and increase apoptosis and decrease cell proliferation in SzS

(A) Heat map depicting levels of members of *miR-17-92* and homologous clusters as measured by microarray and levels of (B) *miR-92*, (C) *miR-106a*, (D) *miR-17-5p* and (E) *miR-19a* in SzS samples ($n = 17$), and controls (CD4⁺ T cells ($n = 7$), CD3⁺ T cells ($n = 6$) and B cells ($n = 6$)) measured by qRT-PCR. P -values relate to SzS versus CD4⁺ (Mann-Whitney independent t -test). Data shown as box-whisker plots. Expression of *miR-17-5p* in SeAx cell line resulted in an increase in levels of (F) apoptosis and decrease in levels of (G) cell proliferation compared to mock transfected control. Values shown are mean values from 3 experiments.

Discussion

A total of 114 miRNAs were identified as being SzS-associated (adjusted $P < 0.05$), of which only 10 were up-regulated in SzS samples. A general decrease in miRNA expression in tumors compared to counterpart normal tissue is consistent with previous findings,^{15,16} although we¹⁰ and others¹⁷ found the opposite to be the case for B-cell lymphomas.

Using the same array as in this study we previously identified 60 miRNAs (40 up-regulated and 20 down-regulated) associated ($P < 0.05$) with B-cell lymphoma (either DLBCL or FL).¹⁰ Surprisingly, a comparison of these miRNAs with SzS-associated miRNAs reveal a great deal of similarity that suggests the presence of a common lymphoma miRNA signature (Table 1-2). A total of 7 of the 10 miRNAs that were up-regulated in SzS were also significantly up-regulated in B-cell lymphoma, while 18 of the 20 miRNAs that were down-regulated in B-cell lymphoma were also significantly down-regulated in SzS (Table 1-2).

The cause of aberrant miRNA expression in SzS, in common with most other cancers, is not readily apparent. Recently we detailed a number of recurrent chromosomal aberrations associated with SzS, albeit in a different cohort of patients.¹⁸ These abnormalities were generally consistent with miRNA expression in this cohort of SzS patients (Table 1-2). For example miR-152, which is up-regulated, is encoded by region of gain (17q21.32) in 70% of SzS cases, whereas the 13q14 locus, frequently deleted in SzS, encodes for *miR-15a* and *miR-16*, both of which were down-regulated ($P < 0.001$; Figure 1G-H). Similarly, *miR-107*, *miR-146b* and *miR-22* which were down-regulated, are encoded by common regions of loss or deletion in SzS (10q23.31, 10q24.32 and 17p13.3, respectively). However, similar to other studies,¹⁹ this correlation did not always hold true as some regions of recurrent gain (i.e. 4p16.1, 8q24.1 and 17q25) encode for miRNAs that were down-regulated (*miR-95*, *miR-30* and *miR-338*, respectively). This suggests that these miRNAs, at least, are regulated by another mechanism. It was recently demonstrated that MYC, which is aberrantly active in SzS,¹⁸ down-regulates a number of miRNAs including *miR-30*.²⁰ Indeed all of the miRNAs shown to be repressed by MYC (i.e. *miR-22*, *miR-26a*, *miR-29c*, *miR-30*, *miR-146a* and *miR-150*) were also down-regulated in SzS samples (Table 2).

To investigate whether SzS-associated miRNAs correlated with gene expression patterns, we interrogated the miRGen database¹² for miRNAs predicted to target the 69 genes that we had previously identified as being up-regulated in a separate cohort of patients with SzS.²¹ Nearly all (97 of 104 [93%]) of down-regulated SzS-associated miRNAs were predicted to target one or more of these genes (median number of targets = 5; Table 2). Furthermore, a comparison between this set of miRNAs with 10,000 randomly generated sets of 104 miRNAs showed a highly significant enrichment for SzS-associated target genes (P -value < 0.001). This suggests that miRNAs play an important role in the regulation

of gene expression in SzS, although it remains possible that the distinct cohorts of patients with SzS used for this analysis are not directly comparable and further analysis should be carried out.

Early detection and treatment are directly correlated with favorable outcome for SzS, but diagnosis is frequently difficult as cutaneous histology is often not diagnostic in erythrodermic skin disease and atypical T cells can be detected in the peripheral blood of erythrodermic patients with inflammatory conditions.²² In order to test the diagnostic ability of the SzS-associated miRNAs, we used the top 10 most discriminatory up- and down-regulated miRNAs in a SVM class-prediction model. This resulted in the correct assignment of samples as either SzS or control for 26 of 27 (96%) of cases and expression levels of just three of these miRNAs (*miR-17-5p*, *miR-150*, and *miR-15a* or *miR-16*) were sufficient to correctly predict all samples. Although microarray data is informative it is not readily available in a clinical setting. Therefore, we analyzed miRNA levels measured by qRT-PCR, a technique available to the majority of clinical diagnostic laboratories, using the k-fold cross-validation predictive model. Consistent with the microarray data, individual expression levels of *miR-150*, *miR-15a*, *miR-16* or *miR-191*, correctly predicted samples all samples in this cohort. To validate these findings and include a more clinically relevant control group (i.e. non-erythrodermic MF) we expanded these analyses to an independent validation set. This time, only *miR-223* levels were found to have useful predictive capacity (90% accuracy, 87% specificity, and 96% sensitivity) that was consistent (90% accuracy, 91% specificity, and 90% sensitivity) when both training and validation sets were combined (i.e. 32 SzS and 30 control samples). Very similar results were obtained using a SVM predictive algorithm instead (SMO function in Weka; data not shown). The discrepancy between the 2 cohorts is largely a result of the inclusion of MF samples, which gave similar miRNA expression values to the SzS samples (Supplementary Figure S2), possibly due to disruption of the peripheral T-cell repertoire that is often associated with non-erythrodermic MF.²³

Recently, expression levels of both *DNM3* and *CDO1* were found to be 100% specific (91 and 82% sensitive, respectively) although this study lacked controls with confounding diagnoses and small numbers were used (11 SzS, 12 healthy controls and 10 patients with inflammatory disorders).¹³ Previously a 5-gene signature was identified that could discriminate between SzS and control samples with 90% accuracy.²⁴ However, this model was not reproducible in the hands of Booken *et al*,¹³ and the higher costs and time associated with measuring 5 genes suggest that a single miRNA assay may prove a more useful classifier for SzS diagnosis.

The most discriminatory miRNA identified by ANOVA analysis of the microarray data was *miR-342* (adjusted $P < 0.001$). This miRNA is intronically encoded within the *EVL* gene.

Both *miR-342* and *EVL* were found to be down-regulated in SzS. It had been shown that the *miR-342/EVL* locus is epigenetically silenced via CpG-island methylation in colorectal cancer.⁹ In contrast, we found no evidence of similar methylation in SzS patient samples. A possible explanation for this discrepancy is that unlike colorectal cancer, which express the *EVL-203* transcript containing the CpG island in proximity (~30bp) to its start codon,⁹ patients with SzS express a much shorter transcript (*EVL-201/2*; data not shown) which initiates approximately 100kbp downstream from this regulatory element.

One intriguing possibility to explain down-regulation of *miR-342* in SzS was that the host gene, *EVL*, is itself a target for miRNA regulation. Of particular interest amongst the miRNAs predicted to target *EVL* was *miR-199a** which is up-regulated in SzS. We tested this hypothesis by luciferase assay and demonstrated an inverse relationship between levels of *miR-199a** and *miR-342* in SzS as inhibition of the former resulted in an increase in endogenous levels of *miR-342* whilst expression of *miR-199a** in Jurkat cells that naturally express high levels of *miR-342* reduced levels of *miR-342*.

Approximately 40% of miRNAs are encoded in intronic regions of encoding genes.²⁵ Although generally believed to be passively co-expressed with their host mRNA recent data suggests the regulation of intronic miRNAs is more complex than previously thought.²⁶ Our data suggests a novel and elegantly simple model for the regulation of intronically encoded miRNAs via modulation of expression levels of miRNAs that target the host mRNA. It should be noted, however, that as *miR-342* is spliced out of the *EVL* transcript before being exported to the cytoplasm, *miR-199a** must function in the nucleus. There is now compelling evidence that many mature miRNAs, are reimported into the nucleus and once there can regulate transcription.²⁷⁻³⁰ It has recently been demonstrated that *miR-199a** is present at higher levels in the nucleus (and nucleolus) than in the cytoplasm of cells.³¹ This is the first evidence for such a regulatory mechanism operating in biological systems and provides a new level of complexity to the poorly understood area of miRNA regulation.

Interestingly, *miR-199a** is also intronically encoded within the *DNM3* gene which is itself specifically expressed in SzS.¹³ Furthermore, *miR-199a** is directly up-regulated by TWIST1 binding,^{32,33} which is up-regulated in SzS²¹ due to frequent gain of the encoding region.¹⁸ These data suggest a model for de-regulation of *miR-342* depicted in Figure 2J.

To investigate the biological relevance of reduced *miR-342* levels in SzS we reconstituted this miRNA in the SeAx cell line. This resulted in a significant increase in apoptosis levels ($P = 0.001$), but did not significantly affect cell proliferation levels as had been reported in colorectal cancer cell lines.⁹ Significantly, the same effect was observed when *miR-199a** expression was suppressed in this model. The presence of distinct yet analogous mechanisms to suppress *miR-342* coupled with a common anti-apoptotic phenotype

suggests that *miR-342* could play a tumor-suppressor role in these 2 cancers.

To investigate how *miR-342* down-regulation might inhibit apoptosis in SzS, we used the miRGen database to identify putative apoptosis-associated target genes (Supplemental Table 7). Of particular interest was *TNFSF11* (also known as *RANKL* or *TRANCE*; supplemental Figure 3A), an anti-apoptotic molecule that was previously identified as being up-regulated in SzS,^{13,21} that was also up-regulated in our cohort of patients with SzS ($P < .001$; supplemental Figure 3B). We found that levels of *TNFSF11* decreased in SzS cells transfected with either *miR-342* or *anti-miR-199a** compared with nontransfected controls (supplemental Figure 3C). *TNFSF11* is highly expressed in peripheral activated and memory but not resting peripheral T cells.³⁴ This pattern is consistent with our previous observation that *miR-342* is more highly expressed in resting than memory T cells.⁴ It has been suggested that *TNFSF11* expression can promote T-cell survival by interacting with dendritic cells, leading to induction of IL-15.³⁴ Interestingly, IL-15 has been shown to protect SzS cells from apoptotic agents.³⁵ Although these data are preliminary, it suggests that *miR-342* down-regulation in SzS mediates its anti-apoptotic affect via up-regulation of *TNFSF11*.

The *miR-17-92* cluster has been proposed to act as both tumor-suppressor or oncogene depending upon the cellular context, although until now there was a lack of *in vivo* evidence to support its tumor-suppressor role.³⁶⁻³⁸ Members of this cluster are widely up-regulated in B-cell lymphomas and solid tumors.^{4,10,39} However, we found that all 13 miRNAs encoded by this cluster and homologous clusters (*miR-106a-363* and *miR-106b-25*) were down-regulated in SzS samples by microarray analysis (average fold-change = 6.1; range 3.64-11.82). These data were validated by qRT-PCR for *miR-106a*, *miR-19a*, *miR-17-5p* and *miR-92* (Figure 3; $P \leq 0.001$). Although the 13q31 locus is deleted in some solid tumors,¹⁹ and it has been reported that *miR-17-5p* is down-regulated in some breast cancer cell lines,³⁸ as far as we are aware this is the first report to describe *in vivo* down-regulation of these miRNAs in malignancy. This finding is particularly intriguing as the *miR-17-92* cluster, in B-cell lymphomas at least, is up-regulated by MYC-binding,⁴⁰ which in turn is aberrantly active in SzS.¹⁸ To explore the possibility that MYC-binding sites⁴⁰ were mutated in SzS patients, we sequenced this and surrounding regions but found no mutations (data not shown). Therefore the causal mechanism for down-regulation of these miRNAs in SzS remains to be determined and is an area we are actively pursuing. Similar to previous reports in breast cancer cell lines,³⁸ ectopic expression of *miR-17-5p* in SzS cells resulted in a decrease in cell proliferation levels coupled with an increase in levels of apoptosis. Whether or not this finding can be extended to other T-cell lymphomas, or is an anomaly of SzS, remains to be discovered; however it is clearly an area of research that warrants further investigation

Cumulatively the above data strongly suggests that miRNAs are important in the pathogenesis of SzS and provides exciting new possibilities for the diagnosis and treatment of this disease.

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5

miRNA expression profiling of mycosis fungoides

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Abstract

MicroRNAs (miRNAs) are small RNA species that regulate gene expression post-transcriptionally and are aberrantly expressed in many malignancies including lymphoma. However, the role of miRNAs in the pathogenesis of T-cell lymphoid malignancies is poorly understood. Previously we examined the miRNA profile of Sézary syndrome (Sz), a leukemia of skin-homing memory T cells. In this study we determined the complete miRNome of mycosis fungoides (MF), the most common type of cutaneous T-cell lymphoma. The miRNA profile of skin biopsies from 19 patients with tumor-stage MF and 12 patients with benign inflammatory dermatoses (eczema and lichen planus) were compared by microarray analysis. We identified 49 miRNAs that are differentially expressed in tumor-stage MF compared to benign inflammatory dermatoses using ANOVA analysis ($P < 0.05$, Benjamini-Hochberg corrected). The majority of the differentially expressed miRNAs (30/49) were up-regulated in tumor-stage MF. The most significant differentially expressed were miR-155 and miR-92a (both up-regulated in tumor-stage MF), while miR-93 showed the highest up-regulation in tumor-stage MF with a fold difference of 5.8. Differential expression of a selection of these miRNAs was validated by miRNA-Q-PCR on additional test groups (tumors and controls). None of the miRNAs up-regulated in tumor-stage MF was previously shown to be up-regulated in Sz, and only 2 of the 19 miRNAs down-regulated in tumor-stage MF were also down-regulated in Sz. Taken together this report is the first describing the miRNA signature of tumor-stage MF.

Introduction

Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma (CTCL) and is characterized clinically by an evolution of patches, plaques to tumors containing malignant skin-homing T cells. Patients with limited plaque-stage disease have a 10-year survival of 97%-98% but in patients with tumor-stage disease the 10-year survival decreases to 42%.¹ In previous studies tumor-stage MF samples were investigated for genomic alterations, gene expression profiles, and promoter hypermethylation to unravel the molecular basis of this disease.²⁻⁵

MicroRNAs (miRNAs) are short non-coding RNA molecules involved in crucial biological processes, including development, immune function, proliferation, apoptosis, and the stress response through negative regulation of the stability and translation of target messenger RNAs (mRNAs).⁶⁻⁸ It is becoming increasingly clear that specific miRNAs contribute to cancer initiation and progression, and that miRNAs may have oncogenic or tumor suppressing properties depending on their target genes.⁹ Also, there is increasing evidence for a role for miRNAs in the pathogenesis of lymphoid malignancies such as lymphoma.¹⁰

Little is known about the role of miRNAs in cutaneous lymphoma. So far our study on the miRNome of Sézary syndrome (Sz), a leukemia of skin-homing T cells, is the only one describing miRNA expression in cutaneous lymphoma.¹¹ In the current study we performed miRNA analysis on tumor-stage MF to gain further insight in the molecular pathogenesis of this disease. Firstly, by identifying aberrantly expressed miRNAs specific for tumor-stage MF by comparing the miRNA profiles of tumor-stage MF with benign inflammatory dermatoses. Secondly, by correlating miRNA expression with previously described copy number alterations characteristic for tumor-stage MF.^{2,4,5} Finally by comparing aberrantly expressed miRNAs of tumor-stage MF with aberrant expression in Sz to identify differences.

Materials and Methods

Patient selection

Tumor-stage MF patients were selected from the database of the Dutch Cutaneous Lymphoma Group (DCLG), reviewed by an expert panel of dermatologists and hematopathologists before entry into this database and classified using the criteria of the WHO-EORTC classification for primary cutaneous lymphomas.¹ Nineteen formalin-fixed, paraffin-embedded (FFPE) MF tumor biopsies containing more than 75% tumor cells were

selected for array analysis and 10 additional cases for miRNA-Q-PCR validation experiments. Clinical characteristics are shown in Table 1. For control material FFPE biopsies of benign inflammatory dermatosis) containing T-cell rich infiltrates were selected for array analysis (eczema (n=5) and lichen planus (n=7) and miRNA-Q-PCR validation experiments (additional eczema (n=5) and lichen planus (n=6) cases). This study was performed in accordance with the Dutch code and Leiden University Medical Center guidelines on leftover material.

miRNA extraction

Total RNA was isolated from 6 x 20 µm sections of tumor biopsies and 8 x 20 µm sections of benign control biopsies using the Ambion total nucleic acid extraction kit according to manufacturers' protocol (Ambion, Warrington UK). To assess the (tumor) infiltrate of slides sectioned for miRNA extraction, HE and CD3 stainings were performed on slides sectioned directly before and after sections used for RNA extraction.

miRNA array analysis

Total RNA (± 3 µg), were labeled and hybridized to miRNA microarrays as previously described¹¹ along with a synthetic human miRNA universal reference pool containing 454 miRNAs as a common reference. The arrays contain 655 human probes (miRBase v.10.1). Probe details can be found at www.microRNAworld.com. Image analysis was carried out by Bluefuse software (BlueGnome, Cambridge, UK). Raw fold ratio data were global loess-normalized within arrays and quantile normalized between arrays using the LIMMA package.¹² The normalized log ratios (average of four replicates per probe) were used for subsequent analysis in Genespring 7.2 (Agilent Technologies, CA, US). MicroRNAs were filtered prior to ANOVA analysis to remove those that had a median intensity >300 fluorescence units background in more than 50% of the arrays. ANOVA analysis was used to identify miRNAs differentially expressed between sample types and *P*-values were adjusted using the Benjamini-Hochberg multiple testing correction method.

miRNA-Q-PCR

For miRNA cDNA synthesis, 300 ng RNA was reverse transcribed using the miRNA reverse transcription kit (Applied Biosystems) in combination with the stem-loop Megaplex primer pool A v2.1 (Applied Biosystems), allowing simultaneous reverse transcription of 377 miRNAs and endogenous controls. MicroRNA-Q-PCR was performed using Taqman miRNA assays and 2x Universal PCR mastermix (Applied Biosystems). All reactions were run on the LightCycler480 (Roche, Almere, the Netherlands), according to manufacturer's protocol (Applied Biosystems). The cycle parameters were as follows:

Table 1 Clinical characteristics of MF patients

Patient	Gender	Age	Stage at diagnosis / biopsy	Current status	Follow-up after diagnosis / biopsy (months)
Array group					
1	M	66	T2N0M0/T3N0M0	AWD	173 / 81
2	M	80	T3N0M0/T3N0M0	DOOC	100 / 100
3	M	57	T2N0M0/T3N0M0	DOD	24 / 16
4	M	54	T3N0M0/T3N0M0	DOD	45 / 45
5	F	65	T3N0M0/T3N0M0	DOD	56 / 19
6	M	67	T2N0M0/T3N0M0	DOD	58 / 7
7	M	57	T2N0M0/T2N0M0	DOD	28 / 28
8	F	49	T2N0M0/T3N1M0	DOD	12 / 4
9	F	66	T1N0M0/T3N0M0	ACR	139 / 10
10	F	84	T3N0M0/T3N0M0	DOD	13 / 9
11	M	65	T3N0M0/T3N0M0	DOD	54 / 7
12	F	61	T2N0M0/T3N0M0	DOD	83 / 39
13	F	44	T3N0M0/T3N0M0	ACR	11 / 9
14	M	63	T2N0M0/T3N0M0	AWD	306 / 16
15	M	75	T3N0M0/T3N0M0	AWD	49 / 49
16	F	87	T2N0M0/T2N0M0	AWD	9 / 9
17	M	62	T3N3M0/T3N3M0	DOD	22 / 22
18	M	60	T2N3M0/T3N0M0	AWD	34 / 18
19	F	50	T3N1M0/T3N1M0	DOD	20 / 20
Validation Group					
1	M	33	T2N0M0/T3N3M0	DOD	55 / 4
2	M	72	T2N0M0/T3N0M0	DOD	41 / 13
3	F	50	T2N0M0/T3N0M0	ACR	147 / 90
4	M	61	T3N0M0/T3N0M0	DOOC	45 / 44
5	M	69	T2N0M0/T3N0M0	DOD	21 / 14
6	M	74	T2N0M0/T3N0M0	DOD	136 / 11
7	M	68	T3N0M0/T3N0M0	DOOC	24 / 17
8	M	76	T3N0M0/T3N0M0	DOD	39 / 39
9	M	80	T2N0M0/T3N0M0	DOOC	87 / 33
10	F	77	T3N0M0/T3N0M0	AWD	74 / 74

DOD denotes died of disease, DOOC died of other causes, ACR alive in complete remission, AWD alive with disease.

