IDENTIFICATION OF NOVEL TARGET GENES IN DIFFERENT SUBTYPES OF CUTANEOUS T-CELL LYMPHOMA

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ACADEMIC DISSERTATION

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in the lecture hall of Skin and Allergy Hospital,
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TABLE OF CONTEST

LIST OF ORIGINAL PUBLICATIONS

I.   Karenko L, **Hahtola S***, Päivinen S*, Karhu R, Syrjä S, Kähkönen M, Nedoszytko B, Kytölä S, Zhou Y, Blazevic V, Pesonen M, Nevala H, Nupponen N, Sihto H, Krebs I, Poustka A, Roszkiewicz J, Saksela K, Peterson P, Visakorpi T, Ranki A. Primary cutaneous T-cell lymphomas show a deletion or translocation affecting NAV3, the human unc-53 homologue. Cancer Res 2005; 65: 8101–8110.

II.   **Hahtola S***, Tuomela S*, Elo L, Häkkinen T, Karenko L, Nedoszytko B, Heikkilä H, Saarialho-Kere U, Roszkiewich J, Aittokallio T, Lahesmaa R, Ranki A. Th1-response and cytotoxicity genes are downregulated in cutaneous T-cell lymphoma. Clin Cancer Res 2006; 12: 4812–4821.

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*These authors contributed equally to the study. Original publications have been reprinted with the permission of their copyright holders. In addition, some unpublished data are presented. Study I is also included in the Ph.D. thesis of Dr. Leena Karenko, University of Helsinki, 2004.

ABBREVIATIONS

aCGH	microarray CGH
APC	antigen presenting cell
BAC	bacterial artificial chromosome
CCR	chemokine receptor
cDNA	complementary DNA
CLA	cutaneous lymphocyte -associated antigen
CGH	comparative genomic hybridization
COBRA-FISH	combined binary ratio labelling FISH
C_t	threshold cycle
CTCL	cutaneous T-cell lymphoma
CTL	T-cell cytotoxicity
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
EORTC	The European Oragnization for Reseach and Treatment of Cancer
EST	expressed sequence tag
FACS	fluorescence-activated cell sorting
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HDAC	histone deacetylase
HPS	hemophagocytic syndrome
IFN	interpheron
IHC	immunohistochemistry
IL	interleukin
KIR	killer cell immunoglobulin-like receptor
LEP	lupus erythematosus profundus
LOH	loss of heterozygosity
MF	mycosis fungoides
MFISH	multifluor-FISH
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NAV3	neuron navigator 3
NHL	non-Hodgkin lymphoma
NSCLC	non-small cell lung cancer

PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PUVA	psoralen + ultraviolet A photochemotherapy
QPCR	quantitative PCR
RNA	ribonucleic acid
RNAi	RNA interference
RxFISH	cross-species color banding
SCC	squamous cell carcinoma
SCLC	small cell lung cancer
SCOMP	single cell comparative genomic hybridization
siRNA	small interfering RNA
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
SPTL	subcutaneous panniculitis like T-cell lymphoma
SS	Sezary syndrome
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper cell
TNF	tumor necrosis factor
TNM	tumor node metastasis –classification
Treg	T regulatory cell
UVB	ultraviolet B radiation
YAC	yeast artificial chromosome

ABSTRACT

Cutaneous T-cell lymphomas (CTCL) represent a group of non-Hodgkin lymphomas showing a growing incidence especially in the Western world. The mechanisms leading to the disease are largely unknown, diagnosis is difficult and therefore often delayed, and no curative therapy exists. CTCL presents with skin symptoms although the malignant cells are not derived of human skin but of human immune system instead. The malignant cells are mature T helper memory cells, and preferentially express cytokines characteristic to T-helper 2 (Th2) type immune response. Chromosomal instability is a typical feature of CTCL. Some secondary cancers occur in CTCL patients more often than in general population, the most common of which are lung cancers and non-Hodgkin lymphomas.

The aim of the study was to identify genes relevant to CTCL pathogenesis to clarify the poorly understood pathomechanisms behind the disease group. The two most common subgroups of CTCL, mycosis fungoides (MF) and Sezary syndrome (SS), as well as the difficult to diagnose subcutaneous panniculitis like T-cell lymphoma (SPTL), were studied. To reveal the molecular pathogenesis underlying CTCL-associated lung cancer, CTCL-associated lung cancer samples were analysed moleculocytogenetically and compared to primary / reference lung cancer samples. Identification of potential novel diagnostic markers as well as target molecules for therapy was a special focus of the study. To achieve this, patient derived material was studied with molecular cytogenetic techniques, microarrays and gene expression analysis.

This study identified the first specific recurrent common gene level aberration in CTCL, namely the deletion / translocation of neuron navigator 3 (*NAV3*) in chromosome 12q21 occurring in 50% of patients with early CTCL and in 85% of patients with advanced CTCL. *NAV3* is hypothesized to function as a non-classical, i.e. haploinsufficient tumor suppressor influencing the differentiation of T-helper cells by increasing the production of cytokine interleukin-2. *NAV3* deletion was observed in many CTCL subgroups (MF, SS, and SPTL), and its demonstration by FISH-technology provides a novel diagnostic aid. Also additional chromosomal hot spots of loss and gain were identifed, with both DNA and RNA copy numbers changing to the same direction. Future studies will concentrate mainly on these areas to search for further target genes in CTCL.

With microarray technology changes in gene expression were identified, which could clarify the CTCL pathogenesis. A panel of genes with a central role in Th1-type immune responses, e.g. *T-bet*, *RANTES*, and *NKG7*, was downregulated in CTCL, thus explaining the previous observation of the Th2 type cytokine profile of CTCL cells. Moreover, overexpression of potential target molecules for antibody-based therapy, e.g. membrane antigens *MS4A4A*, *LIR9* and *CD52*, was identified.

For the first time, CTCL-associated lung cancers were observed to show chromosomal aberrations differing from primary lung cancers. Especially amplifications of chromosome arm 4q and selected receptor tyrosine kinase genes (*KIT*, *PDGFRα*, and *VEGFR2*) in 4q12 found in CTCL-associated lung cancers were of interest, since chromosome arm 4q is frequently deleted in primary lung cancer. These preliminary observations warrant further prospective studies to identify the common underlying factors between CTCL and CTCL-associated lung cancer.

To conclude, *NAV3* gene aberrations are common to many different CTCL subtypes, and the pathways affected by its aberrant function, are currently being studied. Novel insights to CTCL pathogenesis were achieved through the observation that several genes specific for Th1 type immune response are downregulated in CTCL. Moreover, the finding of the difference in genomic aberrations of CTCL-associated and reference lung cancers raises a question whether cancer stem cells also have a role in the pathogenesis of CTCL. Demonstration of *NAV3* deletion by FISH provides a novel diagnostic tool, and overexpression of certain membrane antigens will provide the basis for developing novel therapeutic means.

