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 classification[3]

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/mm^3, 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]

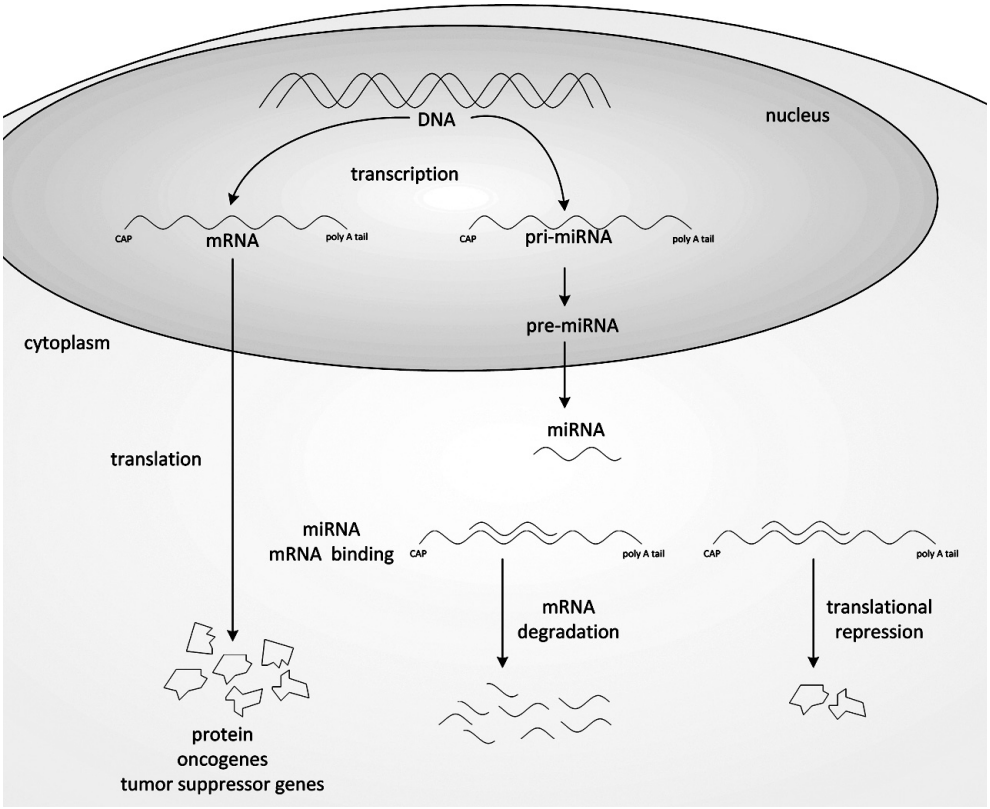

Figure 1 Interactions of DNA, mRNA and miRNA

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, deletions[36] 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.

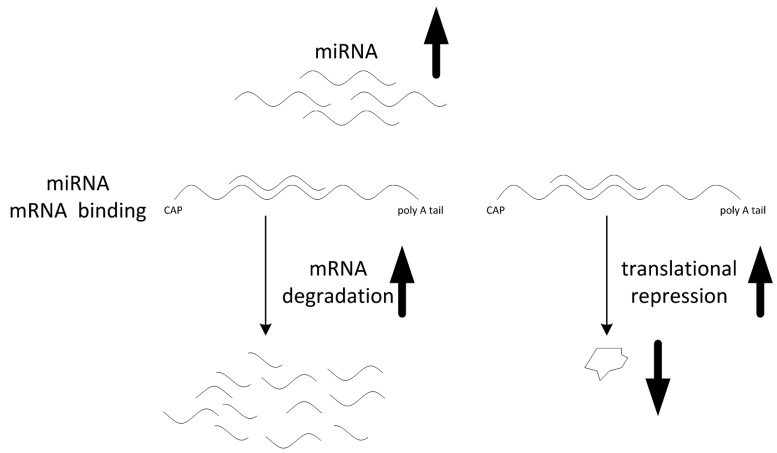

Figure 1b miRNAs can play a role in tumorigenesis by targeting proteins with a tumor suppressing function. Increased miRNA expression can lead to increased mRNA degradation and increased translational repression both leading to decreased protein synthesis

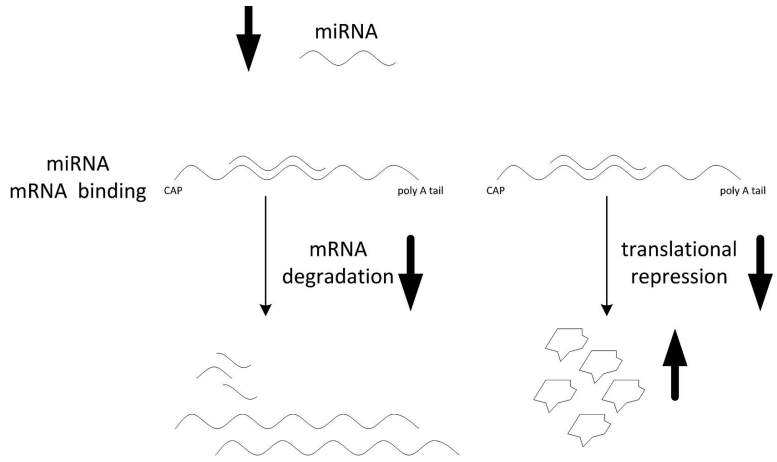

Figure 1c Likewise miRNAs can play a role by targeting oncogenes. Decreased miRNA expression can lead to decreased mRNA degradation and decreased translational repression both leading to increased protein synthesis

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 evolvement 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 cluster[52] 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 phytohematoglutinin 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 RHOF*.[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 β1), *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 *TGFBR2, 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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