10 min at 95 °C, 45 cycles denaturing for 15 s at 95°C and annealing and extension for 60 s at 60 °C. MicroRNA expression was analyzed using the ΔCt method expressed relative to U6. Statistical analyses were performed using the Mann-Whitney independent *t*-test in SPSS (version 17).

Results

Aberrantly expressed miRNAs in tumor-stage mycosis fungoides are predominantly up-regulated in comparison to benign controls

We determined the miRNA profiles from FFPE biopsies of 19 tumor-stage MF patients and 12 inflammatory dermatoses (lichen planus *n*=7, eczema *n*=5) using miRNA microarrays (miRBase v.10.1). Using unsupervised clustering (Figure 1) the majority of tumor and benign tissue samples clustered in different clusters. However, 3 samples from benign inflammatory dermatoses clustered with tumor samples. Clinical data including the extent

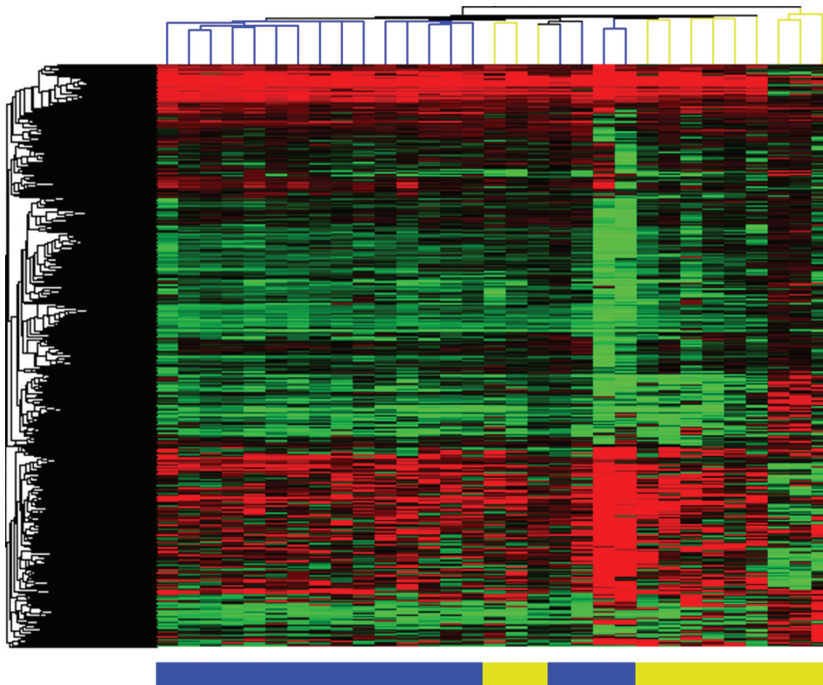


Figure 1 Unsupervised cluster analysis tumor-stage MF and benign inflammatory dermatoses
Heatmap representing miRNA array expression data of MF tumor samples (*n*=19, depicted in blue) and benign inflammatory dermatosis samples (*n*=12, depicted in yellow).

of the lymphoid infiltrate did not correlate with the misclassification of these samples. Comparison of the miRNA expression levels in MF tumors with benign inflammatory dermatoses identified 49 differentially expressed miRNAs with an adjusted P -value < 0.05 (Table 2). The majority (30 miRNAs) were up-regulated in tumor-stage MF, and of these 30 up-regulated miRNAs 16 have a fold change (FC) > 2 . Of the 19 miRNAs that are down-regulated in tumor-stage MF, 7 have a FC < -2 . To validate the array results miRNA-Q-PCR was performed for 6 miRNAs up-regulated in tumor-stage MF (miR-93, miR-155, miR-30b, miR-16 miR-30c and miR-92a) and 1 miRNA down-regulated in tumor-stage MF (miR-383) in an additional group of tumor-stage MF ($n=10$ and benign inflammatory dermatoses (eczema $n=5$ and lichen planus $n=6$) cases. In these experiments miRNA-Q-PCR results are consistent with microarray data and shown in Figure 2. All miRNAs show significant differential expression measured by Mann-Whitney independent t -test ($P < 0.05$).

Discussion

In this study we elaborated the miRNA expression profile of tumor-stage MF using miRNA microarrays. We found 49 miRNAs aberrantly expressed in MF tumors compared to benign inflammatory dermatoses. Remarkably, the majority (30) of the miRNAs is up-regulated, while in most tumors miRNAs are predominantly expressed at lower levels compared to their benign counterparts, though higher expression in the majority of differentially expressed miRNAs has also been described for B-cell malignancies.¹³ We validated differentially expression of 7 miRNAs in additional test groups supporting the robustness of the results.

The miRNA with the highest fold difference is miR-93, which is part of the miR-106b-25 cluster and has been previously described as an oncomir preventing apoptosis and promoting tumor growth,^{14,15} targeting tumor suppressors *integrin-β8*,¹⁴ *FUS1*,¹⁶ and *E2F1*.¹⁷ MiR-93 over expression is reported in a subtype of nodal lymphoma (ALK⁺ ALCL when compared to ALK⁻ ALCL)¹⁸ and in gastric and hepatocellular carcinoma.^{17,19} MiR-93 is hosted by the gene encoding MCM7 (minichromosome maintenance protein 7) which has been reported to be over expressed in MF on the protein level.²⁰ Another up-regulated miRNA is miR-155, which targets *SHIP1*, *PU.1*, *AID*, *SOCS1*, *BACH1*, *CEBPB*, *CSFR*, *TAB2*, *MAF* and *JARID2*, all known to play a role in immune responses and required for T-cell function.^{8,21} MiR-155 up-regulation is described for several types of lymphoma and leukemia, the first reports were on B-cell lymphoma.^{8,22-24} Several members of the miR-17-92 cluster are up-regulated in MF tumor stage (miR-17-5p, miR-20a, miR-92a and miR-92). MiRNAs from the miR-17-92 cluster, targeting *BIM* and *PTEN*, regulate T-cell

survival and selection⁸ and are involved in many malignancies including lymphoma.²⁵ Remarkably, we demonstrate miR-16 up-regulation, while the tumor suppressing function of miR-16 and the corresponding down-regulation is frequently described in tumors and lymphoma.²⁴ Possibly miR-16 plays a different yet unexplored role in MF tumors. Recently the effect of DNA copy number alteration on miRNA expression was shown and has been linked to the initiation, progression and development of malignancies.^{26,27} To investigate the possible effect of copy number alterations on miRNA expression in tumor-stage MF we determined the genomic location (miRBase release September 2010) of differentially expressed miRNAs and correlated those to previously described

Table 2 Differentially expressed miRNAs with chromosomal location

miRNA	Fold change	Adj. <i>P</i> -value	Chromosomal location
hsa-miR-620	-3,07	0,0481	12: 116586365-116586459 [-]
hsa-miR-302d	-2,23	0,0422	4: 113569160-113569227 [-]
hsa-miR-483	-2,19	0,0030	11: 2155364-2155439 [-]
hsa-miR-204	-2,08	0,0010	9: 73424891-73425000 [-]
hsa-miR-323b-5p	-2,07	0,0407	14: 101522556-101522637 [+]
hsa-miR-380-5p	-2,06	0,0303	14: 101491354-101491414 [+]
hsa-miR-383	-2,02	0,0459	8: 14710947-14711019 [-]
hsa-miR-211	-1,96	0,0459	15: 31357235-31357344 [-]
hsa-miR-363*	-1,94	0,0015	X: 133303408-133303482 [-]
hsa-miR-133b	-1,92	0,0033	6: 52013721-52013839 [+]
hsa-miR-485-3p	-1,80	0,0075	14: 101521756-101521828 [+]
hsa-miR-517c	-1,70	0,0377	19: 54244567-54244661 [+]
hsa-miR-199b	-1,64	0,0165	9: 131007000-131007109 [-]
hsa-miR-199a	-1,63	0,0304	19: 10928102-10928172 [-] 1: 172113675-172113784 [-]
hsa-miR-133a	-1,53	0,0475	18: 19405659-19405746 [-] 20: 61162119-61162220 [+]
hsa-miR-99a	-1,48	0,0407	21: 17911409-17911489 [+]
hsa-miR-197	-1,44	0,0155	1: 110141515-110141589 [+]
hsa-miR-218	-1,41	0,0481	4: 20529898-20530007 [+] 5: 168195151-168195260 [-]
hsa-miR-100	-1,40	0,0248	11: 122022937-122023016 [-]
hsa-miR-25	1,28	0,0483	7: 99691183-99691266 [-]
hsa-miR-15b	1,34	0,0407	3: 160122376-160122473 [+]
hsa-miR-195	1,34	0,0325	17: 6920934-6921020 [-]

follow up table 2

miRNA	Fold change	Adj. <i>P</i> -value	Chromosomal location
hsa-miR-221	1,40	0,0066	X: 45605585-45605694 [-]
hsa-miR-103	1,46	0,0389	20: 3898141-3898218 [+] 5: 167987901-167987978 [-]
hsa-miR-107	1,48	0,0176	10: 91352504-91352584 [-]
hsa-miR-181a	1,49	0,0407	9: 127454721-127454830 [+] 1: 198828173-198828282 [-]
hsa-miR-342	1,67	0,0025	14: 100575992-100576090 [+]
hsa-miR-20a	1,69	0,0165	13: 92003319-92003389 [+]
hsa-miR-222	1,70	0,0025	X: 45606421-45606530 [-]
hsa-let-7i	1,78	0,0022	12: 62997466-62997549 [+]
hsa-miR-30c	1,91	0,0075	6: 72086663-72086734 [-] 1: 41222956-41223044 [+]
hsa-miR-191	1,92	0,0040	3: 49058051-49058142 [-]
hsa-miR-24	1,99	0,0038	9: 97848303-97848370 [+] 19: 13947101-13947173 [-]
hsa-miR-17-5p	2,06	0,0021	13: 92002859-92002942 [+]
hsa-miR-342-3p	2,08	0,0004	14: 100575992-100576090 [+]
hsa-miR-146b	2,13	0,0007	10: 104196269-104196341 [+]
hsa-miR-29a	2,26	0,0407	7: 130561506-130561569 [-]
hsa-miR-92b	2,26	0,0007	1: 155164968-155165063 [+]
hsa-miR-320a	2,41	0,0295	8: 22102475-22102556 [-]
hsa-miR-16	2,46	0,0021	13: 50623109-50623197 [-] 3: 160122533-160122613 [+]
hsa-miR-30b	2,51	0,0155	8: 135812763-135812850 [-]
hsa-miR-146a	2,70	0,0005	5: 159912359-159912457 [+]
hsa-miR-92	2,80	<0,0001	13: 92003568-92003645 [+] X: 133303568-133303642 [-]
hsa-miR-142-3p	3,05	0,0077	17: 56408593-56408679 [-]
hsa-miR-92a	3,20	<0,0001	13: 92003568-92003645 [+] X: 133303568-133303642 [-]
hsa-miR-21	3,86	0,0123	17: 57918627-57918698 [+]
hsa-miR-155	4,41	<0,0001	21: 26946292-26946356 [+]
hsa-miR-425-5p	5,54	0,0165	3: 49057581-49057667 [-]
hsa-miR-93	5,83	0,0093	7: 99691391-99691470 [-]

Positive fold changes are up-regulated in MF samples and negative values down-regulated compared to controls. Adj. *P*-value is adjusted *P*-value after Benjamini-Hochberg multiple testing correction.

copy number alterations seemingly characteristic for tumor-stage MF,^{2,4,5} which revealed that 8 up-regulated miRNAs (miR-93, miR-21, miR-142-3p, miR-30b, miR-92b, miR-29a, miR-181a and miR-25) are encoded in genomic regions of gain (Table 3). We could not find any correlation between down-regulated miRNAs and regions of loss identified in tumor-stage MF. Not all miRNAs of a miRNA cluster located in a region of gain are up-regulated suggesting that other mechanisms than copy number effect, such as transcriptional regulation primarily regulate miRNA expression in MF.

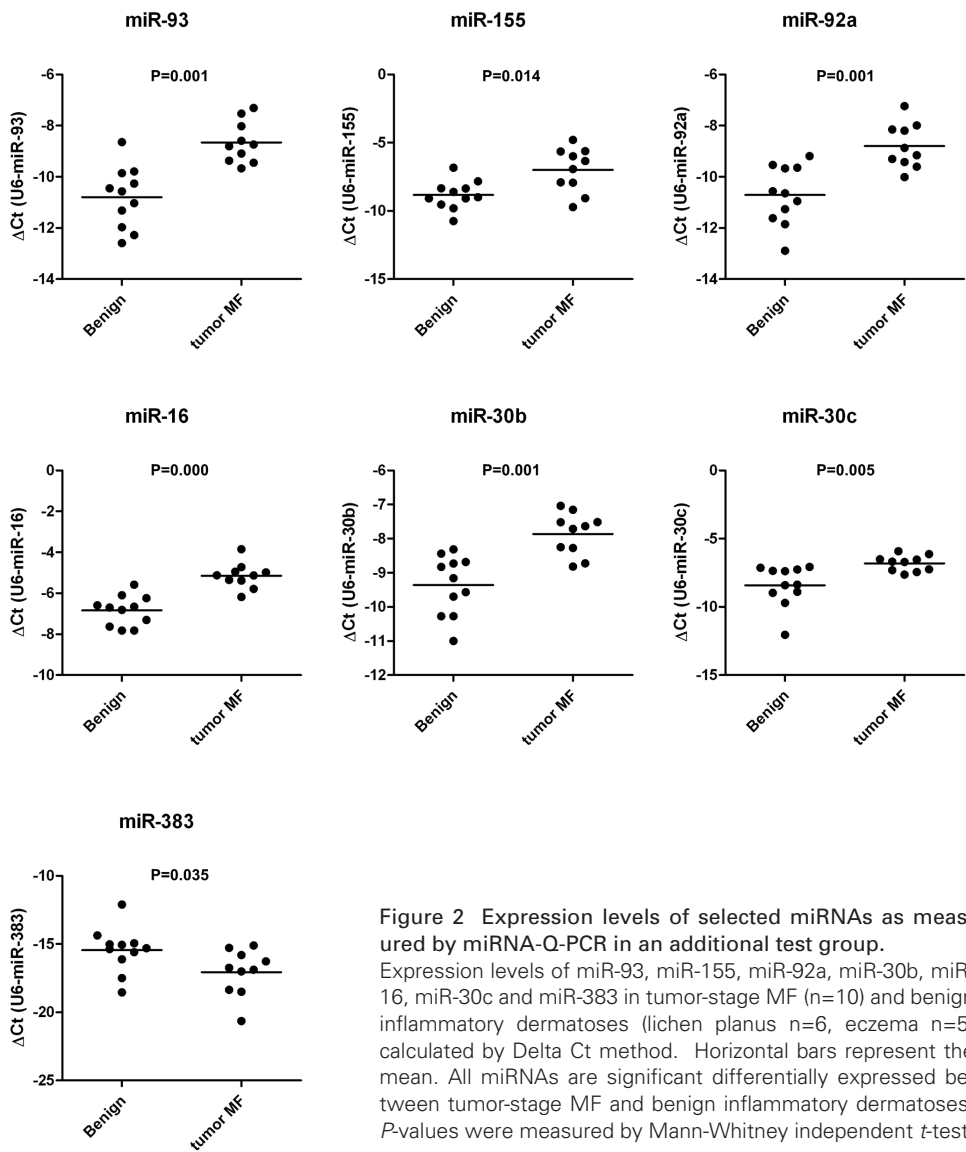


Figure 2 Expression levels of selected miRNAs as measured by miRNA-Q-PCR in an additional test group. Expression levels of miR-93, miR-155, miR-92a, miR-30b, miR-16, miR-30c and miR-383 in tumor-stage MF (n=10) and benign inflammatory dermatoses (lichen planus n=6, eczema n=5) calculated by Delta Ct method. Horizontal bars represent the mean. All miRNAs are significant differentially expressed between tumor-stage MF and benign inflammatory dermatoses, P-values were measured by Mann-Whitney independent t-test.