1. REVIEW OF THE LITERATURE

1.1 Genes, chromosomes and cancer

1.1.1 General introduction

Current concept of cancer development includes the development of chromosomal instability, chromosomal number changes and a series of acquired genetic aberrations affecting genes important for the growth and survival properties of the cell. The most important properties of malignancies are considered to be clonal cell growth and invasive ability (Fearon and Vogelstein, 1990). According to Kinzler and Vogelstein there are five different types of genetic alterations in tumor cells: 1) subtle alterations, such as small deletions, insertions and mutations, 2) chromosome number changes, i.e. aneuploidy, 3) chromosomal translocations, 4) amplifications of small regions of chromosomes or single genes, and 5) exogenous sequences derived from tumor viruses (Kinzler and Vogelstein, 1998). Hanahan and Weinberg (2000) have proposed six principles defining cancer, namely 1) self-sufficiency in growth signals, 2) insensitivity to negative growth signals, 3) capability to evade programmed cell death, 4) capacity for sustained proliferation, 5) angiogenesis, and 6) tissue invasion and metastasis. Several different deregulated genes and pathways are involved in carcinogenesis (Hanahan and Weinberg, 2000). However, two main categories among genes with major effect on tumor initiation are identified, namely oncogenes and tumor suppressor genes (Fearon and Vogelstein, 1990), and a certain gene may have both of these functions (Yang et al., 2007). Today, cancer is recognized as a multistep process in which the number of genetic hits rather than their order is essential. According to Vogelstein and Kinzler (2004) at least five different genetic changes are required for a cancer to develop. Moreover, the genetic defects target more precisely different pathways than different genes (Vogelstein and Kinzler, 2004).

1.1.2 Oncogenes and tumor suppressors

Oncogenes are altered forms of normal cellular genes, called proto-oncogenes. In human cancers, proto-oncogenes are often located adjacent to chromosomal breakpoints and are targets for mutation. According to current knowledge, activation of several oncogenes and inactivation of several tumor suppressor genes are necessary for the acquisition of a complete neoplastic phenotype. Oncogenes regulate main cellular functions, e.g. cell cycle, cell differentiation and cell division. Oncogenes may act by rescuing the cells from senescence and apoptosis, thus blocking the cell differentiation, or by reducing the growth factor requirements resulting in a continuous proliferative response.

Oncogenes are activated through chromosomal translocations, gene amplifications or gain-of function mutations (Bishop, 1991). Oncogenes act dominantly at cellular level, meaning that a mutation in one of the two oncogene alleles is sufficient to lead to its increased activity. To date, more than 100 oncogenes have been identified (Bishop, 1991; Rabbitts, 1994; Futreal et al., 2004).

In contrast to oncogenes, classical tumor suppressor genes act recessively at cellular level, meaning that two mutational events are required for the cancer development (Knudson, 1971). The first mutation may be inherited or somatic, whereas the second mutation is often achieved through a gross chromosomal mechanism, such as deletion, gene conversion, mitotic recombination resulting in loss of heterozygosity (LOH), or through epigenetic mechansims (Devilee et al., 2001; Tomlinson et al., 2001; Jones and Baylin, 2002). Approximately 30 classical tumor suppressor genes have been identified thus far, including well-known genes for the pathogenesis of different cancer types, such as retinoblastoma 1 (*RB1*), von Hippel-Lindau tumor suppressor (*VHL*), neurofibromin 1 (*NF1*), adenomatosis polyposis coli (*APC*), and breast cancer 1 and 2, early onset (*BRCA1* and *BRCA2*) (Futreal et al., 2001).

In addition to the classical tumor suppressors, acting through biallelic inactivation of a tumor suppressor gene, other phenomena, for example haploinsufficiency (Quon and Berns, 2001; Sherr 2004), have been shown to contribute to tumorigenesis. For a haploinsufficient tumor suppressor gene, inactivation of only one copy of the gene is sufficient for the suppressive effect. Examples of tumor suppressor genes acting in haploinsufficient manner include *p27^{Kip1}* (Fero et al., 1998), *p53* (Venkatachalam et al., 1998), *PTEN* (Trotman et al., 2003) and *DMP1* (Inoue et al., 2001). Knock out studies on mice have demonstrated that in *p53* hemizygous mice (p53 +/-) the tumors arise later than in homozygous mice (p53 -/-) but earlier and more frequently than in wild-type mice (p53 +/+) (Venkatachalam et al., 1998). The degree of haploinsufficiency may vary among tumor suppressor genes, ranging from no apparent effect to weak or strong effects (Cook and McCaw, 2000; Quon and Berns, 2001). It is believed that the biallelic inactivation of a tumor suppressor gene contributes to tumor progression and more severe tumor susceptibility (Quon and Berns, 2001; Rossi et al., 2002). However, haploinsufficiency would allow the clonal expansion of cells that are heterozygous for a tumor suppressor gene, and would thus increase the size of the target cell population available for subsequent mutations during the remaining course of tumor progression. Thus, some degree of haploinsufficiency may be required to generate a sufficiently large target cell population for mutagenesis. (Quon and Berns, 2001) It is believed that many classical tumor suppressors may well manifest haploinsufficient effects, particularly when combined with collaborating mutations affecting additional tumor suppressors or oncogenes (Sherr, 2004).

MicroRNAs (miRNAs) are short, noncoding RNAs that posttranscriptionally regulate gene expression. To date, over 500 miRNA genes have been identified in the human genome, and miRNAs have been found to be involved in the pathophysiology

of all types of analysed human cancers by functioning either as tumor suppressors or oncogenes. Additionally, miRNAs alterations may cause cancer predisposition. miRNAs are shown to control key cellular events, such as cell proliferation, differentiation, and apoptosis (Calin et al., 2006). Thus, miRNAs profiling by microarrays or quantitative PCR provides novel diagnostic and prognostic tools for cancer patients. (Barbarotto et al., 2008; Yang et al., 2008)

1.1.3 Cancer stem cells

According to a current prevailing hypothesis, the recurrence and dissemination of many cancers is dependent on a small population of cells forming the primary tumor, namely the cancer stem cells, which apart from being capable of self-renewal and proliferation, also contribute to drug resistance and express typical stem cell markers (Harris, 2004; Dean et al., 2005; Polyak and Hahn, 2006). Cancer stem cells are responsible for initiating and sustaining tumor growth but are predicted to be refractory to current therapies which are designed to eradicate actively cycling cells. Changes in the surrounding specialized microenvironment (niche) of the tumor cells can also directly influence tumor growth (Polyak and Hahn, 2006). Cancer stem cells may arise as a malignant transformation of tissue-specific stem cells or from more differentiated cells that have acquired stem cell characteristics through subsequent de-differentiation. Still another possibility is that bone marrow –derived CD34+ stem cells migrate to sites of tissue damage, where they become tissue-specific stem cells (Passegue et al., 2003; Borue et al., 2004; Bjerkvig et al., 2005; Polyak and Hahn, 2006). There is experimental evidence supporting all these pathways, and it may be, that in different tumour types, different pathways operate (Polyak and Hahn 2006). The first cancer stem cell described was the leukaemia stem cell, and to date the existence of cancer stem cells has been proven in breast, brain, and gastrointestinal tumors (Sirard et al., 1996; Cobakeda et al., 2000; Al Hajj et al., 2003; Singh et al., 2003; Houghton et al., 2004; Li et al., 2007).