Recent data from our group and others demonstrated many differences in gene expression profiles and DNA copy number alterations between MF and Sz, a leukemia of skin-homing T cells, in addition Campbell and colleagues recently suggested that MF and Sz arise from different T-cell subsets, indicating that the molecular pathogenesis of these CTCL may be distinct.^{2,28-30} We compared our MF tumor-stage miRNA profile with the previously generated miRNA profile of Sz.¹¹ Although a similar array platform was used, a direct comparison of MF and Sz miRNA array results was not possible, since different reference miRNAs were used for competitive hybridization in both experiments (synthetic miRNAs in current experiments, and miRNAs isolated from tonsils in previous experiments).¹¹ We therefore compared the list of differentially expressed miRNAs identified in MF tumors (MF vs benign inflammatory dermatoses) with the list of differentially expressed miRNAs in Sz (CD4⁺ T cells of Sz vs CD4⁺ T cells from healthy donors).¹¹ This comparison, showing minimal overlap between MF and Sz, support the notion that Sz and tumor-stage MF are molecularly distinct. In contrast to tumor-stage MF the majority of the miRNAs (104 miRNAs) in Sz is down-regulated compared to its benign counterpart (CD4⁺ T cells). Of the 19 miRNAs down-regulated in tumor-stage MF, only miR-100 and miR-133 are also down-regulated in Sz. The 30 miRNAs up-regulated in MF tumor-stage and the 10 miRNAs up-regulated in Sz show no overlap. Several of the miRNAs up-regulated in tumor-stage MF are down-regulated in Sz, among these are miR-93, miR-155, miR-16 and members of the miR-17-92 cluster.

Table 3 Differentially expressed miRNAs located in a previously described region of DNA copy number alteration in MF

miRNA	Cluster	Cytoband	Chromosomal location	Gain in MF		
				van Doorn et al. ²	Salgado et al. ⁵	Laharanne et al. ⁴
hsa-miR-92b		1q22	1: 155164968-155165063 [+]	x		
hsa-miR-181a	181a-181b	1q32.1	1: 198828173-198828282 [-]		x	
	181a-181b	9q33.3	9: 127454721-127454830 [+]			
hsa-miR-25	106b-93-25	7q22.1	7: 99691183-99691266 [-]	x		
hsa-miR-93	106b-93-25	7q22.1	7: 99691391-99691470 [-]	x		
hsa-miR-29a	29a-29b	7q32.3	7: 130561506-130561569 [-]	x		x
hsa-miR-30b	30b-30d	8q24.22	8: 135812763-135812850 [-]	x		
hsa-miR-142-3p		17q22	17: 56408593-56408679 [-]	x		
hsa-miR-21		17q23.1	17: 57918627-57918698 [+]	x		

In summary this is the first report describing the characterization of the miRNA profile in tumor-stage MF, thereby identifying 49 aberrantly expressed miRNAs. For 8 miRNA encoding genes, DNA copy number alteration could possibly contribute to aberrant expression. This analysis provides a framework for further (functional) studies which will reveal the role of these miRNAs on gene and protein expression and thereby their specific contribution to the pathogenesis of this disease.

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6

Cutaneous anaplastic large cell lymphoma and peripheral T-cell lymphoma NOS show distinct chromosomal alterations and differential expression of chemokine receptors and apoptosis regulators

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Abstract

Primary cutaneous anaplastic large cell lymphoma (C-ALCL) has an indolent clinical course and favorable prognosis. On the contrary, primary cutaneous peripheral T-cell lymphoma not otherwise specified (PTL-NOS) displays aggressive clinical behavior. To identify genomic events relevant in the pathogenesis of these cutaneous T-cell lymphomas (CTCLs), we carried out array-based CGH analysis. Simultaneously, gene expression profiling was conducted to gain insight into gene expression programs associated with the different clinical behavior of these CTCLs.

C-ALCL was characterized by gains on chromosome 7q and 17q and losses on 6q and 13q. PTL-NOS similarly demonstrated gains on 7q and 17q, but was distinguished by gains on chromosome 8 and loss of a focal overlapping region on 9p21. We identified minimal common regions harboring candidate oncogenes and tumor suppressor genes in C-ALCL and PTL-NOS. Genes with a role in lymphocyte chemotaxis, apoptosis and proliferation were overrepresented among genes differentially expressed between these lymphomas. C-ALCL demonstrated higher expression of the skin-homing chemokine receptor genes *CCR10* and *CCR8* which may explain the lower tendency to disseminate to extracutaneous sites. Furthermore, C-ALCL and PTL-NOS showed aberrant expression of distinct genes implicated in apoptosis and proliferation, such as *IRF4/MUM1* and *PRKCQ*, which may account for differences in clinical aggressiveness.

Introduction

Primary cutaneous anaplastic large cell lymphoma (C-ALCL), formerly designated as primary cutaneous CD30-positive large T-cell lymphoma, is a T-cell lymphoma composed of large cells with an anaplastic, pleomorphic or immunoblastic cytomorphology, that show expression of the CD30 receptor.¹ Most patients present with solitary or localized skin tumors that have a tendency to regress spontaneously. C-ALCL has an indolent clinical behavior and rarely shows extracutaneous dissemination. This cutaneous T-cell lymphoma (CTCL) is commonly treated with radiotherapy and has an excellent prognosis with an estimated 5-year survival exceeding 90%.²⁻⁴ In contrast, primary cutaneous peripheral T-cell lymphoma not otherwise specified (PTL-NOS), presents with more generalized skin tumors and displays aggressive clinical behavior.¹ PTL-NOS, formerly termed CD30-negative large T-cell lymphoma, frequently disseminates to extracutaneous sites and is often refractory to chemotherapeutic treatment. Patients with PTL-NOS have an estimated 5-year survival of less than 15%.⁵⁻⁷

The genetic events involved in the pathogenesis of these cutaneous lymphomas are largely unknown. Cytogenetic analyses of C-ALCL have revealed recurrent copy number alterations (CNAs) of several chromosomal regions, including gains on 6p, 7q, and 19, and losses on 6q, 9 and 18.⁸⁻¹² The t(2;5)(p23;q35) translocation inducing the NPM-ALK chimeric protein, a characteristic feature of nodal ALCL, is not or only rarely found in C-ALCL.¹³ To date no studies of chromosomal alterations in primary cutaneous PTL-NOS have been published.

Genetic factors responsible for the differences in clinical behavior of C-ALCL and PTL-NOS, both derived from skin-homing T cells, have not yet been resolved. Our group has previously shown that the FAS receptor is expressed by C-ALCL tumor cells, whereas expression is lost in the majority of PTL-NOS cases, suggesting differences in sensitivity to extrinsic pro-apoptotic signals.¹⁴ In addition, studies have indicated that signaling through the CD30 receptor is implicated in proliferation and apoptosis of lymphoid cells in C-ALCL.^{15,16}

In this study, array-based CGH analysis and gene expression profiling was performed on C-ALCL and primary cutaneous PTL-NOS tumor samples to identify chromosomal aberrations and gene expression patterns, which may contribute to a better understanding of the molecular mechanisms involved in the development and in the differential clinical behavior of these 2 types of CTCL.

Material and Methods

Selection of Patients

Pre-treatment biopsies of 11 patients with C-ALCL and 10 patients with primary cutaneous PTL-NOS were selected for this study (Table 1). In all 21 cases, the biopsies contained more than 75% malignant T cells. The immunophenotypical characteristics of the 21 cases are presented in Supplementary Table S1. All patients had been retrieved from the database of the Dutch Cutaneous Lymphoma Group (DCLG), reviewed by an expert panel of dermatologists and hematopathologists before entry in this database and classified using criteria of the WHO-EORTC classification for the primary cutaneous lymphomas.¹ In all patients routine staging procedures including physical examination, complete and differential blood cell counts and serum biochemistry, CT scan of neck, chest and abdomen and a bone marrow biopsy had been negative. With respect to the group of primary cutaneous PTL-NOS, these cases did not meet the criteria of one of the rare subtypes of primary cutaneous PTL-NOS (aggressive epidermotropic CD8⁺ CTCL; cutaneous gamma-delta T-cell lymphoma; CD4⁺ small/medium pleomorphic CTCL), and none of them had a history of or concurrent patches and plaques suggesting a diagnosis of mycosis fungoides. Of 10 patients with PTL-NOS, 9 presented with generalized ulcerating tumors. CD30 staining was either completely negative (eight cases) or showed expression by a minor proportion (< 25%) of neoplastic T cells (cases 20 en 21). Approval for these studies was obtained from the institutional review board of the LUMC. Informed consent was provided according to the Declaration of Helsinki.

Extraction of RNA and DNA

In all cases, DNA and RNA were isolated from the same frozen tumor biopsy sample for array-based CGH and microarray-based gene expression analysis, quantitative real-time PCR (qPCR), and mutation analysis. RNA was extracted from 25 x 50 μ M frozen sections using the RNeasy kit (Qiagen, Hilden, Germany), yielding 25-60 μ g total RNA. DNA was isolated from 25 frozen sections (20 μ M) using the Genomic-tip 20/G kit (Qiagen), yielding 10-60 μ g genomic DNA.

Array-based CGH analysis

Genome-wide analysis of CNAs was performed using array-based CGH containing approximately 3500 BACs produced at the Leiden University Medical Center. The particular BAC set used to produce the arrays was distributed by the Wellcome Trust Sanger Institute (Hinxton, United Kingdom) and contains large insert clones spaced at approximately 1 Mb density over the full genome, a set of subtelomeric sequences for

Table 1 Clinical characteristics

No	Sex	Age	Extent	Therapy	Result	Site of Relapse	Current status	Follow-up (months)
C-ALCL								
1	M	48	LOC	RT	CR	S; LN;LUNG	DOD	231
2	F	62	GEN	chemo	PR	S; LN	DOD	36
3	F	74	SOL	RT	CR	S	ACR	258
4	F	45	GEN	Chemo	PR	S; LN	DOD	308
5	M	69	LOC	RT	CR		ACR	120
6	M	51	GEN			S	ACR	105
7	M	63	GEN	RT	PR	S	AWD	139
8	M	86	LOC	RT	CR		ACR	29
9	M	70	SOL	RT	CR	S	ACR	12
10	F	79	SOL	RT	CR		ACR	13
11	F	44	LOC	EXC	CR	S	AWD	62
PTL-NOS								
12	M	33	GEN	chemo	PR	S;LN;CNS	DOD	13
13	M	65	SOL	chemo	PR	S;CNS	DOD	8
14	F	80	GEN	chemo	PR	S;LN	DOD	27
15	M	70	GEN	chemo	PR	S	DOD	13
16	M	75	GEN	chemo	PD	BLOOD	DOD	1
17	M	73	GEN	chemo	PR	S;LN	DOD	14
18	M	58	GEN	chemo	PD		DOD	3 #
19	F	65	GEN	chemo	PD	S;LN; BM	DOD	12
20	M	33	GEN	chemo	PD	S;CNS	DOD	19
21	M	59	GEN	chemo	PR		DOD	6 #

Abbreviations used: SOL, solitary lesion; LOC, localized disease; GEN, generalized (multifocal) skin disease; RT, radiotherapy; EXC, excision; CR, complete remission; PR, partial remission; PD, progressive disease; S, skin; LN: lymph node; CNS, central nervous system; BM: bone marrow; ACR, alive in complete remission; AWD, Alive with clinical symptoms of disease; DOD, death by disease; #: died of therapy-related side effects

each chromosome arm, and a few hundred probes selected for their involvement in oncogenesis.¹⁷ Fabrication and validation of the array, hybridization methods and analytical procedures have been described elsewhere in detail.¹⁸ Data were analyzed using CAPweb and visualized using VAMP.¹⁹ Copy number was classified as normal, copy number gain ($\log^2$ ratio > 0.25) or genomic loss ($\log^2$ ratio < -0.25). Identified CNA of regions with copy number variations described in the Database of Genomic Variants (<http://projects.tcag.ca/variation>) were excluded from analysis.

Gene expression profiling

Samples and microarrays (Human Genome U133plus2.0 array, Affymetrix Santa Clara, CA), interrogating over 47000 human transcripts and variants, were processed according to the manufacturer's protocol as described previously.²⁰ The microarray images were quantified utilizing the Genechip operating system (GCOS) v1.2 software (Affymetrix). The 260/280 ratios of isolated RNA were >1.8 for all samples, as measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), confirming RNA purity. RNA integrity was determined by gel electrophoresis, which showed two ribosomal RNA bands with the 28S rRNA band having a higher intensity than the 18S rRNA band in all cases. In addition, we used the internal controls present on the Affymetrix arrays allowing monitoring of RNA quality after hybridization. The 3'/5' GAPDH and 3'/5' beta-actin values were within the limits recommended by Affymetrix (maximally 1.25 and 3 respectively) for all samples.

The average fluorescence intensity was determined for each microarray and then the output of each experiment was globally scaled to a target value of 200. The profiles of normal CD4⁺ T cells were obtained from the GEO database (accession numbers GSM146182-GSM146186) previously published by Piccaluga.²¹ They obtained the CD4⁺ T cells by positive selection using magnetic beads (Miltenyi Biotec). Normalization and variance stabilization was performed using VSN in the R statistical software package.²²

Data analysis

BAC clone and oligonucleotide probe positions were established based on Ensembl (Ensembl is a joint project between European Bioinformatics Institute (EBI), an outstanding of the European Molecular Biology Laboratory (EMBL), and the Wellcome Trust Sanger Institute (WTSI), Hinxton, UK) release 44 (April 2007). Recurrent minimal common regions (MCRs) with CNA affecting at least 35% of analyzed samples were computed in CAPweb using the algorithm proposed by Rouveirol *et al.*²³ MCRs consisting of only subtelomeric clones were not taken into consideration. The nearby borders of adjacent clones were chosen to delineate MCRs. Candidate genes with pathobiological relevance were selected by focusing on genes listed as oncogene or tumor suppressor gene in the Cancer Gene Census list (November 2008).²⁴ Comparative analysis of the gene expression patterns of C-ALCL and PTL-NOS was performed utilizing BRB-arraytools v3.5.0 (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) using the Significance Analysis of Microarray algorithm, with a false discovery rate of 0.1, performing 100 permutations. The DAVID bioinformatics database was used for Gene Ontology (GO) enrichment analysis.²⁵ Genes most significantly differentially expressed with a ratio of geometric means (RGM) higher than 2.5 or lower than 0.4 were analyzed with highest stringency for enriched Gene Ontology clusters. By

means of the gene location, the associated BAC clone was determined, for genes not located in the region of a BAC clone the most proximate clone spotted on the array was taken. For these BAC clones the percentage of CNAs was calculated.

Quantitative real-time PCR

cDNA synthesis was performed on 1 µg total RNA, after treatment with RQ1 DNase I (Promega, Madison, WI), using IScript reverse transcriptase (Bio-Rad, Veenendaal, the Netherlands), oligo(dT)₁₂₋₁₈ and random hexamer priming (Bio-Rad) in a final volume of 20 µl. qPCR was performed with the MyIQ Detection System and the SYBR Green Supermix (Bio-Rad). The cycle parameters for transcripts of interest and for the reference genes *U1A* and *RPS11* used for normalization were as follows: denaturing for 15 s at 97 °C; annealing and extension for 20 s at 60 °C, for 40 cycles. Primer sequences (Invitrogen, Breda, The Netherlands) are given in Supplementary Materials online (Table S2). Data were evaluated using MyIQ software (Bio-Rad) and the second derivative maximum algorithm, while confirmation of the specificity of the PCR product and standard curves were performed as previously described.²⁰ Freshly isolated CD4⁺ T cells of 4 healthy donors were used as controls for qPCR experiments.

Mutation analysis

Mutation analysis of the coding region of the *PRKCQ* gene was performed on cDNA from 8 biopsy samples of patients with PTL-NOS. Primer sequences are listed in Supplementary Data (Table S2). The PCR fragments were purified and directly subjected to sequence reactions. The software Mutation Explorer (SoftGenetics, LLC, State College, PA) was applied for analyzing sequences and comparing with reference sequences from the NCBI database (National Center for Biotechnology Information, Bethesda, MD).

Immunohistochemistry

Immunohistochemical analysis of the protein expression of TNFRSF8/CD30, TRAF1 and IRF4/MUM1 was performed on the 21 C-ALCL and PTL-NOS samples selected for genomic analyses. In addition, paraffin-embedded sections of an independent set consisting of 20 C-ALCL and 5 PTL-NOS samples were subjected to immunohistochemical staining. Immunostaining was performed with antibodies against TNFRSF8/CD30 (DAKO, Glostrup, Denmark), TRAF1 and IRF4/MUM1 using a standard three-step streptavidin-biotin-peroxidase-based technique after antigen retrieval with microwave heating as described previously.²⁶ The antibodies against IRF4/MUM1 and TRAF1 were kindly provided by prof. dr. G. Cattoretti, Institute of Cancer Genetics, Columbia University, New York, USA and prof.dr. H. Dürkop, Institute of Pathology, Charité, Berlin, Germany, respectively.

Results

Recurrent chromosomal alterations in C-ALCL and PTL-NOS with potential biological significance

Tumor biopsy samples of 11 patients diagnosed with C-ALCL and 10 patients with PTL-NOS were analyzed for numerical chromosomal alterations using array-based CGH. Clinical characteristics of the patients are shown in Table 1 and immunophenotypical characteristics of the tumor cells are provided in the supplementary data (Table S1). In DNA isolated from all C-ALCL and PTL-NOS tumor samples numerous chromosomal alterations were present. An overview of the cumulative array-based CGH results in a Frequency of Amplicon, Gain and Loss (FrAGL) plot is shown in for C-ALCL in Figure 1a and for PTL-NOS in Figure 1c.

The overall pattern of chromosomal alterations of C-ALCL is characterized by gains of large regions on chromosome 7q and 17 and losses of regions on chromosome 6q and 13. To delineate chromosomal regions harboring genes with pathobiological relevance we determined the minimal common regions (MCRs), the smallest recurrent chromosomal region with altered probes common to the set of array-based CGH profiles.²³ A total of 30 MCRs present in at least 35% of the patients were identified. MCRs are visualized in the averaged chromosomal pattern of C-ALCL as vertical bands in Figure 1b and are listed in Table 2. Of these recurrent MCRs with CNA, 20 represent gains of chromosomal regions and 10 correspond to losses. The most highly recurrent chromosomal alterations are gain of 7q31 and loss of 13q34 and 6q16-6q21, all affecting 45% of patients. Next, we cross-referenced the genes residing in these 30 MCRs with the Cancer Gene Census, a list of genes for which mutations have been causally implicated in cancer.²⁴ As presented in Table 2a, the MCRs harbored 26 known oncogenes and tumor suppressor genes. The most highly recurrent MCR with gain in C-ALCL at 7q31 harbors a single oncogene, the *MET* gene that encodes the hepatocyte growth factor receptor. Three putative tumor suppressor genes, *CDC16*, *CUL4A* and *PRDM1* reside in the loci with loss on 13q34 and 6q16-6q21.