1.1.3.1 Lung cancer stem cells

For lung cancer, a stem cell population giving rise to lung adenocarcinoma, has recently been identified (Kim et al., 2005). However, small cell lung cancer (SCLC) progenitor cell has not been isolated yet, although there is evidence of the existence of a primitive neuroendocrine cell giving rise to some subsets of SCLC (Watkins et al., 2003). Studies on SCLC mouse model have also shown a possible dysplastic precursor lesion containing small foci of neuroendocrine cell proliferation and expressing markers for neuroendocrine differentiation (Meuwissen et al., 2003; Calbo et al., 2005). Also, it has been shown recently, that fetal lung mesenchymal stem cells can differentiate into neural cells in addition to the mesenchymal differentiation (Fan et al., 2005).

1.1.3.2 Cancer stem cells in the skin

To date, there is emerging evidence of the stem cell origin of some cutaneous malignancies, especially squamous cell carcinoma and melanoma (Kamstrup et al., 2007). Squamous and basal cell carcinomas are believed to arise from stem cells locating in the hair follicles and, to a lesser degree, in the basal layer of interfollicular epidermis (Blanpain et al., 2004; Tumbar et al., 2004). Studies on mouse models have demonstrated the squamous cell carcinomas to occur when the oncogenic *HRAS* gene is expressed in the stem cells within the hair follicle but not, if other cells of the epidermis are targeted (Brown et al., 1998). Another study reported that mice, in which the interfollicular epidermis had been removed, developed carcinomas as often as the intact mice (Morris et al., 2000). In basal cell carcinoma, a subset of cells escaping from the blockage of the Hedgehog signalling pathway and thus maintaining their capacity of tumor formation, has been recognized (Hutchin et al., 2005). As melanoma cells are capable of expressing neural markers (Rasheed et al., 2005), the association between nervous system tumors and melanoma in certain individuals, is believed to represent an underlying abnormality in neural crest stem cells (Fang et al., 2005). Moreover, metastatic melanoma has been shown to recur from a common progenitor cell (Wang et al., 2006).

No CTCL progenitor cell has been isolated so far, although there are preliminary reports of the initial malignant transformation to occur in bone marrow in a low-grade cutaneous lymphoma, namely lymphomatoid papulosis (Gniadecki et al., 2003; Gniadecki, 2004). It has been speculated that bone marrow –derived stem cells would migrate to the site of chronic skin inflammation (like Parapsoriasis en plaques), often preceding CTCL, and then either fuse with mutated lymphocytes in the skin or undergo malignant transformation by themselves (Kamstrup et al., 2007).

1.1.4 Epigenetic progenitor origin of cancer

In accordance with the cancer stem cell model, a recent theory suggesting the epigenetic progenitor origin of human cancer has been proposed (Feinberg et al., 2006). Epigenetic refers to heritable information related to gene function not encoded in the nucleotide sequence (Baylin and Ohm, 2006; Feinberg et al., 2006). Epigenetic mechanisms include global changes such as histone modifications and gene-specific hypo- or hypermethylation of CpG dinucleotide islands resulting in gene activation or silencing, respectively (Herman et al., 2003; Issa et al., 2004). According to the epigenetic progenitor model of cancer, cancer arises in three steps (Feinberg et al., 2006). First, polyclonal epigenetic alterations occur in stem / progenitor cells within a certain tissue. These early epigenetic alterations predispose cells to genetic abnormalities. Second, an initiating monoclonal genetic mutation occurs within the subpopulation of the epigenetically disrupted cells. Third, genetic and epigenetic instability leads to tumor increased tumor evolution.

1.2 Primary cutaneous T-cell lymphoma (CTCL)

Primary cutaneous lymphomas represent a heterogeneous group of non-Hodgkin lymphomas (NHL) with homing preference to skin. After the gastrointestinal NHL's, skin lymphomas are the second most common type of NHL's. The annual incidence of CTCL is estimated 2,5 per 100000 for men but it is continuously increasing and affecting even younger people (Weinstock and Horn, 1988; Hartge et al., 1994; Siegel et al., 2000; Väkevä et al., 2000). CTCL is characterized by poorly known etiopathogenesis, difficult diagnosis and lack of curative therapy.

1.2.1 Classification of cutaneous T-cell lymphomas

The classification of cutaneous lymphomas is currently based on the World Health Organization - European Organization of Research and Treatment of Cancer (WHO-EORTC) classification (Willemze et al., Blood, 2005). The majority (75%) of the skin lymphomas are T-cell lymphomas, whereas skin lymphomas of B-cell origin comprise approximately 25% of all cutaneous lymphomas. The basis of the classification is to divide skin lymphomas into T-cell and B-cell origin, and thereafter into indolent and aggressive clinical behaviour. The most common form of cutaneous lymphomas is mycosis fungoides (MF), comprising 44% of all cutaneous lymphomas. The WHO-EORTC classification of cutaneous T-cell lymphomas is shown in Table 1, and the subtypes that were studied in this thesis are characterized in more detail below.

Table 1. WHO-EORTC classification of cutaneous T-cell lymphomas with disease frequency and survival		
Indolent clinical behaviour	Frequency, %*	Disease-specific 5-year survival, %
Mycosis fungoides (MF)	44	88
Folliculotropic MF	4	80
Pagetoid reticulosis	<1	100
Granulomatous slack skin	<1	100
Primary cutaneous anaplastic large cell lymphoma	8	95
Lymphomatoid papulosis	12	100
Subcutaneous panniculitis-like T-cell lymphoma	1	82
Primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma	2	75
Aggressive clinical behaviour		
Sezary syndrome	3	24
Primary cutaneous NK/T-cell lymphoma, nasal-type	<1	NA
Primary cutaneous aggressive CD8+ T-cell lymphoma	<1	18
Primary cutaneous γ/δ T-cell lymphoma	<1	NA
Primary cutaneous peripheral T-cell lymphoma, unspecified	2	16

* Data are based on 1905 patients with a primary cutaneous lymphoma registered at the Dutch and Austrian Cutaneous Lymphoma Group between 1986 and 2002.

NA = not available

1.2.1.1 Mycosis fungoides (MF)

Mycosis fungoides is the most common form of cutaneous lymphomas comprising almost half of all primary cutaneous lymphomas. MF typically affects older adults (median age at diagnosis 55-60 years), and male-to-female ratio is 1.6-2.0:1 (Zackheim et al., 1999; van Doorn et al., 2000; Kim et al., 2003; Willemze et al., 2005).