The pattern of CNAs of PTL-NOS is predominated by gains of large regions on chromosome 7, 8 and 17 (Figure 1c). Thirty-four MCRs affecting at least 35% of patients were identified, including 30 gains and only 4 losses (Fig 1d, Table 2). The most highly recurrent MCRs with CNA are 7q36 affected by gain in 60% of patients and 7q21-7q22, 8p12-8q12, 8p21.1-8q21.3, and 8q22-8q24.2, each showing gain in 50% of patients. Forty-three known cancer-associated genes, listed in Table 2b, are located in MCRs with recurrent CNA. Although no confirmed oncogenes are located on 7q36, it harbors the *FASTK* gene encoding an anti-apoptotic kinase expressed by T cells.²⁷ The *MYC* oncogene, previously

shown to be amplified and over expressed in Sézary syndrome and also in aggressive B-cell lymphomas, is located in the MCR with gain on 8q22-8q24.2.^{28,29}

Similarities and differences between chromosomal alterations in C-ALCL and PTL-NOS

The cumulative patterns of chromosomal alterations of C-ALCL and PTL-NOS show many overlapping features. C-ALCL and PTL-NOS are concordant with respect to gain of large chromosomal regions on 7q and 17. These chromosomes contain several MCRS with CNA that affect both of these lymphomas, such as gains on 7q21-7q22 and 17q21-17q25. Other MCRs that C-ALCL and PTL-NOS have in common are gain of 6p21.3 and losses on 8p21-22 (Table 2).

However, clear differences exist between the CNA patterns of C-ALCL and PTL-NOS. Most strikingly, gains on chromosome 8 affect the majority of patients with PTL-NOS, but are almost absent in C-ALCL. On the other hand, C-ALCL tumor samples were distinguished by frequent losses on chromosome 6 and 13. A distinction of potential relevance concerns the 9p21 locus, which is not affected by loss in any of the C-ALCL patients, but is deleted in 50% of PTL-NOS patients (Fig 1c). The deleted region is quite large in most patients and contains a MCR located on 9p21.3 harboring the *CDKN2A* tumor suppressor gene.

Gene expression patterns of C-ALCL and PTL-NOS show marked differences

To gain more insight into the gene expression programs of the tumor cells that underlie the difference in clinical behavior of these entities, gene expression profiling was performed. Supervised gene expression analysis revealed that 547 probe sets targeting 358 genes were significantly differentially expressed. Of these genes 325 were relatively higher expressed in C-ALCL and 33 showed higher expression in PTL-NOS. A heatmap, indicating gene expression intensities across the samples, showing the 91 most differentially expressed genes with a ratio of geometric means (RGM) exceeding 2.5 is depicted Figure 2a and 2b. To illustrate the expression of these 91 genes in benign CD4⁺ T cells, we made use of published transcriptome data acquired using the same microarray platform.²¹ A heatmap illustrating transcript abundance can be found as supplementary data (Supplementary Figure S1). Expression of the majority of genes differentially expressed between C-ALCL and PTL-NOS is low in benign CD4⁺ T cells. A complete list of the differentially expressed genes can be found in Supplementary Table S3. Genes with the relatively highest expression in C-ALCL are *CCR10* and *TNFRSF8/CD30*. PTL-NOS demonstrated higher expression of several genes including *Protein Kinase C theta (PRKCQ)*, *Fyn binding protein* and several *GIMAP* genes.

As copy number alterations contribute to oncogenesis by altering the expression of resident genes, we assessed the possible relationship between altered expression and the presence of gains and losses of specific chromosomal regions in these lymphomas. For the most significantly differentially expressed, the frequency of gain or loss of the

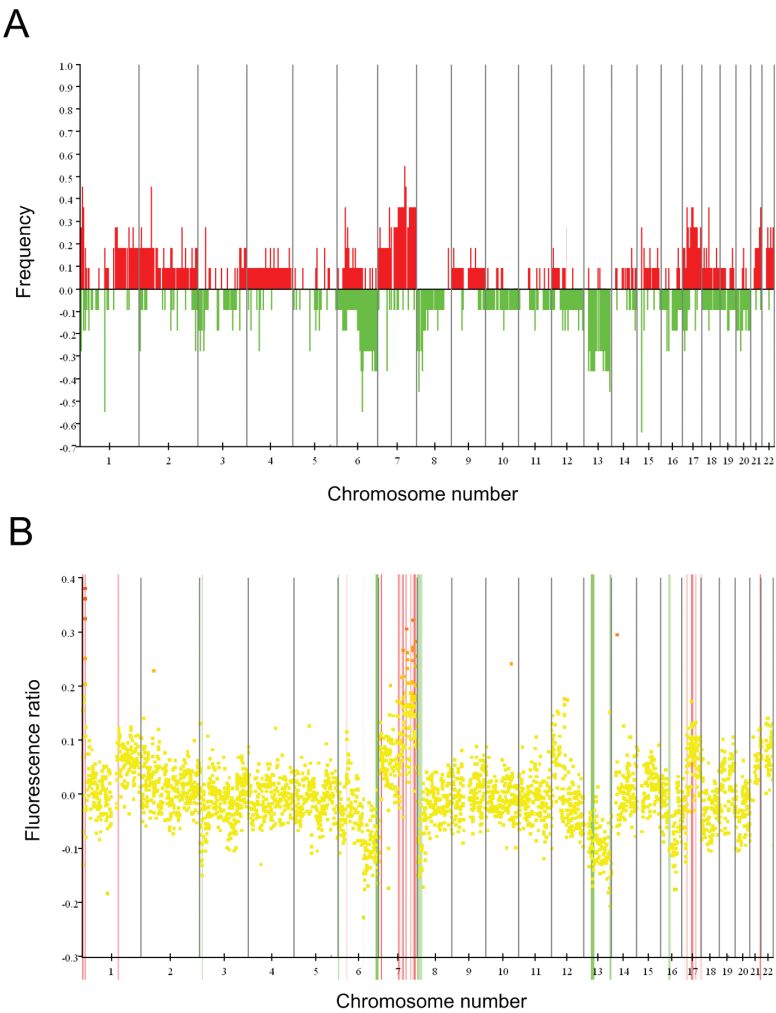
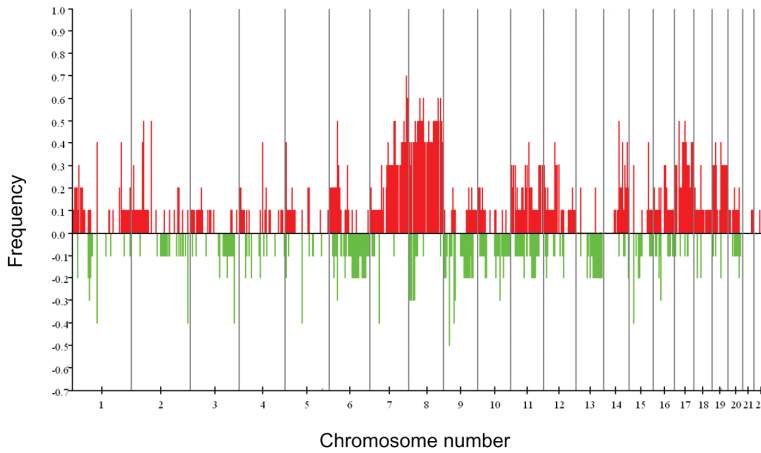


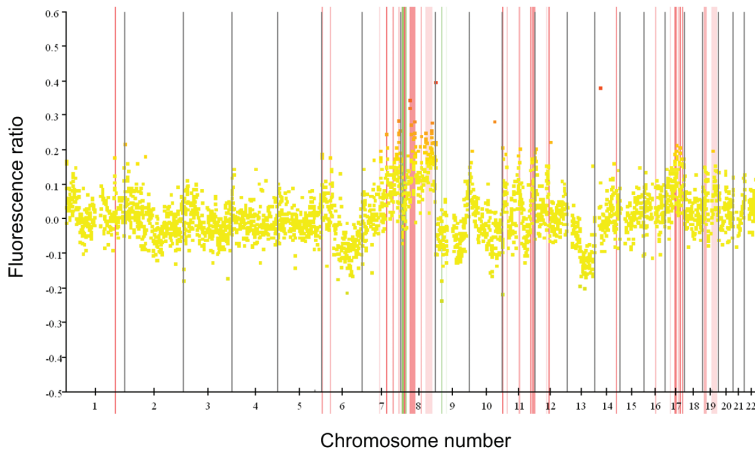
Figure 1 Visualisation of the array-based CGH data using VAMP
Chromosomes are indicated on the horizontal axis; excluded are the X and Y chromosomes. Gains are depicted in red and losses in green. (A) Frequency of Amplicon, Gain and Loss (FrAGL) plot for C-ALCL tumor samples. (B) Averaged CGH pattern of C-ALCL tumor samples. MCRs with loss occurring in at least 35% of patients are indicated as green vertical lines and MCRs with gain as red vertical lines. (C) FrAGL plot for PTL-NOS tumor samples. (D) MCRs with loss occurring in at least 35% of patients indicated as green vertical lines and MCRs with gain indicated as red vertical lines in the averaged CGH pattern of PTL-NOS tumor samples.

corresponding chromosomal region in the two lymphomas was evaluated. As apparent in Figure 2, many genes relatively over expressed in C-ALCL were located in a chromosomal region that was frequently affected by gain in C-ALCL (*TNFRSF8/CD30*, *CCR7*, and *CCR10*), or less often in a region with loss in PTL-NOS (*TMOD1*).

C



D



Conversely, the *GIMAP 1, 4* and *6* genes, relatively over expressed in PTL-NOS, reside in chromosomal regions commonly showing gain in this malignancy. The number of samples per diagnostic group was insufficient to perform a comprehensive integrative analysis by correlating expression of each individual gene to the presence of CNA of the corresponding chromosomal region.

Pathway analysis reveals that C-ALCL and PTL-NOS differ in particular with respect to expression of genes involved in chemotaxis, apoptosis, and lymphocyte proliferation

Next, to interpret the comparative gene expression data and extract information regarding biological processes and signaling pathways that may distinguish these lymphomas, we performed knowledge-based pathway analysis. Gene Ontology analysis of the most differentially expressed genes revealed significant enrichment of 6 GO clusters, these are depicted in Figure 2c with their respective *P*-values. Genes belonging to these enriched clusters are marked in Figure 2a and b. The lymphoma types C-ALCL and PTL-NOS in particular differed in expression of gene clusters with a role in chemokine receptor activity (*CCR10, CCR7, CNTNAP1, CCR8*), apoptosis (*TNFRSF8/CD30, JMY, RFFL, TMEM23/SGMS1, TRAF1, HIP1, PMAIP1, CDKN2C/p18*) and lymphocyte proliferation (*PRKCQ*). This result is consistent with the observed difference in extracutaneous dissemination and treatment resistance of these CTCLs.

Quantitative real-time PCR and immunohistochemistry confirm gene expression results

To validate the results of gene expression analysis, transcript abundance of selected genes was measured using qPCR in the C-ALCL and PTL-NOS tumor samples as well as in CD4⁺ T cells from healthy individuals used as benign reference (Figure 3). Genes with a role in chemotaxis (*CCR10, CCR7*), apoptosis (*TNFRSF8/CD30, TRAF1*), T-cell activation and proliferation (*IRF4/MUM1, PRKCQ*) were analyzed.

In addition, expression levels of the cytokine receptor gene *IL23R* and of the oncogenic Polycomb gene *EZH2* were quantified. qPCR analysis confirmed significant differential expression found in microarray-based gene expression analysis of all tested genes. Expression levels differed between C-ALCL and PTL-NOS with fold changes ranging from 3.8 (*TRAF1*) to 131.5 (*CCR7*). The genes encoding the chemokine receptors *CCR10* and *CCR7* appeared to be selectively expressed in tumors of C-ALCL patients. Moreover, the expression of *IRF4/MUM1*, a transcription factor that regulates T-cell apoptosis, was markedly higher in C-ALCL than in PTL-NOS or CD4⁺ T cells. Expression of *TRAF1*, encoding a protein that relays signals from *TNFRSF8/CD30*, was significantly higher in C-ALCL than

in PTL-NOS. Conversely, transcript levels of *PRKCQ* were higher in PTL-NOS than in C-ALCL and CD4⁺ T cells (fold change 10.9 and 4.6 respectively). The *PRKCQ* gene is exclusively expressed by T cells and functions as a downstream target of the T-cell receptor, relaying signals required for activation and survival following stimulation by antigen.³⁰⁻³² Abundant expression of *PRKCQ* has been noted previously in T-cell leukemias and lymphomas.³³ For these reasons we considered it a prime candidate oncogene in PTL-NOS. As oncogenes, in particular kinases, can be activated through aberrant over expression as well as through activating mutations, we performed analysis of the coding region of *PRKCQ* for mutations potentially resulting in constitutive activity of its kinase domain. This failed to reveal any mutations in the included PTL-NOS tumor samples (data not shown).

In addition, the expression of *IRF4/MUM1* and *TRAF1* was investigated on the protein level using immunohistochemistry. Stainings were carried out on samples included in this study for genomic profiling as well as on additional C-ALCL cases not included in this study. Staining of more than 50% of the neoplastic T cells for IRF4/MUM1 and TRAF1 was observed in 31 of 31 (100%) and 26 of 31 (84%) cases of C-ALCL, but not in any of the 15 PTL-NOS cases. Results of exemplary stainings are shown in Figure 4. In a report on TRAF1 expression, Assaf and colleagues³⁴ described strong TRAF1 expression in only 1 of 28 (4%) C-ALCL cases. Kempf and colleagues have described IRF4/MUM1 expression in only 2 out of 10 cases of C-ALCL.³⁵ Consistent with our results, more recent immunohistochemical studies have demonstrated IRF4/MUM1 and TRAF1 expression in 80-100% of C-ALCL cases.³⁶⁻³⁸

Table 2A Minimal common regions C-ALCL

Chr	Cytogenetic band	CNA	Clone Start	Clone End	Position Start	Position End	# Cases affected	Genes
6	6q16-6q21	Loss	RP3-454N4	RP3-454N4	104974085	107020676	5	PRDM1*
7	7q31	Gain	RP11-328M22	RP11-126C19	110975058	118629320	5	MET
13	13q34	Loss	RP11-310D8	RP11-265C7	110681700	114142980	5	CDC16*, CUL4A*
1	1p36.2	Gain	RP11-285P3	RP11-285P3	12462984	15425500	4	
6	6q25-6q27	Loss	RP3-336G18	RP1-137D17	156122661	170373241	4	MLLT4, FGFR1OP
7	7q21	Gain	RP11-28I21b	RP5-1093O17	80528144	84729683	4	
7	7q21	Gain	RP11-212B1	RP5-1084H12	85485924	91973205	4	AKAP9
7	7q21	Gain	RP11-101N13	RP5-1145A22	92090477	97314287	4	
7	7q21-7q22	Gain	RP4-550A13	RP11-44M6	97314794	100976355	4	
7	7q32-7q34	Gain	RP11-193I17	RP11-269N18	130792900	139186224	4	TIF1
7	7q34	Gain	RP11-237G17	RP5-894A10	140558165	142236215	4	
7	7q35	Gain	RP5-819O4	RP4-811H12	144233517	147084269	4	
7	7q35-7q36	Gain	RP5-1136G13	RP4-800G7	147259180	149650854	4	
8	8p22-8p21	Loss	RP11-369E15	RP11-529P14	18644291	22394183	4	
13	13q12-13q14	Loss	RP11-550P23	RP11-351K3	29008462	45700578	4	FOXO1A, BRCA2, LHFP, LCP1
17	17q12-17q21	Gain	RP11-94L15	RP11-58O9	34979298	36236729	4	ERBB2, RARA
21	21q22	Gain	RP5-1031P17	RP1-171F15	39702870	41559383	4	
1	1p36.3	Loss	RP4-785P20	RP11-49J3	1145847	7059893	3	
1	1q21-1q23	Gain	RP1-13P20	RP11-15G16	148424834	161212962	3	NTRK1, PRCC, TPM3, SDHC, ARNT, FCGR2B, MUC1
3	3p26-3p25	Loss	RP11-167K17	RP11-329A2	6636703	8869825	3	
6	6p21.3	Gain	RP5-1077I5	RP1-93N13	32200774	33467588	3	
7	7p22-7p21	Gain	RP4-733B9	RP5-1100A7	6234587	12919070	3	PMS2

follow up table 2A

Chr	Cytogenetic band	CNA	Clone Start	Clone End	Position Start	Position End	# Cases affected	Genes
8	8p23-8p22	Loss	RP11-104F14	RP11-19N21	1706678	17782553	3	
16	16p11.2-16q11.2	Loss	RP11-274A17	RP11-274A17	32345186	45067244	3	
17	17p13	Loss	RP11-186B7	RP11-199F11	7063252	7563870	3	TP53
17	17p11.2	Gain	RP5-836L9	RP11-121A13	19748613	21055083	3	USP6
17	17q21-17q22	Gain	RP11-361K8	RP11-312B18	43043105	49753469	3	COL1A1
17	17q23	Gain	RP11-178C3	RP11-156L14	54872929	59626448	3	CLTC, NACA, BRIP1
17	17q25	Gain	RP11-87G24	GS-362-K4	72271167	78774742	3	MSF
17	17p11.2	Gain	RP1-162E17	RP1-162E17	18114679	19211694	3	

Start and end positions are delineated by the nearby borders of adjacent clones. Genes are the cancer census genes situated in the subsequent MCRs. In addition, genes indicated with an asterisk are referred to in the results and discussion sections.

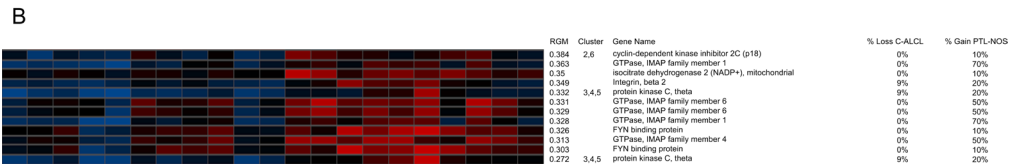
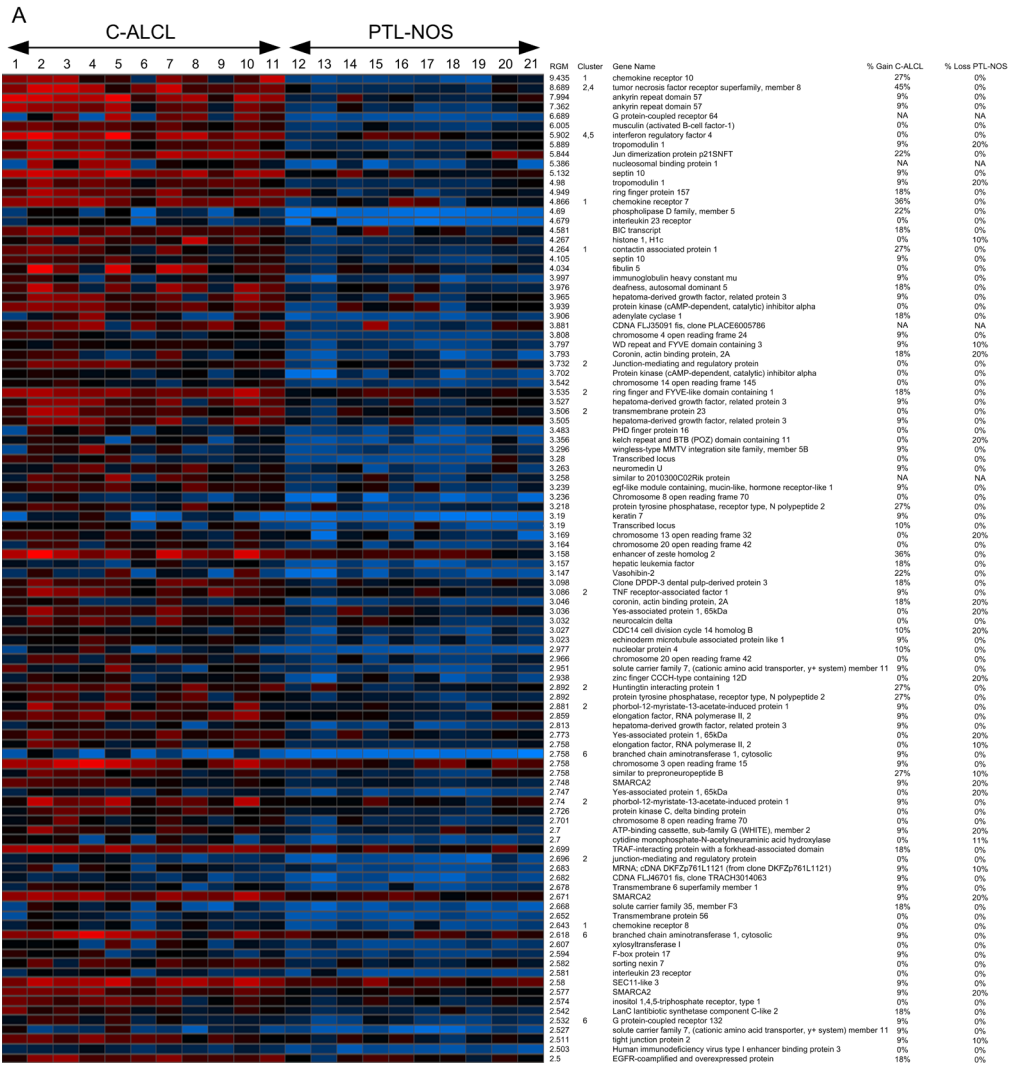
Table 2B Minimal common regions PTL-NOS

Chr	Cytogenetic band	CNA	Clone Start	Clone End	Position Start	Position End	# Cases affected	Genes
7	7q36	Gain	RP4-548K24	RP4-548D19	149838701	151558264	6	FASTK *
7	7q21-7q22	Gain	RP4-550A13	RP5-1059M17	97314794	101175494	5	
8	8p12-8q12	Gain	RP11-44K6	RP11-114M5	38488992	61676214	5	FLAG1
8	8q21.1-8q21.3	Gain	RP11-34M16	RP11-120I21	82095683	87610633	5	
8	8q22-8q24.2	Gain	RP11-21E8	RP11-3O20b	101431725	130562466	5	MYC, EXT1
7	7q11.2	Gain	RP11-313P13	RP11-107L23	71274704	76190020	4	HIP1
7	7q32	Gain	RP11-128A6	RP5-999D10	127266861	129658047	4	SMO
8	8p23	Gain	GS-580-L5	RP11-104F14	0	2817096	4	
8	8p23-8p22	Gain	RP11-589N15	RP11-809L8	10796421	18447618	4	PCM1
9	9p13-9p12	Loss	RP11-211N8	RP11-211N8	40774565	46771375	4	
12	12q13	Gain	RP5-1057I20	RP11-302B13	46299979	48258682	4	
17	17p11.2	Gain	RP11-189D22	CTB-1187M2	17521462	19614077	4	USP6
17	17q21	Gain	RP5-1110E20	RP5-1169K15	35920940	41095565	4	BRCA1, ETV4,
17	17q21	Gain	RP11-220N20	RP11-506D12	41565972	46429569	4	COL1A1
17	17q25	Gain	RP11-478P5	RP11-313F15	69639765	77988928	4	MSF
1	1q32	Gain	RP11-383G10	RP11-534L20	202933682	205087972	3	SLC45A3
6	6p21.3	Gain	RP1-93N13	RP11-175A4	32498505	33876274	3	
8	8p23	Loss	RP3-461F17	RP11-177H2	2304174	11802558	3	
8	8p22-8p21	Loss	RP11-161I2	RP11-529P14	18450010	22394183	3	
9	9p21	Loss	RP11-149I2	RP11-149I2	21158465	22479496	3	CDKN2A- p14ARF, CDKN2A -p16(INK4a)
11	11p15	Gain	CTC-1277H1	CTC-1277H1	16774089	18095206	3	
11	11q13	Gain	RP11-126P21	RP5-901A4	66035373	68072319	3	

follow up table 2B

Chr	Cytogenetic band	CNA	Clone Start	Clone End	Position Start	Position End	# Cases affected	Genes
11	11q13	Gain	RP11-554A11	RP11-31L22	68278585	72378220	3	CCND1, NUMA1
11	11q23-11q25	Gain	RP11-35P15	RP11-215D10	116368601	120790891	3	MLL, CBL, ARHGEF12, PCSK7, BM-PR1A, DDX6, PAFAH1B2
11	11q24-11q25	Gain	RP11-432I22	GS-770-G7	124761526	134156487	3	FLI1
12	12p13	Gain	GS-124-K20	RP11-264F23	0	5163995	3	CCND2
12	12q13	Gain	RP11-474N8	RP11-181L23	55007887	56286203	3	DDIT3
14	14q31-14q32	Gain	RP11-257P13	RP11-257P13	88987033	91389787	3	
16	16q12.1	Gain	RP11-305A7	RP11-147B17	47397787	49618230	3	CYLD
17	17q22_27	Gain	RP11-372K20	RP11-112J9	49922957	52616475	3	HLF
17	17q22-17q23	Gain	RP11-567L7	RP11-332H18	54280512	57301021	3	CLTC, NACA, BRIP1
17	17q23-17q24	Gain	RP11-156L14	RP11-89H15	58038015	61192768	3	
19	19p13.3-19p13.1	Gain	RP5-859H16	RP11-943H6	5138666	19023191	3	ELL, TPM4, BRD4, LYL1, MLLT1
19	19q12-19q13.4	Gain	CTC-325L16	GS-325-I23	36979850	63806651	3	ERCC2, BCL3, AKT2, CEBPA, CIC, TFPT, ZNF331

Start and end positions are delineated by the nearby borders of adjacent clones. Genes are the cancer census genes situated in the subsequent MCRs. In addition, genes indicated with an asterisk are referred to in the results and discussion sections.



C

Cluster	Name	Enrichment score	P-value
1	C-C chemokine receptor activity	1.79	7.91E-05
2	apoptosis	1.65	0.011865
3	positive regulation of lymphocyte proliferation	1.55	0.011155
4	positive regulation of cytokine biosynthetic process	1.38	0.017689
5	lymphocyte activation	1.1	0.057936
6	G1/S transition of mitotic cell cycle	1.1	0.015366

Discussion

C-ALCL and primary cutaneous PTL-NOS are two distinct types of CTCL that are both derived from skin-homing T cells, but show marked differences in clinical behavior and prognosis. Array-based CGH analysis allowed identification of recurrent chromosomal alterations harboring candidate oncogenes and tumor suppressor genes in these lymphoma types. Although C-ALCL and PTL-NOS both demonstrated gains of large regions on chromosome 7 and 17, C-ALCL was distinguished by additional losses on chromosome 6 and 13 and PTL-NOS by gains on chromosome 8 and losses on chromosome 9. Detailed analysis of CNAs in C-ALCL showed that the most highly recurrent MCR with gain was located on 7q31 and loss on 6q16-6q21 and 13q34, each affecting 45% of the patients. The focal MCR with gain on 7q31 harbors the *MET* oncogene. *MET* amplification has previously been shown to result in its increased expression in nodal ALCL³⁹ and deregulated expression has been noted in acute T-cell leukemia.^{40,41} The MCR with loss on 6q16-6q21 contains the *PRDM1/BLIMP-1* gene encoding a transcription factor that is implicated in T-cell homeostasis and differentiation. In mice lacking PRDM1/Blimp-1 activity accumulation of CD4⁺ T cells is observed.^{42,43} Other cancer-associated genes located in regions with recurrent CNA with potential relevance in the pathogenesis of C-ALCL include *FOXO1A* and *BRCA2* on 13q12-13q14 (loss in 36%), *PRDM16/MEL1* on 1p36 (gain in 27%) and *TP53* on 17p13 (loss in 27% of patients). We have identified previously unreported recurrent chromosomal alterations in C-ALCL and confirmed several gains and losses found in the few studies in which conventional CGH was applied. Consistent with our results, Zettl *et al.* observed gain of regions on 7q and 6p in 2 of 11 C-ALCL samples.¹¹ Mao described gains of 1p, 5, 6, 7, 8p and 19, showing partial overlap with our findings.⁹ In a group of 7 patients with relapsing C-ALCL Prochazkova and colleagues observed recurrent gain of regions on chromosome 9 and losses on chromosome 6 and 18.¹⁰ Furthermore in nodal ALCL recurrent CNAs affecting 7q and 13q have been described.^{11,44}

Figure 2 Comparative analysis of gene expression profiles of 11 C-ALCL and 10 PTL-NOS tumor samples

Heatmap depicting gene expression intensities of significantly differentially expressed genes (SAM algorithm, false discovery rate 0.1). Values are visualized according to the scale bar that represents the difference in expression relative to the mean expression. Red represents high, black represents intermediate and blue represent low expression. (A) Genes showing higher expression in C-ALCL compared to PTL-NOS with a Ratio of Geometric Means (RGM) higher than 2.5. (B) Genes expressed at lower levels in C-ALCL than in PTL-NOS with a RGM less than 0.4. The last two columns show the percentage of C-ALCL and PTL-NOS cases affected by chromosomal alterations at the locus where each differentially expressed gene resides. (C) Results of Gene Ontology analysis, revealing gene clusters involved in the biological processes for which significant enrichment was discovered. Genes belonging to these clusters are indicated in a separate column "cluster" in (A) and (B); the numbers refer to the clusters designated in (C).

In PTL-NOS the most frequently affected MCR with gain was 7q36. This focal region contains the anti-apoptotic *FASTK* gene that is over expressed in association with chromosomal gain in mycosis fungoides.^{27,45} Similar to primary cutaneous PTL-NOS studied herein, tumor cells of PTL-NOS presenting in lymph nodes have been reported to be affected by recurrent gains on chromosome 7q22-qter,¹¹ 17q11–q25 and 8q.⁴⁶ A major difference between the chromosomal alterations observed in PTL-NOS and C-ALCL concerns the occurrence of gains on chromosome 8. These were highly recurrent in PTL-

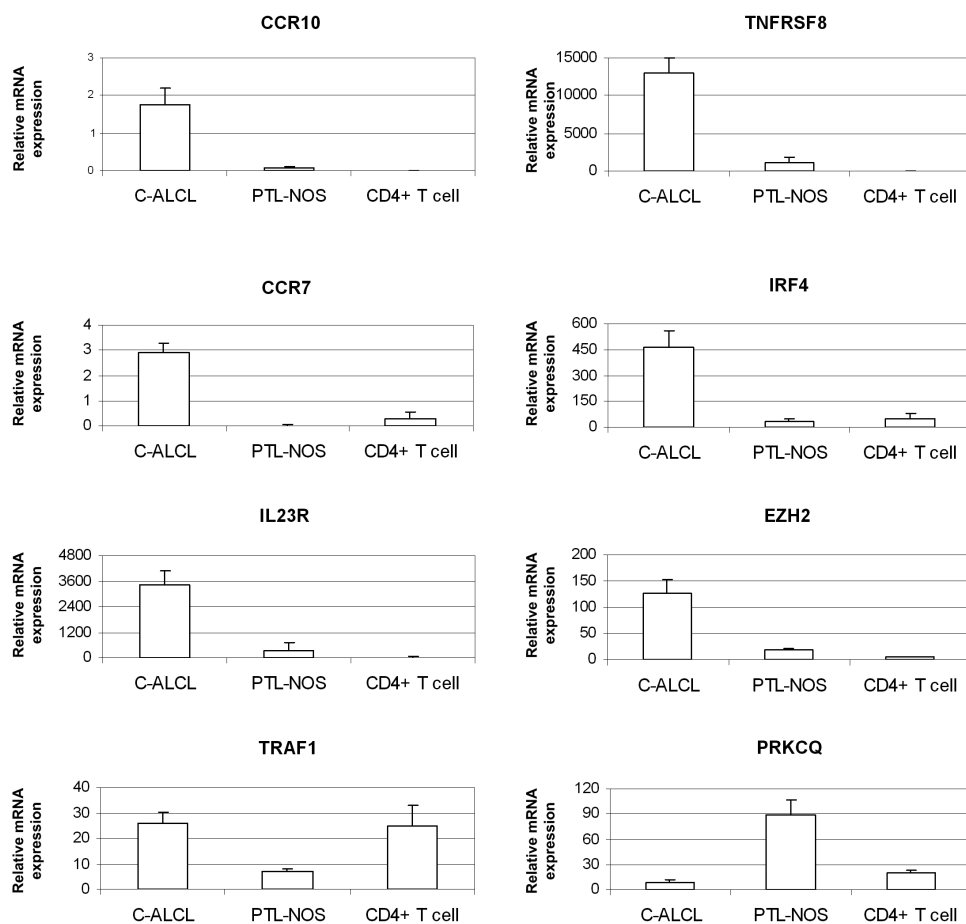


Figure 3 Expression levels of selected genes as measured by qPCR

Cumulative mRNA expression data of C-ALCL (n=11) and PTL-NOS (n=10) (mean $\pm$ S.E.M.). The mRNA expression was measured relative to *RPS11* and *U1A* used as reference genes. Fold change for transcript levels of genes with increased expression in C-ALCL compared to PTL-NOS: *TNFRSF8* 12.9, *IL23R* 9.7, *EZH2* 6.5, *IRF4* 13.0, *CCR10* 25.0, *CCR7* 131.5 and *TRAF1* 3.8. The fold change for the level of *PRKCQ* expression, over expressed in PTL-NOS compared to C-ALCL, was 10.9.

NOS, but did not or scarcely affect patients with C-ALCL. A MCR with gain on 8q22-8q24.2 affecting 50% of PTL-NOS patients contains the *MYC* oncogene that is amplified in patients with Sézary syndrome and aggressive B-cell lymphomas.^{28,29} Gain of chromosome 8q was previously recognized in aggressive CTCLs with shorter survival.^{12,47} Furthermore, we found that the 9p21 locus was affected by loss in 50% of the patients with PTL-NOS, whereas it was not deleted in any of the included C-ALCL patients. The 9p21 region contains the *CDKN2A* tumor suppressor gene, loss of which is associated with an unfavorable prognosis in patients with nodal and cutaneous lymphomas.⁴⁸ Our results contrast with those of Boni and colleagues, who detected loss of 9p21 in a subset of patients with C-ALCL.⁸

Secondly, gene expression analysis revealed marked differences in expression patterns of gene sets in C-ALCL and PTL-NOS. In an effort to unravel these patterns of differentially expressed genes and search for biological functions and signaling pathways distinct in these lymphomas, we applied unbiased Gene Ontology analysis. This demonstrated significant enrichment for gene clusters implicated in chemokine receptor activity, apoptosis, lymphocyte proliferation and several other biological processes. These observed differences may be associated with the differential clinical behavior of C-ALCL and PTL-NOS.

Chemokine receptors determine homing patterns of T cells and serve to mark specific T-cell subsets. C-ALCL tumor biopsies showed higher expression of the chemokine receptor genes *CCR10*, *CCR8* and *CCR7* relative to PTL-NOS. Interestingly, the genes encoding CCR10 and CCR7 are located in chromosomal regions that are frequently affected by copy number gain in C-ALCL. CCR10 marks a subset of memory T cells with skin-homing capacity.⁴⁹ Binding of its ligand CCL27, which is selectively produced in the skin, recruits T cells to the cutaneous microenvironment.⁵⁰ CCR10 expression has been described previously in mycosis fungoides and Sézary syndrome, but not specifically in C-ALCL or PTL-NOS.⁵¹⁻⁵³ Moreover, CCR8 is preferentially expressed by T cells resident in the skin.⁵⁴ The higher tendency of PTL-NOS lymphoid cells to display extracutaneous dissemination may result from lower affinity of these cells for the cutaneous microenvironment due to low expression of the skin-homing receptors *CCR10* and *CCR8*.