Clinically MF presents classically with the evolution of skin lesions from patches to plaques and tumors mainly affecting trunk and other sun-protected areas (Alibert, 1806; Bazin, 1852; Figure 1A). The diagnosis of MF is difficult, since it often resembles eczema or mild psoriasis in its earliest stages. Often long-lasting observation of the clinical picture together with multiple skin biopsies are required to reach the MF diagnosis. Histologically, MF is characterized by the epidermotropic infiltration of small to medium-sized malignant T-lymphocytes with a cerebriform nucleus (Figure 1B). In early MF lesions, the number of the morphologically malignant cells is low and they are surrounded by reactive lymphocytes. Thus, reaching the diagnosis often implies multiple consecutive biopsies (Olsen et al., 2007). Clusters of malignant cells in papillary dermis (Pautriers' microabscesses) are highly specific, although uncommon feature of MF. The immunophenotype of malignant cells is usually CD3+, CD4+, CD8-, CD45RO+, CD30-, although some variants with other immunophenotypic features also exist. The malignant cells are reported to be mature Th1 memory cells (Saed et al., 1994). The T-cell receptor genes are clonally rearranged in most cases, but for diagnostic purposes the TCR-gene rearrangement analysis in unspecific (Lukowsky et al., 2000; Sawabe et al., 2000)

Further staging of MF is achieved by criteria proposed by North American MF Cooperative Group (Bunn et al., 1979; Girardi et al., 2004) recently revised by the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the EORTC (Olsen et al., 2007). MF is divided into four stages based on skin, nodal, visceral and blood involvement as defined by TNM (tumor-node-metastasis) classification (Bunn et al., 1979).

MF has an indolent clinical course and the disease progresses slowly. The estimated 5-year survival is 88% (Willemze et al., 2005). In advanced stages progression to a CD30+ or CD30- large cell T-cell lymphoma may be present (Cerroni et al., 1992). The treatment is based on the stage (IA-IVB) of the disease. Aggressive therapy does not improve the prognosis or remission time. Skin-directed therapy usually leads to remissions in the early stages. The duration of remissions is variable and most patients will suffer from a relapse. MF confined to skin is treated with photo(chemo)therapy: UVB irradiation and PUVA, whereas combination chemotherapy is recommended for systemic CTCL (stage IV). (Dummer et al., 2003; Whittaker et al., 2003; Trautinger et al., 2006)

1.2.1.2 Sezary syndrome (SS)

Sezary syndrome (SS) is the leukaemic form of CTCL, where malignant T lymphocytes circulate in the blood (Sezary et al., 1938). SS can develop from pre-existing MF or arise de novo. Clinically, SS is characterized by pruritic erythroderma, lymphadenopathy, palmoplantar hyperkeratosis, alopecia, and onychodystrophy (Figure 1C). The diagnostic criteria of Sezary syndrome aim especially at differentiating it from bening erythrodermic conditions, and include one or more of the following: an absolute Se-

zary cell (Figure 1D) count of least 1000 cells/mm³, the ratio of CD4/CD8 > 10, loss of any T-cell antigen (CD2, CD3, CD4, CD5), or the demonstration of a T-cell clone in peripheral blood by moleculocytogenetic methods or showing a clonal T-cell receptor rearrangement (Vonderheid et al., 2002; Willemze et al., 2005). Histologically, SS resembles MF, although epidermotropism may be absent and the malignant T-cells may have lost their surface antigens to variable extent. The immunophenotype is similar to MF, but circulating Sezary cells may show loss of CD7 and CD26 (Vonderheid et al., 2002; Willemze et al., 2005). The tumor cells are usually CD3+, CD4+, CD8-, CD45RO+, CD30-, and mature Th2 memory cells (Vowels et al., 1992, Saed et al., 1994; Dummer et al., 1996; Willemze et al., 2005). The prognosis in SS is poor, 5-year survival being only 24% (Table 1). No curative therapy exists, but therapy recommendations include extracorporeal photopheresis either alone or in combination with e.g. interpheron-alpha, or interpheron alpha either alone or in combination with PUVA. New treatment modalities are developed continuously. The most recent options for refractory MF or SS include the rexinoid bexarotene, CD52 antibody alemtuzumab, and histone deacetylase inhibitor vorinostat. Bexarotene and alemtuzumab have been in clinical use in Finland during the last five years, and vorinostat has recently been accepted to treat CTCL in the USA and is in clinical trials in Finland. (Dummer et al., 2003; Whittaker et al., 2003; Rafandi et al., 2006; Trautinger et al., 2006; Gniadecki et al., 2007; Mann et al., 2007)

1.2.1.3 Subcutaneous panniculitis-like T-cell lymphoma (SPTL)

Subcutaneous panniculitis-like T-cell lymphomas (SPTL) are a rare and poorly characterized subgroup of cutaneous T-cell lymphomas (CTCL). The latest WHO-EORTC classification separated for the first time two different subgroups within SPTL (Willemze et al, 2005). Of these, only SPTL, with α/β T-cell phenotype (SPTL-AB), presenting with cells usually expressing CD8 and restricted to the subcutaneous tissue, should be considered SPTL. In the γ/δ T-cell phenotype SPTL (SPTL-GD), the infiltrating malignant cells usually are CD8- and CD56+, may show (epi)dermal involvement, and should be classified among the cutaneous γ/δ T-cell lymphomas (Willemze et al., 2005; Willemze et al., 2008).

Clinically, SPTL presents with solitary or multiple subcutaneous nodules and plaques, predominantly affecting the lower extremities and trunk (Figure 1E). Initial systemic symptoms like fever, fatigue and weight loss are frequent, and hemophagocytic syndrome (HPS) may be present (Gonzalez et al., 1991), indicating a rapidly progressive course and worse prognosis (Marzano et al., 2000; Willemze et al., 2008). Histologically, SPTL is characterized by subcutaneous infiltrates of pleomorphic, small to medium-sized T lymphocytes rimming the individual fat cells, while the epidermis and dermis are typically uninvolved (Figure 1F). Additionally, necrosis, karyorrhexis, leukocytoklasia, and cytophagocytosis are often present. The immunophenotype is usually CD3+, CD4-, CD8+, CD56-, and cytotoxic proteins are frequently expressed.

The disease response to therapy is usually favourable with a 5-year survival of more than 80% (Massone et al., 2004, Willemze et al., 2007), without HPS even 91% (Willemze et al., 2008).
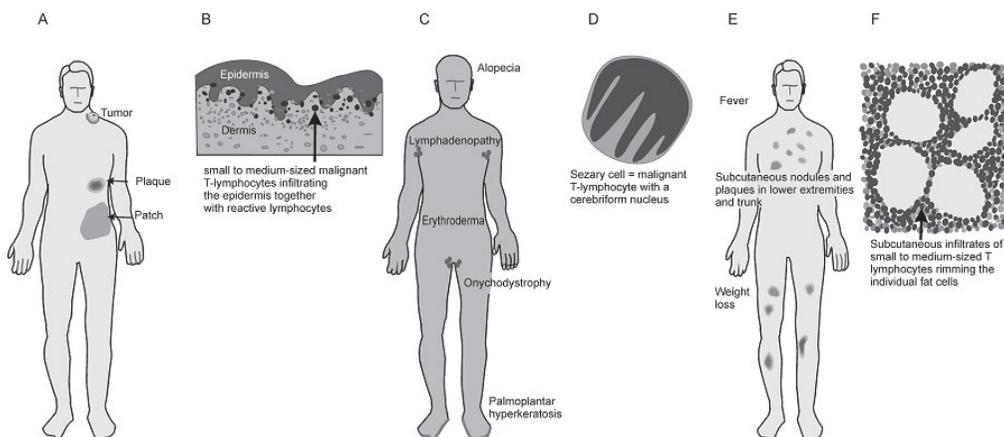
Figure 1. Clinical and histological characteristics of mycosis fungoides (1A-B), Sezary syndrome (1C-D), and subcutaneous panniculitis-like T-cell lymphoma (1E-F).

1.2.2 Chromosomal, genetic and transcriptional aberrations characterizing CTCL

1.2.2.1 Chromosomal aberrations in CTCL

Chromosomal aberrations can be classified as numerical or structural. Numerical aberrations are the most common cytogenetic changes (Krämer et al., 2002). They are caused by defective segregation of chromosomes and can be seen as multiples of haploid chromosome number or extra or missing chromosomes. Structural chromosome aberrations include deletions, translocations, inversions, and multiplications of parts of the chromosome.

Studies on chromosomal aberrations of CTCL patients' chromosomes have provided large number of information on both clonal and non-clonal nature of the chromosome changes. Any chromosome can be aberrated, numerically or structurally. Early conventional cytogenetic techniques, like G-banding have been complemented with multicolor-FISH (including multifluor-FISH, MFISH; spectral karyotyping, SKY; and COmbined Binary RAtio labelling FISH, COBRA-FISH), and cross-species color banding (RxFISH).

The first cytogenetic studies utilized mainly G-banding technique and were performed mostly on blood lymphocytes. Whang-Peng and coworkers reported numerical chromosome abnormalities especially in chromosomes 11, 21, 22, and 8, structur-

al aberrations in chromosomes 1, 6, and 7, but the continuum went on involving all chromosomes (Whang-Peng et al., 1979 and 1982). These chromosome abnormalities were frequently detectable before morphologically neoplastic cells are encountered in blood (Whang-Peng et al., 1982). Karenko and coworkers described that numerical aberrations of chromosomes 6, 13, 15, and 17, marker chromosomes, and structural aberrations of chromosomes 3, 9, and 13 were increased in mycosis fungoides (MF) compared with healthy controls. The detection of a chromosomal clone preceded relapse or progression of the disease (Karenko et al., 1997), and especially aberrations of chromosomes 8 and 17 associate with active or progressive disease (Karenko et al., 2003).

Multicolor-FISH is a novel technology revealing structural chromosome aberrations not detectable with conventional cytogenetic methods, including balanced, complex translocations (see 3.2.3). In CTCL, frequently aberrated chromosomal areas include chromosomes 10 (in 7/9 patients), 6 (6/9), 3, 7, 9, 17, and 19 (5/9), 1 and 12 (4/9), in which the majority of the abnormalities were structural (Batista et al., 2006). Moreover, recurrent breakpoints were observed in 1p32-p36, 6q22-q25, 17p11.2-p13, 10q23-q26, and 19p13.3 (Batista et al., 2006), regions often showing DNA copy number losses in CGH studies (see 1.2.2.2; Karenko et al., 1999; Mao et al., 2002; Fischer et al., 2004).

Cross-species colour banding (Rx-FISH, Müller et al., 1997, 1998, 2002; Teixeira et al., 2000) is a coarse whole-genome screening method based on probes made of primate chromosomes, the DNA of which hybridizes to different human chromosomes forming bands. Espinet and coworkers used this technology in addition to the conventional cytogenetics, and revealed aberrations in chromosomes 10, 1, 6, 8, 9, 11, and 17 to be frequent in SS patients (Espinet et al., 2004).

Still another novel technology, COBRA-FISH (Tanke et al., 1999), which is based on the simultaneous use of combinatorial (binary) labelling and ratio labelling, has recently been used in CTCL research (Vermeer et al., abstract, 2006). Recurring structural chromosomal alterations in SS involved deletion of 10q24 (3 of 7 cases) and breakpoints at 17p11 (3 of 7 cases). (Vermeer et al., abstract, 2006)

Based on these studies on CTCL patients' chromosomes, the most common chromosomal aberrations involve chromosomes 1, 6, 10, and 17. However, these aberrations are diverse.

1.2.2.2 DNA copy number gains and losses in CTCL

Since it is difficult to cultivate true CTCL cells, moleculocytogenetic methods which do not require cell cultivation, like comparative genomic hybridization (CGH) based on competitive hybridization of tumor and reference DNA on normal metaphase chromosomes or arrayed DNA fragments (see 3.2.1 and 3.3.1), are useful. Conventional chromosomal CGH has revealed DNA copy number losses of chromosome arms 1p, 10q, 13q, 17p, 6q, and 19; and gains of 4q, 7, 8q, 17q, and 18 (Karenko et al.,

1999; Mao et al., 2002; Fischer et al., 2004). In the German study, gain of chromosome arm 8q and loss of 6q and 13q correlated with a significantly shorter survival, whereas some more frequent aberrations (loss in 17p and gain in 7) did not influence the prognosis (Fischer et al., 2004).

Genomic microarrays have been used to study the gene-level copy number aberrations leading to CTCL. Mao and coworkers identified several oncogene copy number gains with AmpliOnc I DNA Array containing 57 oncogenes, the most significant of which was the amplification of *JUNB*, detected in 5 of 7 cases with MF or SS studied. *JUNB* was also overexpressed in a larger series of CTCL patients (Immunohistochemistry or RT-PCR; Mao et al., 2003). The CGH of peripheral blood lymphocyte DNA of 21 SS patients on an array of approximately 3500 BAC-sequences (sensitivity 1Mb for deletions) performed by Vermeer and coworkers, revealed amplifications at 5p15 (57%), 8q11 (48%), 8q24 (71%), 17q11 (71%), 17q21 (86%), 17q25 (71%), 19q13 (29%) and 20q11 (24%) and deletions at 4q31 (38%), 5q22 (43%), 6q24 (29%), 10q24 (52%) and 17p12 (67%). Amplification of *MYC* (8q24) and deletion of *p53* (17p13), genes of interest to CTCL pathogenesis, were confirmed at transcriptional level by quantitative PCR (Vermeer et. al., abstract, 2006).

1.2.2.3 Epigenetic changes in CTCL

Recently, increasing evidence of the role of epigenetic changes has been recognized also in CTCL. As lymphomas in general show more frequent pattern of tumor suppressor gene promoter hypermethylation compared to other cancers (Esteller et al., 2001 and 2003), and as CTCL favourably responds to histone deacetylase (HDAC) inhibitor therapy (Piekarz et al., 2004), the epigenetic gene silencing is speculated to be important in CTCL. In CTCL, promoter hypermethylation of genes encoding *CDKN2A* (Navas et al., 2000 and 2002; Scarisbrick et al., 2002, Gallardo et al., 2004), *CDKN2B* (Scarisbrick et al., 2002; Gallardo et al., 2004), *MLH1* (Scarisbrick et al., 2003), *MGMT* (Gallardo et al., 2004); *BCL7A*, *PTPRG*, *TP73*, and *THBS4* (van Doorn et al., 2005) has been reported, and in some cases revealed to associate with progressed disease (Navas et al., 2002; Scarisbrick et al., 2002; Gallardo et al., 2004). The first drug belonging to histone deacetylase inhibitor group (vorinostat) has been recently approved in the U.S. for the treatment of CTCL not responding to other systemic modes of therapy (Mann et al., 2007). Vorinostat inhibits HDAC by binding to a zinc ion in the catalytic domain of the enzyme (Yoo et al., 2006) resulting in closed chromosomal configuration and transcriptional repression (Bolden et al., 2006).