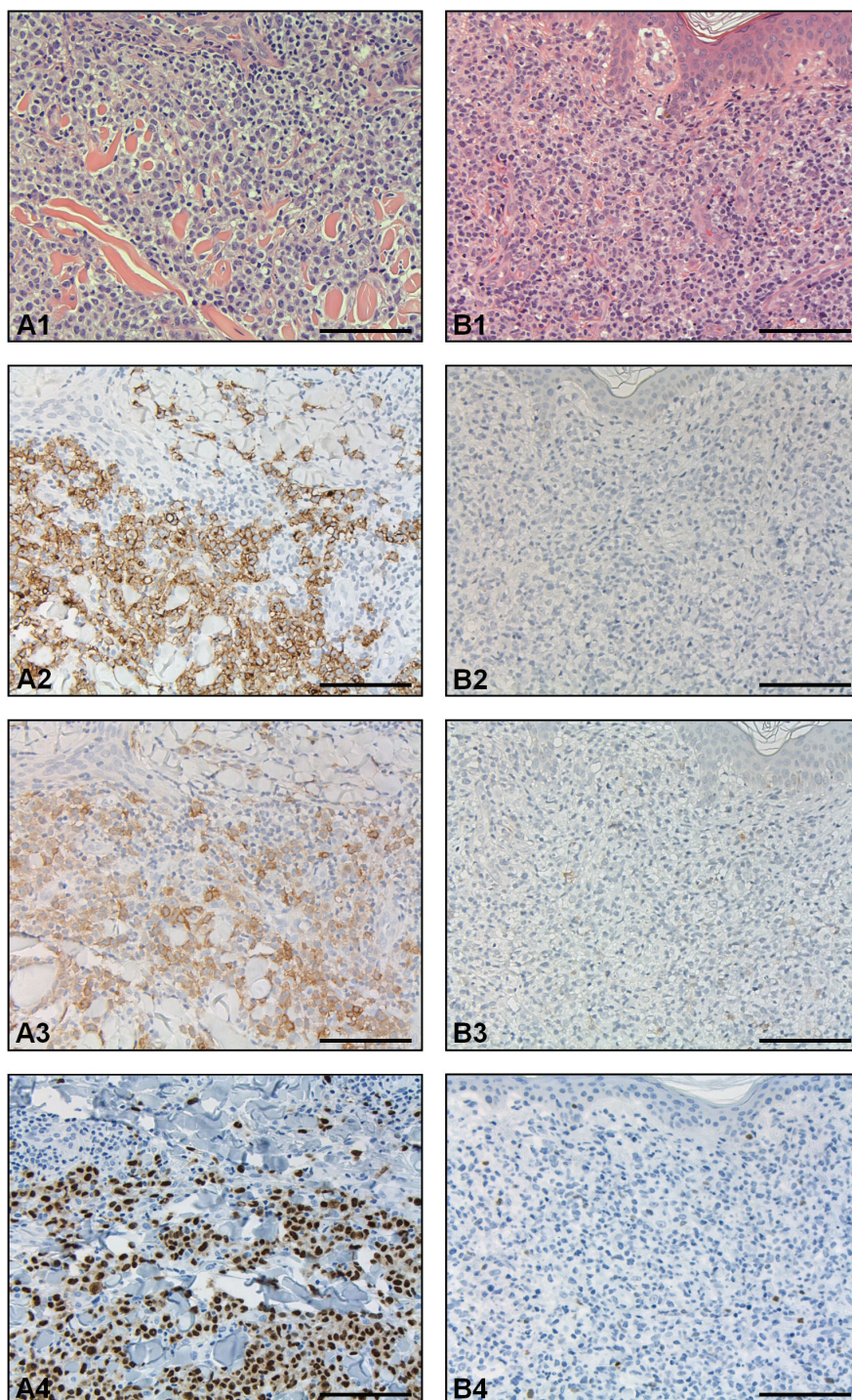
Unbiased comparative analysis of the gene expression programs of C-ALCL and PTL-NOS tumors revealed differential expression of a gene cluster involved in apoptosis. Defective apoptosis signaling is presumed to have an important role in the pathogenesis of C-ALCL and PTL-NOS, as the homeostasis of mature T cells from which these lymphomas are derived is predominantly governed by selective induction of cell death.⁵⁵ Both C-ALCL and PTL-NOS lymphoid cells are both assumed to show apoptosis impairment compared to benign CD4⁺ T cells from which these lymphomas are derived. The occurrence of spontaneous tumor regression in a subset of patients with C-ALCL and the higher

sensitivity of C-ALCL to therapy suggest that apoptosis impairment in this lymphoma type is less pronounced than in PTL-NOS. Accordingly, C-ALCL and PTL-NOS showed altered expression of different sets of apoptosis-regulatory genes. C-ALCL showed relative high expression of *IRF4/MUM1*, *TNFRSF8/CD30* and *TRAF1* and diminished expression of *CDKN2C/p18*. qPCR analysis and immunohistochemistry confirmed increased expression of *IRF4/MUM1* and *TRAF1* by C-ALCL lymphoid cells, also relative to normal CD4⁺ T cells. *IRF4/MUM1* protects CD4⁺ T cells against pro-apoptotic stimuli and activation-induced cell death.⁵⁶ Moreover, *TRAF1*, involved in the intracellular signal transduction of CD30 and other TNF receptors, is thought to protect T cells from apoptosis induced by various stimuli.⁵⁷ Therefore, aberrant expression of *IRF4/MUM1* and *TRAF1* may result in increased resistance to apoptosis in C-ALCL lymphoid cells. On the contrary, PTL-NOS was characterized by high expression of *PRKCQ* and diminished expression of *FAS* and *Caspase 10*. The *PRKCQ* gene relays signals required for T-cell activation and survival following stimulation by antigen.³⁰⁻³² It has been suggested that *PRKCQ* may have oncogenic activity in T-cell malignancies and may serve as a therapeutic target using PKC inhibitors.^{32,33,58} The *PRKCQ* gene was over expressed relative to C-ALCL as well as to normal CD4⁺ T cells. Additional mutational analysis failed to detect activating mutations in these PTL-NOS tumor samples. Loss of expression of the FAS receptor is another mechanism through which PTL-NOS tumor cells can acquire resistance to pro-apoptotic stimuli. The FAS mRNA expression data from this study are consistent with our previous results showing that protein expression of the FAS receptor is lost in the majority of PTL-NOS tumor cells, whereas it is expressed in C-ALCL.¹⁴

Taken together, C-ALCL and PTL-NOS have distinct patterns of chromosomal abnormalities, which may in part explain their different clinical behavior. We were able to identify several candidate oncogenes and tumor suppressor genes residing in MCRs with highly recurrent gains and losses in these T-cell lymphomas. The more aggressive clinical behavior of PTL-NOS may in part be related to chromosomal gains of regions on chromosome 8 and losses affecting 9p21, as decreased p16 and p14^{ARF} expression resulting from such loss has been found to predict poor prognosis in various lymphomas.⁵⁹ Importantly, the distinct clinical behavior of these CTCLs is paralleled by differences in their gene expression programs linked to T-cell homing, apoptosis and proliferation.

Figure 4 Immunohistochemical staining of TNFRSF8/CD30, IRF4/MUM1 and TRAF1 in C-ALCL and PTL-NOS

HE stainings demonstrating the morphology of C-ALCL (A1) and PTL-NOS (B1) tumor cells. The tumor cells of C-ALCL strongly express TNFRSF8/CD30 (A2), TRAF1 (A3) and IRF4/MUM1 (A4). PTL-NOS tumor cells do not or only scarcely show positive staining for TNFRSF8/CD30 (B2), TRAF1 (B3) and IRF4/MUM1 (B4). Bar = 100µm.



Lymphoid cells of patients with C-ALCL demonstrate higher expression of skin-homing receptors, which may explain their higher affinity for the skin and lower tendency to disseminate to extracutaneous sites than PTL-NOS tumor cells. Furthermore, C-ALCL and PTL-NOS are characterized by dysregulated expression of different sets of apoptosis-regulating genes, which may account for differences in treatment resistance and tendency to progress.

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7

General discussion

Studies in this thesis have been aimed to characterize the molecular mechanisms involved in the development and progression of different types of cutaneous T-cell lymphomas (CTCL), namely mycosis fungoides (MF), Sézary syndrome (Sz), primary cutaneous anaplastic large cell lymphoma (C-ALCL) and primary cutaneous peripheral T-cell lymphoma not otherwise specified (C-PTCL-NOS). In this final chapter, the results of these studies, together with those from recent literature, will be summarized and discussed. We will first discuss the results of array-based comparative genomic hybridization (aCGH) and gene expression profiling in tumor-stage MF (T-MF) and Sz. Since the relationship between both conditions is a matter of ongoing debate, we will focus on the similarities and differences between the two disease entities. In the second part, we will discuss the results of our studies on miRNAs in T-MF and Sz and the possible implication of miRNAs in the pathogenesis of these lymphomas. The third part of this chapter addresses two other types of CTCL, C-ALCL and C-PTCL-NOS. We performed aCGH and gene expression profiling to gain insight into the possible mechanisms underlying the different clinical behavior of these types of CTCL. The discussion will conclude with future perspectives.

Mycosis fungoides & Sézary syndrome

MF is the most common type of CTCL and generally has an indolent course with slow progression from patches to more infiltrated plaques and eventually tumors.¹ Sz is a malignant disease characterized by a triad of erythroderma, generalized lymphadenopathy and the presence of neoplastic T cells in the skin, lymph nodes and peripheral blood.¹ Sz has often been considered to represent a leukemic phase or variant of MF, with both malignancies originating from activated, skin-homing, memory T cells with cerebriform nuclei. For this reason, MF and Sz share the same classification and staging system and patients with these conditions are often included in the same clinical trials. However, in the recent WHO-EORTC classification and in the WHO classification of 2008 MF and Sz are included as separate disease entities based on their distinct clinical features and disease behavior (see Table 1, **Chapter 1**).^{1,2} There are also differences in the histopathologic findings of involved skin and lymph nodes in the two conditions. However, whether Sz syndrome should indeed be regarded as a separate type of CTCL or represents a leukemic phase of MF is still a matter of debate. We approached this issue by performing a detailed analysis of numerical chromosomal alterations present in the genomes of MF and Sz. The genomic architecture, especially the occurrence of highly recurrent pathogenic genetic alterations, can be characteristic of a tumor type. To further examine the notion that Sz

and MF might be distinct disease entities, we carried out a series of genomic analyses described in **Chapter 2**. We started with the delineation of recurrent numerical chromosomal alterations in malignant T cells from tumor-stage MF (T-MF) samples using aCGH. We subsequently evaluated whether this pattern corresponded to the highly recurrent gains and losses previously observed in Sz.³ An additional goal was to identify chromosomal regions that may have prognostic value. Finally, we sought to identify candidate oncogenes and tumor suppressor genes residing in chromosomal regions with recurrent copy number alteration by integration with gene expression data.

The detailed genomic profiles of chromosomal imbalances of MF tumor cells displayed marked differences with those previously identified in Sz cells using identical methods (See Table 2, **Chapter 2**). Numerical chromosomal alterations most frequently observed in T-MF include gain of 7q21-36 and 1p36.2 as well as loss of 5q13 and 9p21, whereas Sz is characterized by gain of 17q22-25 and 8q22-24, and loss of 17p13 and 10q25. Notably, several aberrations commonly observed in T-MF, such as 7q11.2, 7q21-7q22, 7q32-7q35 and 7q36 are not or infrequently seen in Sz, arguing against the notion that Sz represents an advanced stage of MF. Conversely gains involving 17q23, 17q22-17q23, 17q24-17q25 and 8q24.1-8q24.2 (harboring *MYC*) are most common in Sz and less frequent in T-MF (see Table 2, **Chapter 2**). Subsequently, we investigated the possible relationship between chromosomal alterations and clinical behavior in T-MF. We identified three chromosomal regions with a prognostic value, namely: loss of 9p21 harboring the *CDKN2A* tumor suppressor gene, gain of 8q24.3 and gain of 1q21-1q22.

Consecutive studies by others largely confirmed our results on gross chromosomal alterations in T-MF in independent patient cohorts^{4,5} supporting the validity of our findings (see Table 1). In addition, Salgado *et al.* confirmed the correlation of 9p21 loss and 8q24.3 gain with a poor prognosis in a larger group of T-MF patients.⁵ Laharanne and colleagues also described prognostic value for 9p21 loss and 8q gain. However, this was only significant for a large group of CTCL patients studied (including T-MF, Sz and C-ALCL), while similar correlations could not be made for separate entities, possibly due to subgroup size.⁴ Additional studies may reveal whether it will be possible to identify patients prone to disease progression by determining 9p21 loss and/or 8q24.3 gain in early-stage MF. Integration of DNA copy number alterations with gene expression data of 22 T-MF cases by investigating regions of DNA copy number gain for up-regulated genes and regions of loss for down-regulated genes revealed 253 transcripts up- or down-regulated in respective regions of gain or loss. Of these 253 transcripts, 23 are established cancer-associated genes reported in the literature. The most frequently altered minimal common region (MCR) of DNA copy number alteration (CNA), gain of 7q36 (Table 1), contains the anti-apoptotic gene *FASTK*,⁶ which is associated with increased expression.

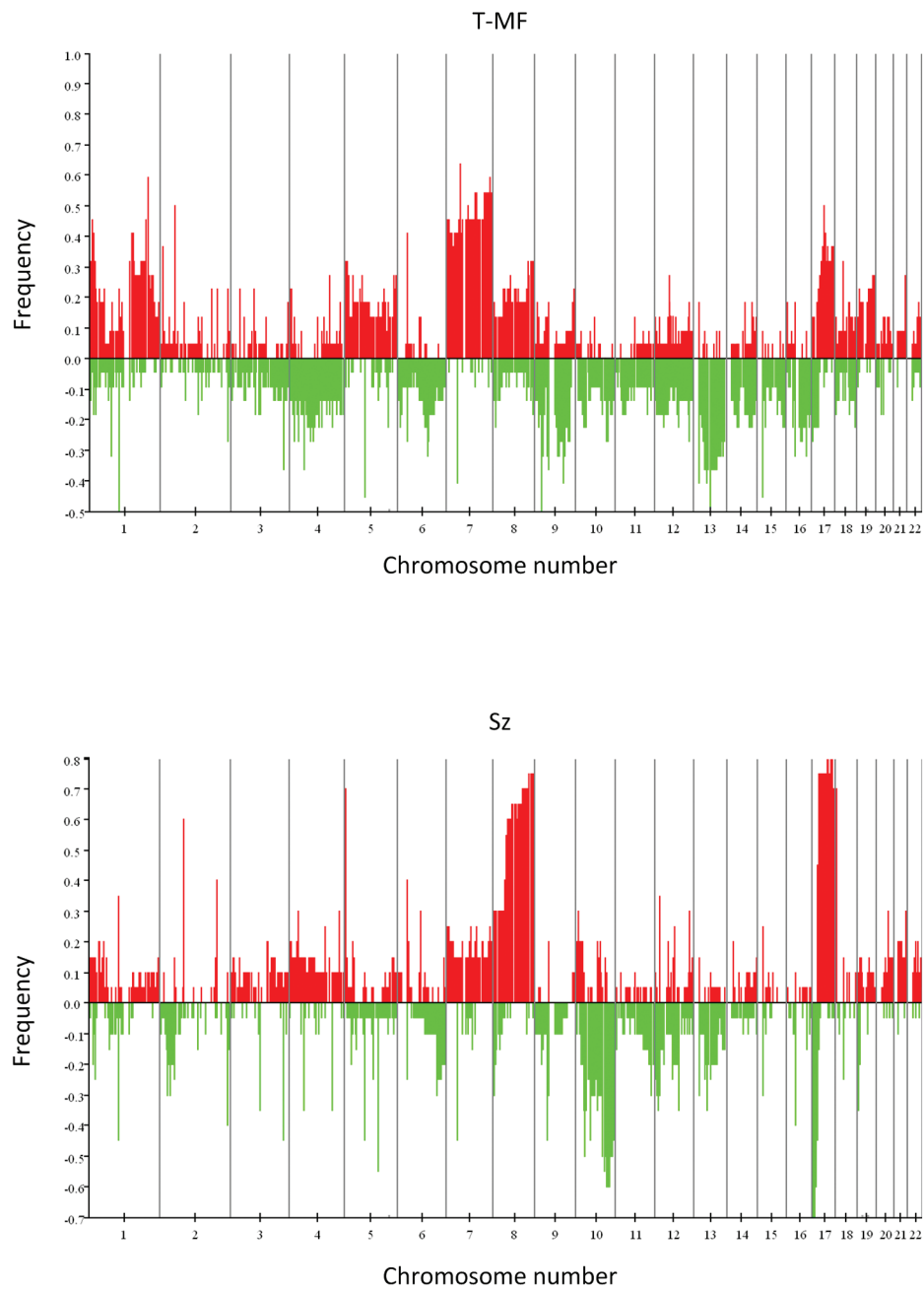
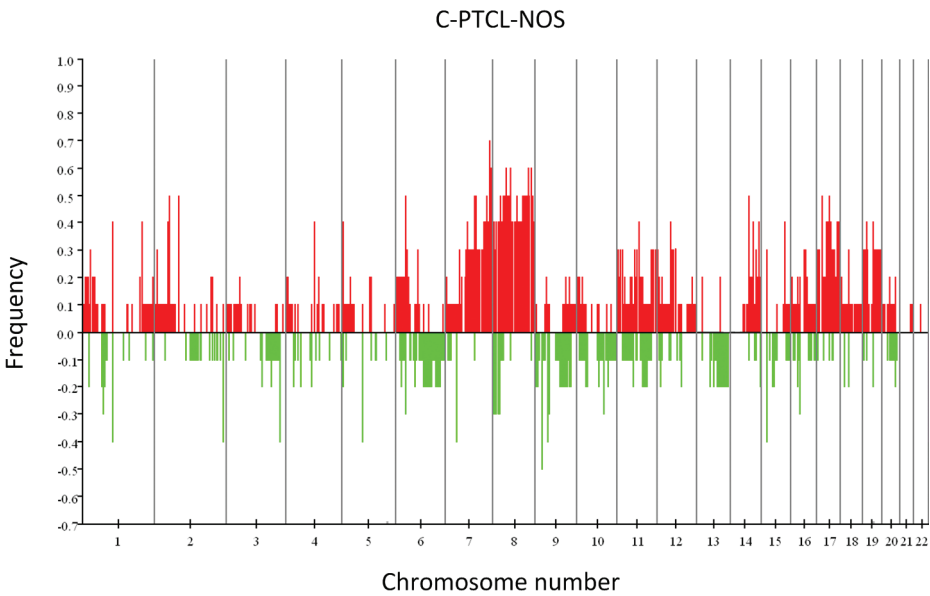
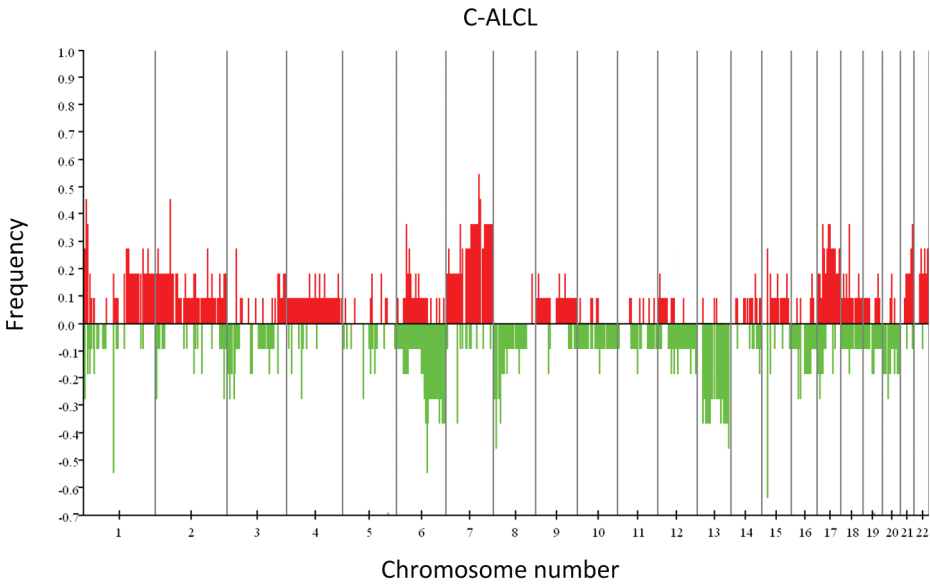


Figure 1 Comparison of Frequency of Amplicon, Gain, and Loss plots of Sz, T-MF, C-ALCL and C-PTCL-NOS



The tumor suppressor gene *CDKN2A*, located in a CNA with prognostic value (9p21), showed decreased expression consistent with previous and subsequent studies.⁷⁻⁹ Also consistent with previous reports, we found recurrent loss of 13q14 and diminished expression of the tumor suppressor gene *RB1*,¹⁰ which is located in this region.