1.2.2.4 Gene expression profiling of CTCL

Recently, gene expression profiling by DNA microarray technology has been performed, and several novel genes possibly having a role in CTCL pathogenesis have been discovered. Tracey and coworkers identified an expression profile suggesting upregulation of genes involved in TNF signaling pathway (e.g. *TRAF1*, *BIRC3*, *TNFSF5*)

among 29 MF skin samples when compared to inflammatory dermatoses with the CNIO OncoChip array (Tracey et al., 2003). Kari and coworkers (2003) found overexpression of many Th2-specific transcription factors (like *GATA-3* and *JUNB*), as well as *RHOB*, *ITGB1* (integrin β1), and *PRG2* (proteoglycan 2), while underexpressed genes included *CD26*, *STAT4*, and *IL-1* receptors among 48 frozen PBMC samples of SS analyzed with a cDNA array containing 4500 genes. Altogether, a panel of 8 genes was identified that could distinguish SS from normal controls, and 10 genes were able to classify patients into short term and long term survivors (Kari et al., 2003). In the blood samples of 10 Dutch SS patients, analyzed with Affymetrix U95Av2 array, decreased expression of some tumor suppressor genes such as *TGFBR2* (TGF- β receptor II) was shown, while *EPHA4* and *TWIST* were overexpressed. The latter two were highly expressed also in some lesional skin samples of MF (van Doorn et al., 2004).

1.2.2.5 Protein-level aberrations characterizing CTCL

The lack of accurate diagnostic tests for CTCL has lead to efforts to identify CTCL-cell specific markers that would easily be applicable for diagnostic purposes. One of such novel molecular markers is T-plastin, a cytoplasmic protein regulating actin assembly and cellular motility, which is expressed on Sezary cells but not on T-helper cells from healthy individuals or patients with non-malignant dermatoses (Su et al., 2003). Some of the members of killer cell immunoglobulin-like receptors (KIR) that are normally expressed on a minor population of circulating NK and CD8+ T lymphocytes, namely CD158A/KIR2DL1, CD158B/KIR2DL3, and CD158K/KIR3DL2, as well as vimentin have also been suggested as diagnostic markers for circulating Sezary cells (Poszepczynska-Guigné et al., 2004; Huet et al., 2006; Ortonne et al., 2006, Marie-Cardine et al., 2007).

The most important molecular genetic and epigenetic features of CTCL reported in the literature are summarized in Table 2.

Table 2. Overview of the most important molecular genetic and epigenetic changes reported in CTCL				
Gene	Mechanism	CTCL subtype	Presumed consequence	Reference
BCL2	Deletion, underexpression	MF, SS	Altered apoptosis	Nevala 2001; Kari 2003; Mao 2004
BCL7a	Promoter hyper-methylation	MF	Tumor suppression	van Doorn 2005
BIRC3	Overexpression	MF	Defective apoptosis, impaired TNF signaling	Tracey 2003
Caspase-1	Overexpression	MF, SS	Th2 up	Yamanaka 2006
CD158a	Overexpression	SS	Regulation of immune responses	Marie-Cardine 2007
CD158b	Overexpression	SS	Regulation of immune responses	Marie-Cardine 2007
CD158k	Overexpression	SS	Regulation of immune responses	Bagot 2001
CD26	Underexpression	SS	Skin homing	Kari 2003; Narducci 2006; Jones 2001
CD40	Overexpression	MF, SS	Impaired TNF signaling	Storz 2001; Kari 2003
CD40L	Overexpression	MF	T-cell proliferation	Tracey 2003
CDKN2A, p16	Promoter hyper-methylation, LOH	MF, SS	Cell cycle regulation, tumor suppression	Navas 2000 and 2002; Scarisbrick 2002; van Doorn 2005
CDKN2B, p15	Promoter hyper-methylation, LOH	MF, SS	Cell cycle regulation, tumor suppression	Navas 2002; Scarisbrick 2002; van Doorn 2005
CTSB	Amplification	MF, SS	Oncogenesis	Mao 2003
CX3CR1	Overexpression	SS	Defective apoptosis	Kari 2003
EphA4	Overexpression	SS	Oncogenesis	van Doorn 2004
Fas	Point mutation, underexpression	MF, SS	Defective apoptosis, tumor suppression	Dereure 2000 and 2002; Nagasawa 2004; Kari 2003
GATA-3	Overexpression	SS	Th2 up	Kari 2003
HRAS	Amplification	MF, SS	Oncogenesis	Mao 2003
IL1R1	Underexpression	SS	Defective apoptosis	Kari 2003
ITGB1	Overexpression	SS	Skin homing	Kari 2003
JUNB	Amplification, overexpression	MF, SS	Th2 up	Mao 2003; Kari 2003
MLH1	Promoter hyper-methylation	MF	Diminished DNA repair	Scarisbrick 2003
MMP-9	Overexpression	MF	Angiogenesis	Vacca 1997
MYC	Amplification, overexpression	MF, SS	Oncogenic transcription factor	Mao 2003; Vermeer 2006
p53	Point mutation, deletion, underexpression	SS	Tumor suppression, cell cycle regulation	McGregor 1999; Vermeer 2006
PAK1	Amplification	MF, SS	Oncogenesis	Mao 2003
PLS3	Overexpression	SS	Actin interactions	Su 2003; Kari 2003
PTEN	Deletion	MF	Tumor suppression	Scarisbrick 2000
PTPRG	Promoter hyper-methylation	MF	Tumor suppression	van Doorn 2005
RAF1	Amplification	MF, SS	Oncogenesis	Mao 2003
RhoB	Overexpression	SS	Actin interactions	Kari 2003
STAT4	Underexpression	SS	Th1 down	Kari 2003
TGFβR2	Underexpression	SS	Defective tumor suppression	van Doorn 2004
THBS4	Promoter hyper-methylation	MF	Tumor suppression	van Doorn 2005
TP73	Promoter hyper-methylation	MF	Tumor suppression	van Doorn 2005
TRAF1	Overexpression	MF	Defective apoptosis, impaired TNF signaling	Tracey 2003
Twist	Overexpression	SS	Defective apoptosis, oncogenesis	van Doorn 2004

## 1.2.3 The biology of T-lymphocytes normally and in relation to CTCL

1.2.3.1 Normal T lymphocyte development

Normally, naïve T lymphocytes develop and proliferate in thymus where they select their antigen specificity by rearranging their T-cell receptor gene structure, and specialize to naïve cytotoxic (CD8+) or naïve helper (CD4+) T-cells (Petrie, 2003; Takahama, 2006). After maturation in thymus, they circulate in blood and migrate to peripheral lymphoid organs, where they are activated by antigen presenting cells (APC; i.e. dendritic cells, macrophages, and B lymphocytes). T helper progenitor cells differentiate into Th1 or Th2 direction in response to the cytokines secreted by the APC's and the antigenic stimulus (Mosmann et al., 1986; Del Prete et al., 1991; Lahesmaa et al., 1992). Recently, still another subset of T-helper cells, termed Th17, has been recognised (Harrington et al., 2006; Weaver et al., 2006). The various factors influencing T helper cell differentiation are presented below and in Figure 2. Th1 cells function in cell mediated immunity and delayed hypersensitivity reactions and an increased Th1 response plays a role in tissue damage and in various autoimmune conditions. Th2 lymphocytes, instead, maintain the humoral immune response and are involved in the pathogenesis of asthma and atopic diseases. Th17 cells function during infections against extracellular bacteria and in some autoimmune diseases. (Abbas et al., 1996; Mosmann and Sad, 1996; Ray and Cohn, 1999; Romagnani, 1996; Wills-Karp, 1999; Singh et al., 1999; Reiner et al., 2007)

1.2.3.2 T helper cell differentiation

When unpolarized T helper progenitor cells encounter antigen presenting cells in lymph nodes, they interact through T-cell receptor of the T cell and MHCII class molecule of the APC. Various activation signals lead to the differentiation of the T cells either to Th1 or Th2 type T helper cells. The most important issue influencing the Th differentiation is the surrounding cytokine milieu, composed mainly of interleukins (Sher and Coffman, 1992). APC's secrete cytokines, which together with the costimulatory molecules on the surface of T cells and APC's lead to the differentiation of T helper cells into Th1 and Th2 types. The major Th1 polarizing cytokine is interleukin (IL) -12, which acts via STAT4, and induces Th1 differentiation and production of Th1 specific cytokines, such as interferon (IFN) -γ, IL-2, and tumor necrosis factor (TNF) –β (Seder and Paul 1994; Jacobson et al., 1995). Also, IFN-α plays a key role in the Th1 differentiation by regulating STAT1. IL-4 is known to be the major cytokine leading to the Th2 phenotype of the unpolarized Th cells. IL-4 acts through STAT6, and results in the Th2 phenotype and production of cytokines IL-4, IL-13, IL-5, IL-9, IL-6, and IL-10. (Hou et al., 1994; Schindler et al., 1994; Seder and Paul 1994) All the factors that contribute to the T helper cell differentiation are not yet fully known, and the process is complex and several factors are known to inhibit

each other. IL-1 and IL-18 are costimulatory molecules that can increase production of both Th1 and Th2 type cytokines. IFN-α/β and IL-27 are involved in the initiation and maintenance Th1 response, whereas IL-13, IL-6, and IL-21 are required for the Th2 type immune response. Besides cytokines, also transcription factors influence T helper cell differentiation. Nuclear transcription factor T-bet leads to the Th1 phenotype by inducing and enhancing IL-12 signaling. On the other hand, GATA-3, that is activated through STAT6 or by autoactivation or by T cell activation signals, increases Th2 type cytokine production and even turns the Th1 committed T cells back to the Th2 direction. Th17 development is induced by the synergistic action of of IL-6 and IL-1β. (Zhang et al., 1997; Zheng and Flavell, 1997; Szabo et al., 2000; Bettelli et al., 2006; Acosta-Rodriguez et al., 2007.)
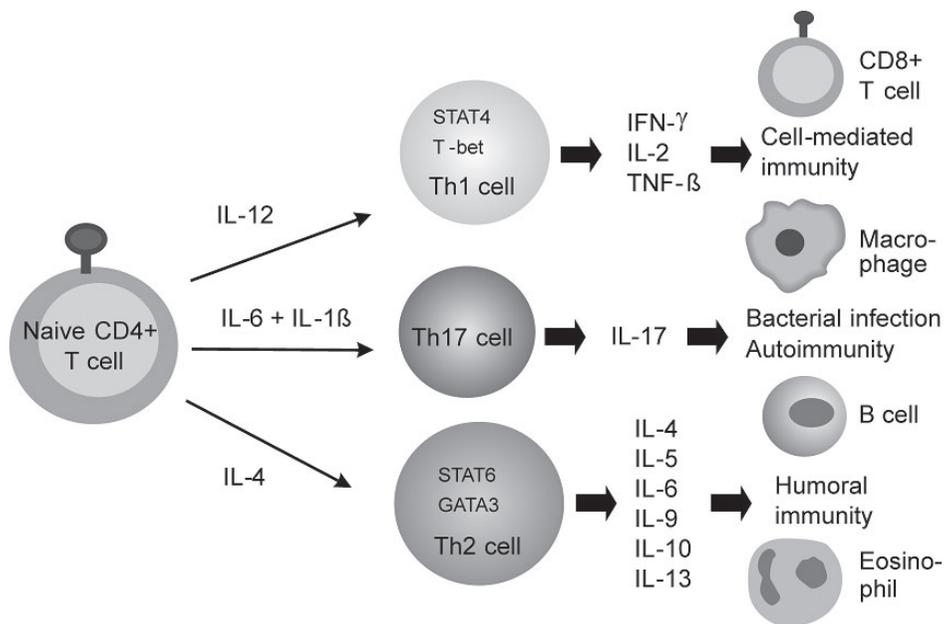
Figure 2. T-helper cell differentiation into Th1 and Th2 cells.
Differentiation of activated T helper cells to Th1 or Th2 direction is induced by IL-12 or IL-4 via STAT4 and STAT6 signaling, respectively, whereas synergistic action of IL-6 and IL-1β favors Th17 development. The cytokines produced by each T helper cell group and their functions in immune responses are shown. Modified from Rengarajan et al., 2000.

1.2.3.3 T lymphocytes in CTCL

The cause, compartment and timing of malignant transformation of the T-lymphocytes in CTCL are not known (Veelken et al., 1995; MacKie et al., 1998; Burg et al., 2001). The migration of Th memory cells to the skin is dependent on the interactions

of a large variety of molecules expressed on T-cells and other cells of the skin (Schön et al., 2003, Pals et al., 2007). While maturing, the T-cells start to express cutaneous lymphocyte-associated antigen (CLA), which through interactions with E-selectin leads their migration to skin (Meijer et al., 1989; Picker et al., 1990). Other molecules leading to skin homing are chemokine receptors 4 and 10 (CCR4 and CCR10), which have been shown to be expressed on CTCL cells often in combination with CXCR3 (Ferenzi et al., 2002; Schön et al., 2003; Notohamiprodjo et al., 2005; Wenzel et al., 2005). Interestingly, MF and SS show different homing signatures, corresponding to the different dissemination patterns of the two subgroups. The tumor cells in MF express low levels of peripheral lymph node homing receptor L-selectin and CCR7. During MF progression, the development of lymph node involvement is accompanied by a loss of skin-specific chemokine receptors and upregulation of CCR7 (Kallinich et al., 2003). In Sezary syndrome, on the contrary, the malignant T-cells coexpress the cutaneous and peripheral lymph node homing signatures, i.e. L-selectin and CCR7 as well as CLA and CCR4 (Sokolowska-Wojdylo et al., 2005), thus explaining the preference of early lymph node involvement in SS.