In **Chapter 3** we generated a molecular signature of T-MF based on gene expression data. We employed a bioinformatic approach involving meta-analysis of publicly available gene expression data sets combined with gene expression data described in **Chapter 2**. Results for a selection of genes were further refined and validated by quantitative PCR and inclusion of additional controls. With this approach we identified a profile specific for T-MF consisting of 989 aberrantly expressed genes, the majority of which (718 genes) were higher expressed in T-MF compared to normal skin, inflamed skin, and normal T cells. As expected, the signature contains genes reflecting the proliferative character of this T-cell malignancy including altered expression of cell cycle and kinetochore regulators. Moreover, we found reduced expression of *NFKBIZ*, an inhibitor of the NF- κ B signaling pathway, possibly explaining enhanced activity of NF- κ B characteristic for CTCL,¹¹ and up-regulation of NF- κ B target genes. Furthermore, the MF tumor profile provided novel insights in the immunophenotype and skin-homing properties of this lymphoma, and revealed expression of possible therapeutic targets. Comparing these data with the Sz expression profile as determined by van Doorn and colleagues¹² shows that only 4 overlapping genes (*ACP2*, *ARPC4*, *ATP5J2*, *PTPRN2*) are up-regulated in T-MF and Sz. Likewise, a minimal overlap (6 out of 53) was observed between T-MF and the list of differentially expressed genes in Sz versus normal CD4⁺ T cells determined by Booken and colleagues (*CRIP1*, *KIR3DL2*, *CHN1*, *IL32*, *TNFSF11* and *CDCA7*)¹³ or Sz versus skewed Th2 cells (an overlap of only 12 genes out of 135 identified as being differentially expressed).¹⁴ Although these differences would support the notion that Sz and MF are different disease entities, these results should be considered to represent not more than an indication, as a similar lack in overlap was found when comparing our results with earlier gene expression studies in MF.¹⁵⁻¹⁷ This lack of consistency between results of MF gene expression studies is most likely due to the different platforms used to measure gene expression levels, dissimilar statistical methods employed as well as choice of controls for comparisons in the different studies. Recently, Campbell and colleagues proposed that Sz and MF are distinct diseases because they arise from different T-cell subsets.¹⁸ Whereas MF derives from the effector memory T cells, Sz arises from the central memory subset of CD4⁺ T cells. To conclude, the results of studies identifying DNA copy number alterations in Sz and T-MF support the notion that they are separate disease entities. Gene expression studies

also indicate differences between Sz and T-MF, however due to differences in experimental design solid conclusions regarding this matter cannot be drawn.

miRNA expression in Sz and T-MF

In **Chapter 4** we studied the miRNA expression profile of Sz by miRNA microarrays, identifying 114 differentially expressed miRNAs compared to normal CD4⁺ T cells. The majority (104 out of 114) of Sz-associated miRNAs were down-regulated and their expression pattern was generally consistent with previously reported genomic copy number abnormalities. However, similar to other studies,¹⁹ this correlation does not always hold true, implying the existence mechanisms of miRNA expression regulation other than copy number effect. To examine the gene regulatory function of dysregulated miRNAs, the previously identified list of up-regulated genes¹² was correlated with the down-regulated miRNAs by assessing which up-regulated genes were predicted targets of down-regulated miRNAs. Almost all (97 out of 104) of the down-regulated miRNAs were predicted to target one or more of these genes. Down-regulation of miR-342 for example coincides with over expression of its putative target *TNFSF11*, a gene encoding an anti-apoptotic protein.^{12,13} Transfection with miR-342 decreased the levels of *TNFSF11* and induced apoptosis in Seax cells, suggesting that down-regulation of miRNA-342 prevents apoptosis by up-regulation of *TNFSF11*. Reintroduction of miR-17-5p, part of the miR-17-92 cluster often described for its oncomir function,²⁰⁻²² in Seax cells resulted in increased apoptosis, and decreased proliferation implying a tumor suppressive role for miR-17-5p. Taken together these results suggest that altered miRNA expression plays a role in the pathogenesis of Sz.

To investigate the role of miRNAs in tumor-stage MF we initially determined the miRNA expression pattern of MF tumors by comparison with benign inflammatory dermatoses. Accordingly, we extracted a miRNA signature characteristic for T-MF, presented in **Chapter 5**. In contrast to Sz, we found that for T-MF that the majority (30 out of 49) of the differentially expressed miRNAs are up-regulated compared to the chosen benign controls. For most of the identified dysregulated miRNAs a role in cancer is described and several up-regulated miRNAs (miR-93, miR-155 and miR-17-92) have been validated functionally as oncomirs.^{20,22-25} In order to gain more insight into the correlation between gene expression and miRNA expression in T-MF, we subsequently searched for enrichment for miRNAs regulating genes that belong to the T-MF expression signature and identified 13 up-regulated miRNAs such as miR-93, miR-21 and miR-92a.

Although the same arrays were used to investigate miRNA profiles in Sz (**Chapter 4**) and

T-MF (**Chapter 5**), a direct comparison between miRNA expression patterns in these two diseases was not possible because different reference RNAs (activated tonsils versus synthetic RNA) and different miRNA sources (CD4⁺ T cells versus skin biopsies) were used. A preliminary screening comparing the lists of differentially expressed genes for both diseases showed minimal overlap, which favors the hypothesis that the two are different disease entities. However, due to the differences in control groups solid conclusions cannot be drawn.

In a recent study of our group using deep sequence technology, we not only confirmed increased expression of miR-214 and miR-199a* in Sézary cells compared to CD4⁺ T cells from healthy controls, but also compared to CD4⁺ T cells isolated from patients with erythroderma secondary to atopic dermatitis,²⁶ further suggesting a potential role for these miRNAs as diagnostic classifiers. Narducci *et al.* also identified miR-214 and miR-199a* up-regulation using a commercial assay-based miRNA expression detection platform. In this study, they also confirmed up-regulation of miR-7 and decreased expression of miR-342, miR-223, miR-92, miR-181a, and miR-191 in Sz.²⁷ Ralfkiaer *et al.* studied a heterogeneous group of CTCL in search of a classifier between CTCL and benign control samples. Due to the use of such a combined group, an overall comparison of these results with our data is not possible. Nevertheless, they did demonstrate high miR-155 expression in MF samples by miRNA-Q-PCR.²⁸ MiR-155 over expression in MF compared to normal skin samples was recently confirmed by Maj *et al.* also using miRNA-Q-PCR.²⁹

C-ALCL and C-PTCL-NOS

Few molecular genetic studies have been performed on CTCL types other than MF and Sz. In **Chapter 6** we describe the results of aCGH and gene expression profiling of C-ALCL and C-PTCL-NOS. Although both CTCLs present with skin tumors, the two lymphomas display a markedly different clinical course. C-ALCL shows a tendency towards spontaneous regression, uncommonly disseminate to extracutaneous sites and have an excellent prognosis with a 5-year survival exceeding 90%.³⁰⁻³² In contrast, C-PTCL-NOS quickly disseminates to extracutaneous sites, and has a poor prognosis with a 5-year survival of less than 15%.³³⁻³⁵ The aim of these studies was to find possible explanations for the different clinical behavior of these two entities.

C-ALCL and C-PTCL-NOS showed distinct patterns of DNA copy number alterations. C-ALCL was characterized by gains on chromosome 7q and 17q and losses on 6q and 13q. C-PTCL-NOS similarly demonstrated gains on 7q and 17q, but was distinguished by

gains on chromosome 8 and loss of minimal region on 9p21 (harboring the *CDKN2A* tumor suppressor gene). Detailed analysis of CNAs in C-ALCL showed that the most highly recurrent MCR with gain was located on 7q31 (harboring the *MET* oncogene) and loss on 6q16-6q21 (harboring transcription factor *PRDM1/BLIMP-1* implicated in T-cell homeostasis and differentiation) and 13q34, each affecting 45% of the patients. In C-PTCL-NOS the most frequently affected MCR with gain was 7q36 (harboring the anti-apoptotic gene *FASTK*); other frequent MCRs were 7q21-7q22, 8p12-8q12, 8p21.1-8q21.3 and 8q22-8q24.2. When comparing all CTCLs, one finds there was overlap between T-MF and C-PTCL-NOS tumors as both shared gains on 7q36, 7q21-7q22, 17q21, 17q22-17q23 and loss of 9p21 and in similar frequencies; however large discrepancies were shown on chromosome 8 and 13 (see Figure 1 and Table 1). Losses on chromosome 13 were frequently found in T-MF but rarely in C-PTCL-NOS. Gains on chromosome 8 were demonstrated at high frequencies in C-PTCL-NOS and Sézary syndrome, but at low frequencies in T-MF. Interestingly, although both C-PTCL-NOS and T-MF showed frequent loss of 9p21 with an established correlation with poor prognosis in T-MF (**Chapter 2**), C-ALCL lacked this loss, which might provide an explanation for the relatively good prognosis of C-ALCL.

Subsequent studies investigating C-ALCL with aCGH describe similar aberrations,^{4,36} but discrepancies were also found including gains on 7q and losses on 16q³⁶(see Table 1). When comparing the minimal common regions of C-ALCL with those identified in systemic ALK⁻ ALCL by conventional CGH, overlap is found at 6q16-q21 and 17q12-q21, but differences are found at locations such as 1q41-qter, 6q21-6q22, 13q21-13q22, 13q32-q33.^{37,38} Systemic ALK⁺ ALCL shows characteristic losses of chromosome 4 and 11^{37,38} not identified in C-ALCL and in low frequencies in ALK⁻ ALCL. In summary, different types of ALCL show different DNA copy number alterations.

C-PTCL-NOS

To date no other studies investigated the genomic profile of C-PTCL-NOS. Nodal PTCL-NOS show a heterogeneous pattern of alterations possibly reflecting the heterogeneous character of the group.³⁹ ACGH shows recurrent gains in chromosome 7q, 8q, 17q and losses in chromosome 5q, 6q, 9p, 10q, 12q and 13q.^{38,40-42} Similar to C-PTCL-NOS, gains on chromosome 8q, including 8q24 containing the *MYC* locus, were also described for nodal PTCL-NOS.^{38,42} Gain of chromosome 8q was described previously to indicate a shorter survival in CTCL.^{43,44} Besides loss of 9p21, gain of 8q could explain the more aggressive character of C-PTCL-NOS. However, the implication of other DNA copy number alterations on the pathogenesis requires further investigation.

Subsequently we studied the gene expression profile of C-ALCL and C-PTCL-NOS.

The lymphoma types C-ALCL and C-PTCL-NOS in particular differed in expression of gene clusters with regards to a role in chemokine receptor activity, apoptosis and lymphocyte proliferation. C-ALCL showed increased expression of the T-cell-homing receptors CCR10 and CCR8, which might explain their higher affinity for the skin and lower tendency to disseminate to extracutaneous sites. C-ALCL and C-PTCL-NOS lymphoid cells are both assumed to demonstrate apoptosis impairment compared to benign CD4⁺ T cells from which these lymphomas are derived. The occurrence of spontaneous tumor regression in a subset of patients with C-ALCL and the higher sensitivity of C-ALCL to therapy suggest that apoptosis impairment in this lymphoma type is less pronounced than in C-PTCL-NOS. C-PTCL-NOS is characterized by diminished expression of pro-apoptotic genes *FAS* and *Caspase 10*, which may contribute to the more clinically aggressive behavior of C-PTCL-NOS. Another class of genes dysregulated in C-PTCL-NOS possibly contributing to aggressiveness are those involved in proliferation. An example of this class is *PRKCQ*, exclusively expressed by T cells and a down-stream target of the T-cell receptor, transducing signals required for activation and survival.⁴⁵⁻⁴⁷ Moreover *PRKCQ* could be a therapeutic target using protein kinase inhibitors.⁴⁸ With gene expression analysis we confirmed high CD30 expression and showed high expression of *IRF4* and *TRAF1* mRNA in C-ALCL, which is in agreement with increased protein expression.⁴⁹ Wozniak and Piris noted that CD30/*IRF4*/*TRAF1* all act through the NF- κ B axis,⁵⁰ which is impaired in other types of CTCL. We also compared the gene expression profile of C-PTCL-NOS with that of MF tumors, and noticed that they were highly similar.

Conclusion and future perspectives

Our studies of DNA copy number alterations and gene expression in CTCL support the notion that MF and Sz should be considered as separate diseases. Therefore MF and Sz cases should be stratified accordingly in clinical trials. Our studies provide clues regarding the molecular pathogenesis of the different types of CTCL underlying the clinical behavior and prognosis. Interestingly, we found that T-MF and C-PTCL-NOS tumors share several chromosomal alterations and show highly similar gene expression profiles. Our miRNA array analysis identified many aberrantly expressed miRNAs in Sz and T-MF. Though our genome-wide studies provided many new insights, exact mechanisms explaining aberrant expression and the functional consequences of altered gene and miRNA expression in CTCL remain to be revealed. Therefore the following lines of inquiry for future research are proposed.

Table 1 Schematic overview of results of aCGH studies investigating Sz^{2,4}, T-MF^{4,5}, C-ALCL^{5,36} and C-PTCL-NOS

Number of cases	T-MF		Sz		C-ALCL		C-PTCL-NOS	
	This thesis n=22	Salgado et al n=41	Laharanne et al n=24	Vermeer et al n=20	Laharanne et al n=16	This thesis n=11	Sánchez-S. et al n=19	This thesis n=10
1p gain	1p36.2 (45%)					1p36.2 (36%)	1p36.32 (16%) 1p36.31 (16%)	
1q gain	1q21-22 (41%) 1q31-32 (45%)	1q31.2-q32.2 (15%)	1q25-31 (35%)			1q21-23 (27%)		1q32 (30%)
1p loss						1p36.3 (27%)	1p36.3 (30%)	
2q loss				2q37 (40%)				
3p loss						3p26-p25 (27%)		
5q loss	5q13 (45%)			5q13 (45%)				
6p gain						6p21.3 (27%)		6p21.3 (30%)
6q loss		6q21.3 (17%)				6q16-q21 (45%) 6q25-q27 (36%)	6q27 (40%)	
7p gain	7p22-p21 (45%) 7p15-p14 (41%) 7p14-p13 (45%)		7p22.1-11.2 (50%)			7p22-p21 (27%)	7p11.1-q11.2 (45%)	
7q gain	7q11.2 (50%) 7q21-22 (55%) 7q32-35 (55%) 7q36 (59%)	7q33.3-q35 (55%) 7q31 (50%)	7q21 (60%) 7q31 (50%)			7q21-22 (36%) 7q31 (45%) 7q32-34 (36%) 7q35-36 (36%)	7q11.2 (40%) 7q21-q22 (50%) 7q32 (40%) 7q36 (60%)	
7p loss				7p14 (45%)				
8p gain				8p11.2-p11.1 (40%)				8p23-8p22 (40%) 8p12-8q12 (50%)
8q gain	8q24.2 (32%) 8q24.3 (36%)	8q24.21 (32%)	8q11.2-q12 (60%) 8q12-21.1 (65%) 8q22-23 (70%) 8q24.1-24.2 (75%) 8q24.2-24.3 (75%)		8q23-q24.3 (41%)			8q21.1-21.3 (50%) 8q22-24.2 (50%)

follow up table 1

Number of cases	T-MF		Sz		C-ALCL		C-PTCL-NOS
	This thesis n=22	Salgado et al n=41	Laharanne et al n=24	Vermeer et al n=20	Laharanne et al n=16	Sánchez-S. et al n=19	
8p loss							This thesis n=10
9q gain		9q34 (17%)					8p23 (30%) 8p22-21 (30%)
9p loss	9p21 (41%) 9p13-p11.1 (32%)	9p21.3 (42%)	9p21 (30%)	9p13.1-p12 (45%)			9p21 (30%) 9p13-p12 (40%)
9q loss	9q21 (32%) 9q22-q31 (32%)	9q31.2 (30%)	9q21-q22 (35%)				
10p gain		10p14 (17%)					
10p loss		10p11.22 (17%)	10p11.2 (30%)	10p11.2 (40%)	10p12-p11.2 (41%)		
10q loss				10q22 (40%)			
				10q23.31 (50%)			
				10q23.32 (50%)	10q22-q24 (47%)		
			10q26 (40%)	10q24-q25 (60%)	10q25-q26 (41%)		
				10q25.2 (60%)			
11p gain				10q25-q26 (50%)			11p15 (30%)
11q gain							11q13 (30%) 11q23-q25 (30%)
11q loss					11q22-q23 (30%)		
12p gain							12p13 (30%)
12q gain							12q13 (40%)
13q loss	13q14-q31 (36%)	13q14.11 (20%)	13q34 (40%)	13q14 (35%)		13q14.3 (21%) 13q21.32 (21%) 13q33.3 (26%)	
14q gain							14q31-14q32 (30%)

follow up table 1

Number of cases	T-MF		Sz		C-ALCL		C-PTCL-NOS		
	This thesis n=22	Salgado et al n=41	Laharanne et al n=24	Vermeer et al n=20	Laharanne et al n=16	This thesis n=11	Laharanne et al n=18	Sánchez-S. et al n=19	This thesis n=10
16p gain								16p13.3 (21%)	
16p loss						16p11.2-q11.2 (27%)	16p11.2-16p11.1 (45%)	16p13.13 (21%) 16p13.12 (21%)	
16q loss		16q23.2 (17%) 16q24.3 (17%)	16q21-q22 (30%) 16q23-q24 (35%)					16q11.2 (32%) 16q12.1 (37%) 16q21 (32%) 16q22.1 (37%) 16q24.3 (37%)	16q12.1 (30%)
17p gain				17p11.2 (45%)		17p11.2 (27%)			17p11.2 (40%)
17q gain	17q21 (41%) 17q22-q23 (32%) 17q25 (36%)	17q21.1 (37%)	17q12 (30%)	17q21.31 (70%) 17q23 (85%) 17q24-q25 (80%)	17q23-q24 (35%)	17q12-q21 (36%) 17q21-q22 (27%) 17q23 (27%) 17q25 (27%)			17q21 (40%) 17q22 (30%) 17q22-q23 (30%) 17q23-q24 (30%) 17q25 (40%)
17p loss		17p13.1 (27.5%)		17p13.3- p13.1(55%) 17p13.1 (75%) 17p12 (70%)	17p13-q11.1 (47%)	17p13 (27%)		17p13.1 (21%)	
19p gain								19p13.3-p13.1 (30%)	
19q gain								19q12-q13.4 (30%)	
20p loss							20p11.1-20q11.2 (30%)		
20q loss								20q13.13 (21%)	
21q gain						21q22 (36%)			

In addition to more detailed identification of genetic alterations (e.g. using exome, whole genome or RNA deep sequence analysis), confirmation of identified dysregulated genes and miRNAs has proven to be essential and further studies confirming abnormal expression of single genes and miRNAs are recommended to be followed by functional studies. Functional studies, either the down-regulation of specific genes or miRNAs with inhibitors, or transfection of cells with the gene or miRNA of interest, followed by investigation of the effects on target gene expression, apoptosis and proliferation could elucidate the specific role genetic alterations play in the molecular pathogenesis of CTCL. It is recommended to study the effects of *NFKB1Z* transfection in Myla cells on other members NF- κ B pathway and on proliferation and apoptosis. Likewise, it would be interesting to study the effect of inhibition of *FASTK* in MF and *PRKCQ* in C-PTCL-NOS. Primary miRNA candidates would be miR-17, being up-regulated in MF and down-regulated in Sz, whether inhibition induces apoptosis and diminishes proliferation in Myla cells. Other primary candidates for inhibition would be miR-214 in Sz and miR-155 in MF. These *in vitro* studies could show the validity of the therapeutic targets and be the subsequent step to *in vivo* studies. Further research assessing the miRNA expression levels, for example miR-16, miR-17 and miR-93, between Sz and T-MF directly by investigating isolated tumor cells, as well as identification and examination of (the expression levels of) target genes could provide clues regarding the different mechanisms of action in Sz and T-MF. Furthermore, studying dysregulated genes and miRNAs in patch and plaque-stage MF could potentially teach us more about disease progression. In **Chapter 3** a start was made for some genes, but a more thorough validation is recommended, such as an independent patient group, with additional controls, and immunohistochemistry permitting evaluation of protein expression in tumor cells within a background of tumor infiltration lymphocytes.

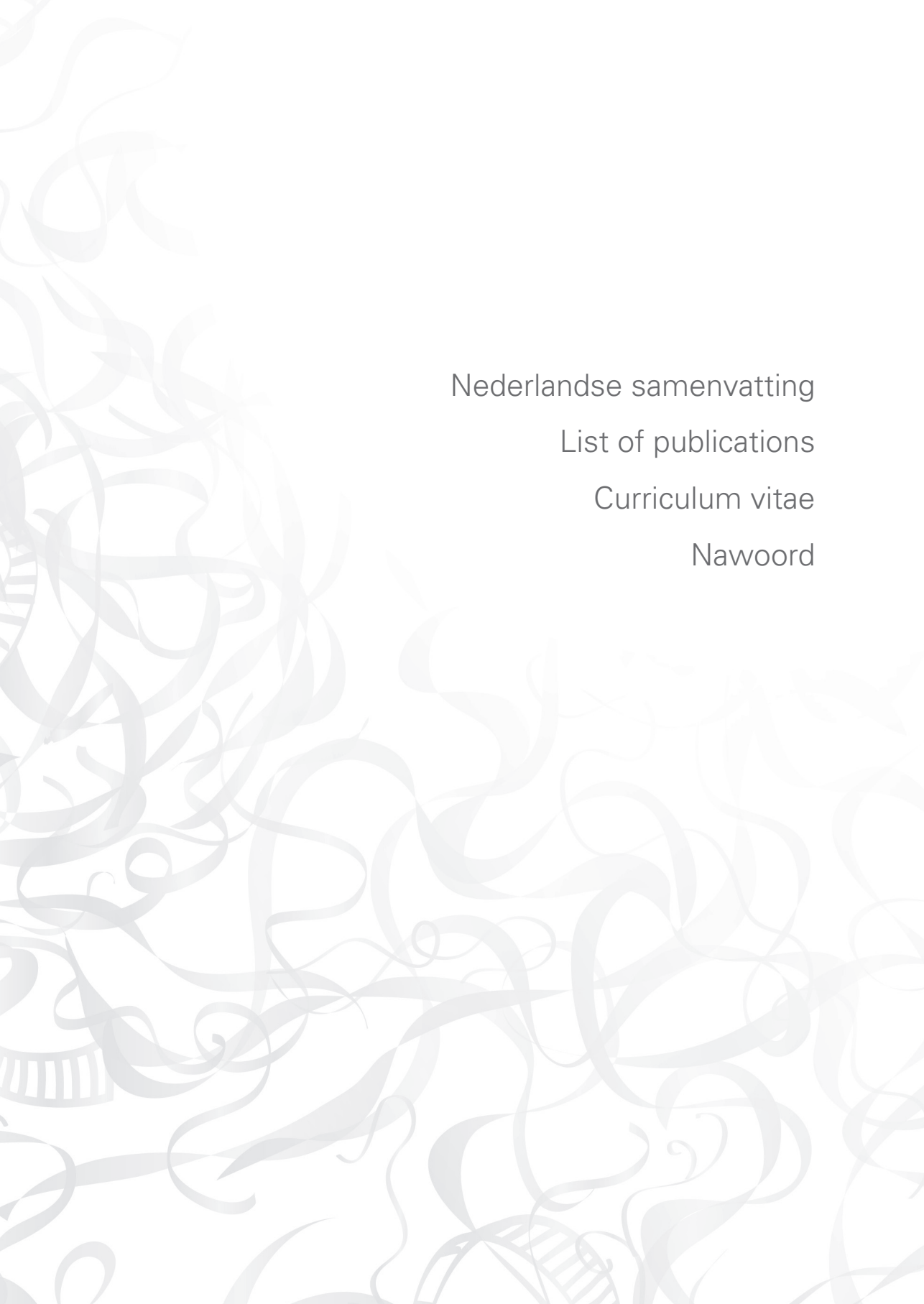
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Nederlandse samenvatting

List of publications

Curriculum vitae

Nawoord

Nederlandse samenvatting

De studies in dit proefschrift zijn gericht op het in kaart brengen van de moleculaire mechanismen betrokken bij de ontwikkeling en progressie van verschillende cutane T-cel lymfomen (CTCL), te weten Sézary syndroom (Sz), mycosis fungoides (MF), primair cutaan anaplastisch grootcellig lymfoom (C-ALCL) en primair cutaan perifere T-cellymfoom, niet anders gespecificeerd (C-PTCL-NOS).

Mycosis fungoides is het meest voorkomende type CTCL en heeft doorgaans een gunstig ziektebeloop met langzame progressie van patches en plaques tot uiteindelijk huidtumoren. Sézary syndroom is een maligne ziekte gekarakteriseerd door een trias van erythrodermie, gegeneraliseerde lymfadenopathie en de aanwezigheid van neoplastische T-cellen in de huid, de lymfklieren en het perifere bloed. Sézary syndroom wordt vaak beschouwd als een leukemische fase of variant van MF, omdat beide maligniteiten voortkomen uit geactiveerde, huid-homende CD4⁺ T-cellen. Om die reden delen MF en Sz dezelfde classificatie en stadiëring, en worden patiënten met deze aandoeningen vaak geïncludeerd in dezelfde klinische trials. Echter in de meest recente WHO-EORTC classificatie en in de WHO classificatie uit 2008 worden MF en Sz als aparte ziektebeelden onderkend gebaseerd op hun verschillende klinisch beeld en gedrag. Echter de discussie of Sz zou moeten worden beschouwd als een apart type CTCL of de leukemische fase van MF, is nog niet gesloten.

In **hoofdstuk 2** worden numerieke chromosomale afwijkingen in tumor stadium MF geïdentificeerd met array-CGH en vergeleken met die gevonden bij Sz. Een aantal chromosomale afwijkingen die zeer frequent voorkomen in tumor stadium MF, werden niet of in slechts lage frequentie gevonden in Sz. Deze bevinding pleit tegen de hypothese dat Sz een vervolgstadium is van MF. Daarnaast worden drie afwijkingen geïdentificeerd met prognostische significantie in tumor stadium MF. Deletie van 9p21 en toename van 8q24.3 en 1q21-1q22 zijn geassocieerd met een slechte prognose. Voor MF zijn numerieke chromosomale afwijkingen geïntegreerd met genexpressie resultaten om genen te identificeren, die bijdragen aan tumorigenese en mogelijk in de toekomst kunnen dienen als therapeutische targets. Voorbeelden zijn *FASTK* en *SKAP1*, gelokaliseerd in regio's van toename en de tumor suppressor genen *RB1* en *DLEU1* gelegen in regio's van verlies.

Met datzelfde doel zijn daaropvolgend in **hoofdstuk 3** de MF genexpressie profielen vergeleken met normale huid, ontstoken huid en normale T-cellen teneinde afwijkende expressie van genen, anders dan ten gevolge van chromosomale afwijking, vast te stellen.

Met deze aanpak werden 989 genen met afwijkende expressie geïdentificeerd, waarvan het overgrote deel (718 genen) verhoogd tot expressie komt in vergelijking tot normale huid, ontstoken huid en normale T-cellen. Zoals verwacht, weerspiegelt het genexpressie profiel, met afwijkende expressie van celcyclus en kinetochoor regulatoren, het proliferatieve karakter van deze T-celmaligniteit. Daarnaast biedt het genexpressie profiel inzicht in het immunofenotype en de huid-homende eigenschappen van dit lymfoom. Tevens worden potentiële therapeutische targets en diagnostische markers geïdentificeerd. Daarbij verklaart verlies van NF- κ B remmer, *NFKBIZ*, mogelijk de toegenomen activiteit van NF- κ B karakteristiek voor CTCL en de toegenomen expressie van NF- κ B targetgenen.

In **hoofdstuk 4** wordt het microRNA (miRNA) profiel van Sz bestudeerd. Daarbij worden 104 miRNAs met verhoogde expressie en 10 miRNAs met verlaagde expressie ten opzichte van normale T-cellen geïdentificeerd. De expressie van miRNAs is grotendeels consistent met eerder beschreven numerieke chromosomale afwijkingen. Om de genregulerende functie van miRNAs, die afwijkend tot expressie komen, te onderzoeken is de lijst van miRNAs, die verlaagd tot expressie komen gecorreleerd aan de lijst van verhoogd tot expressie komende genen. De volgende wijze is gevolgd, van verhoogd tot expressie komende genen is onderzocht welke voorspelde targetgenen van verlaagd tot expressie komende miRNAs zijn. Van bijna alle verlaagd tot expressie komende miRNAs wordt voorspeld dat zij aangrijpen op een of meer genen verhoogd tot expressie in Sz. Een voorbeeld is miR-342; expressie van miR-342 is verlaagd in Sz cellen. Een van de transcripten waarvan de expressie door miR-342 wordt onderdrukt is *TNFSF11*, dat een anti-apoptotische functie heeft. De verlaagde expressie van het miR-342 zou dan ook tot hogere expressie van het anti-apoptotische eiwit TNFSF11 en verminderde apoptose in Sézary cellen leiden. Consistent hiermee is de bevinding dat ectopische expressie van miR-342 in een Sz cellijn leidt tot inductie van apoptose gepaard gaande met lagere expressie van *TNFSF11*. Herintroductie van miR-17-5p, onderdeel van het miR-17-92 cluster, in Sz cellijn resulteert in toegenomen apoptose en afgenomen proliferatie van de cellen, hetgeen een tumor onderdrukkende rol van miR-17-5p impliceert.

In **hoofdstuk 5** wordt de rol van miRNAs in tumor stadium MF onderzocht. In vergelijking tot inflammatoire dermatosen komen 49 miRNAs afwijkend tot expressie in tumor stadium MF. In tegenstelling tot Sz komt het overgrote deel van de afwijkend tot expressie komende miRNAs verhoogd tot expressie (30 van de 49 miRNAs). Voor de meeste afwijkende miRNAs is een rol in tumorigenese beschreven en van een aantal verhoogd tot expressie komende miRNAs (miR-93, miR-155 en miR-17-92) is functionele validatie

als oncomir gepubliceerd. Geen van de miRNAs verhoogd tot expressie in tumor stadium MF komt verhoogd tot expressie in Sz. Slechts twee miRNAs komen verlaagd tot expressie in zowel tumor stadium MF als Sz. Acht miRNAs komen verhoogd tot expressie en zijn gelegen in regio's van chromosomale toename, de chromosomale afwijkingen dragen wellicht bij aan de toegenomen expressie van deze 8 miRNAs.

In **hoofdstuk 6** worden de resultaten beschreven van array-CGH en genexpressie analyses van C-ALCL en C-PTCL-NOS. Hoewel beide CTCL zich presenteren met huidtumoren hebben deze lymfomen duidelijk een verschillend beloop. C-ALCL laat een neiging tot spontane regressie zien, zaait zelden uit naar extracutane lokalisaties en heeft een gunstige prognose met een 5-jaarsoverleving van meer dan 90%. C-PTCL-NOS daarentegen zaait snel uit naar extracutane lokalisaties en heeft een slechte prognose met een 5-jaarsoverleving van minder dan 15%. Voor zowel C-ALCL als C-PTCL-NOS zijn de numerieke chromosomale afwijkingen en genexpressie profielen bepaald om de respectievelijk goede en slechte prognose te kunnen verklaren. C-ALCL is gekarakteriseerd door toename op chromosoom 7q en 17q en verlies van 6q en 13q. C-PTCL-NOS laat ook toename zien van 7q en 17q, maar verschilt door toename op chromosoom 8 en verlies van 9p21. Genen betrokken bij lymfocyt chemotaxie, apoptose en proliferatie zijn oververtegenwoordigd in de lijst van differentieel tot expressie komende genen tussen C-ALCL en C-PTCL-NOS. C-ALCL laat hogere expressie zien van chemokine receptoren CCR10 en CCR8, wat mogelijk een verklaring biedt voor de verminderde neiging tot disseminatie naar lymfklieren en interne organen. Tevens laten C-ALCL en C-PTCL-NOS aberrante expressie zien van verschillende genen betrokken bij apoptose en proliferatie, zoals IRF4 en PRKCQ, wat mogelijk het verschil in klinische agressiviteit verklaart.

Hoofdstuk 7 geeft een samenvatting van de resultaten beschreven in voorgaande hoofdstukken en de bevindingen worden bediscussieerd. Tevens worden er aanbevelingen voor verder onderzoek gedaan.

List of publications

Cucurbitacin I inhibits Stat3 and induces apoptosis in Sézary cells.

van Kester MS, Out-Luiting JJ, von dem Borne PA, Willemze R, Tensen CP, Vermeer MH.
Journal of Investigative Dermatology, 2008; 128(7):1691-5.

Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome.

van Doorn R, **van Kester MS**, Dijkman R, Vermeer MH, Mulder AA, Szuhai K, Knijnenburg J, Boer JM, Willemze R, Tensen CP.
Blood, 2009; 113(1):127-36.

Cutaneous anaplastic large cell lymphoma and peripheral T-cell lymphoma NOS show distinct chromosomal alterations and differential expression of chemokine receptors and apoptosis regulators.

van Kester MS, Tensen CP, Vermeer MH, Dijkman R, Mulder AA, Szuhai K, Willemze R, van Doorn R.
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Ballabio E, Mitchell T, **van Kester MS**, Taylor S, Dunlop HM, Chi J, Tosi I, Vermeer MH, Tramonti D, Saunders NJ, Boultonwood J, Wainscoat JS, Pezzella F, Whittaker SJ, Tensen CP, Hatton CS, Lawrie CH.
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MicroRNA-21 expression in CD4⁺ T cells is regulated by STAT3 and is pathologically involved in Sézary Syndrome.

van der Fits L, **van Kester MS**, Qin Y, Out-Luiting JJ, Smit F, Zoutman WH, Willemze R, Tensen CP, Vermeer MH.
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Deep sequencing analysis reveals that the miR-199a2/214 cluster within DNM3os represents the vast majority of aberrantly expressed microRNAs in Sézary syndrome.

Qin Y, Buermans HPJ, **van Kester MS**, van der Fits L, Out-Luiting JJ, Osanto S, Willemze R, Vermeer MH, Tensen CP.

Journal of Investigative Dermatology, 2012;132(5):1520-2.

A meta-analysis of gene expression data identifies a molecular signature characteristic for tumor-stage mycosis fungoides.

van Kester MS, Borg MK, Zoutman WH, Out-Luiting JJ, Jansen PM, Dreef EJ, Vermeer MH, van Doorn R, Willemze R, Tensen CP.

Journal of Investigative Dermatology, 2012;132(8):2050-9.

Primary cutaneous anaplastic large cell lymphoma shows a distinct miRNA expression profile and reveals differences from tumor-stage mycosis fungoides

Benner MF, Ballabio E, **van Kester MS**, Saunders NJ, Vermeer MH; Willemze R, Lawrie CH, Tensen CP.

Experimental Dermatology, 2012; 21(8):632-4.

Curriculum Vitae

Marloes van Kester werd geboren op 6 juni 1984 te Rotterdam. Zij groeide op in het Westland en doorliep de middelbare school aan het ISW te Naaldwijk. Na het behalen van het eindexamen gymnasium in 2002, begon zij in datzelfde jaar aan de studie geneeskunde aan het Leids Universitair Medisch Centrum. Gedurende de studie geneeskunde behaalde zij in 2006 de propedeuse van de studie bio-farmaceutische wetenschappen. In 2006 begon zij in het kader van een wetenschapsstage aan het onderzoek naar het effect van Cucurbitacine I op Sézary cellen onder leiding van dr. C.P. Tensen en prof.dr. M.H. Vermeer. Dat onderzoek werd in datzelfde jaar gevolgd door het promotieonderzoek beschreven in dit proefschrift onder leiding van dr. C.P. Tensen, dr. R. van Doorn en prof.dr. R. Willemze. In 2007-2008 maakte zij als Think Global Initiative Project Coordinator deel uit van het "team of officials" van the International Federation of Medical Students' Associations. De Rene Touraine Fondation kende in 2008 een European fellowship aan haar toe, teneinde de samenwerking tussen Europese laboratoria te faciliteren. Dit heeft haar in staat gesteld een deel van de experimenten beschreven in dit proefschrift uit te voeren in het laboratorium van dr. Charles Lawrie in Oxford. In januari 2011 is zij begonnen aan de co-schappen, waarbij zij het co-schap kindergeneeskunde volgde aan het St Vincentius ziekenhuis in Paramaribo, Suriname. Momenteel is zij bezig aan haar semi-artsstage aan de afdelingen dermatologie van het Diaconnessenhuis Leiden en het Leids Universitair Medisch Centrum.

Nawoord

Hierbij wil ik van de gelegenheid gebruik maken om iedereen die betrokken is geweest bij de totstandkoming van mijn proefschrift te bedanken. Graag wil ik een aantal mensen in het bijzonder noemen.

Mijn promotor Rein Willemze, dank voor het vertrouwen in deze student geneeskunde en de kans om het promotieonderzoek uit te voeren. Mijn co-promotoren Kees Tensen en Remco van Doorn, dank voor jullie begeleiding en kritische blik. Maarten Vermeer, dank voor het introduceren in het lymfoomonderzoek en je deskundige adviezen.

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