Local T-cell growth factors, mainly interleukins, maintain T-cell proliferation in the skin. Autocrine IL-2, keratinocyte-derived IL-7, and APC-derived IL-15 are the most important growth factors promoting growth and survival of CTCL cells in vitro (Dalloul et al., 1992; Döbbeling et al., 1998). In later stages of CTCL, tumor cells may become independent of these exogenous signals, e.g. by autocrine production of IL-15 or by interactions via STAT (signal transducers and activators of transcription) molecules (Asadullah et al., 2000; Qin et al., 1999, 2001). Disturbances in STAT signalling pathways have been observed in CTCL, e.g. constitutive STAT3 activation (Zhang et al., 1996), loss of STAT1 and STAT4 protein expression (Tracey et al., 2002; Kari et al., 2003), and dysregulation of the balance between full-length and truncated forms of STAT5 (Mitchell et al., 2003), which affects the cell cycle progression (Moriggl et al., 1999). Despite the availability of the above-mentioned cytokines, it has proven to be difficult to cultivate CTCL cells in vitro. The lack of true CTCL cell lines may be explained by the complicated T-cell signalling, not yet fully understood, as well as some unknown epidermal stimuli, that the cells require.

Based on initial studies on cytokine profiling, it has been revealed that mycosis fungoides exhibits a Th1 type cell-mediated cytokine profile whereas Sezary syndrome expresses a Th2-type profile (Saed et al., 1994, Vowels et al., 1992). In later studies, the Th2 type cytokine profile of SS was confirmed, but reports on MF showed nonconsistent findings with cytokine profile favouring Th1 or Th2 polarization or no polarization (Vowels et al., 1994; Dummer et al., 1996; Harwix et al., 2000). Besides cytokine profiling, also transcription factor level characteristics of CTCL have been revealed. Overexpression of *GATA-3* and underexpression of *STAT4* have been reported in Sezary syndrome (Kari et al., 2003), whereas *STAT4* overexpression characterizes MF (Tracey et al., 2003).

Recently, reduced expression of IL-17 produced by the Th17 cells, has been observed in late stages of MF with blood involvement (Chong et al., 2008).

Types of Th cells other than Th1, Th2 and Th17 have been identified. These are called regulatory T cells (Tregs, previously called suppressor T cells) mediating immunosuppressive functions. Several subsets of Tregs exist, e.g. CD4+CD25+ Tregs, CD4+CD25- Tregs, Tr1 Tregs, Th3 Tregs, and NK Tregs (Beissert et al., 2005) mediating various functions, most importantly suppression of autoimmunity. According to one theory, CTCL would be a malignant proliferation of T regulatory cells (Berger et al., 2005). On the other hand, another theory suggests lack of functional regulatory (CD4+CD25+FOXP3+) T-cells in SS (Tiemessen et al., 2006).

1.2.4 CTCL-associated secondary cancers

Until now only a few epidemiological studies about CTCL-associated secondary cancers have been published. Kantor and coworkers discovered in 1989 an increased risk of lung and colon cancer, as well as other non-Hodgkin lymphomas among CTCL patients in a study covering a 10-year period in American population (Kantor et al., 1989). Väkevä and coworkers performed a study on Finnish CTCL patients, based on the information collected from the Finnish Cancer Registry during the years 1953-1995 (Väkevä et al., 2000). In a cohort of 319 Finnish CTCL patients, 12 patients were diagnosed to have lung cancer apart from CTCL. Half of the lung cancers were of microcellular and one fourth of squamous cell origin. Thus, lung cancer, especially small cell lung cancer (SCLC), was more common than in the general population with over 8,5-fold risk. Also, the risk of non-small cell lung cancer and other non-Hodgkin lymphomas was increased. The average time from CTCL diagnosis to lung cancer diagnosis was 6 years, but half of the SCLC cases occurred within one year after diagnosis of CTCL suggesting possible common biological factors. Environmental factors or treatment of CTCL could not explain the increased incidence of lung cancer. (Väkevä et al., 2000)

1.2.4.1 Lung cancer

Lung cancer, being mainly caused by cigarette smoking, is a leading cause of cancer-related deaths worldwide, with approximately 1.2 million deaths annually (Parkin et al., 2005). Also in Finland, most cancer-deaths occur due to lung cancer and it is the second most common cancer among Finnish males and the fifth most common cancer among Finnish females. In Finland and other developed countries, the incidence of lung cancer is decreasing among men but increasing among women. (http://www.cancerregistry.fi) Malignancies of the lung are divided into small cell (SCLC) and non-small cell lung cancers (NSCLC), the latter consisting of squamous cell carcinoma (SCC, epidermoid carcinoma), adenocarcinoma, and large cell carcinoma. Especially SCLC is a rapidly proliferating and early metastasizing malignancy with poor

survival; median survival after treatment is 1 year (Worden et al., 2000). Recent studies have shown the genetic background to be different among these two cancer types (Wistuba et al., 2001; Fong et al., 2003; Kaminski et al., 2004).

Interestingly, CTCL and SCLC share some histological similarities, as SCLC is composed of small, lymphocyte-like cells, with scarce cytoplasm and molded nuclei growing typically underneath the bronchial epithelium (Kumar et al., 1997). SCLC is considered to be derived from the neuroendocrine cells of the lung (Bunn et al., 1985), and shows different expression of genes related to neuroendocrine cell differentiation and/or growth compared to non-cancerous lung tissue cells (Taniwaki et al., 2006). Typically, in lung carcinomas, multiple chromosome aberrations can be observed indicating genomic instability. SCLC and NSCLC differ from each other based on the molecular cytogenetic findings, although some common abnormalities also exist. The most common shared CGH findings include losses of genetic material from chromosome arms 3p, 4q, 5q, 8p, 13q, and 17p, and gains of 3q, 5p, and 8q, while SCLC cases frequently show losses from 10q and 16q, and gains of 19q, whereas NSCLC is characterized by 9p losses and 1q gains (Balsara and Testa, 2002). When comparing previously reported molecular cytogenetic findings of CTCL and lung cancer, especially SCLC shows some similarities with CTCL. Both CTCL and lung cancer (SCLC and NSCLC) frequently show DNA copy number losses of chromosome arms 13q and 17p, and gains of 8q. Additionally, CTCL and SCLC share the characteristic 10q loss (Karenko et al., 1999; Balsara and Testa, 2002; Mao et al., 2002, 2003; Fischer et al., 2004). This might suggest common underlying genetic factors, possibly even a common cancer stem cell (Huntly et al., 2005; Kim et al., 2005; see 1.1.3).

2. AIMS OF THE STUDY

The aim of the study was to identify novel chromosomal and genetic alterations in cutaneous T-cell lymphomas leading to the clarification of CTCL pathogenesis and classification, develop novel accurate diagnostic tools and identify candidate molecules for targeted therapy. The more detailed aims were to

    I.   identify DNA copy number and gene expression aberrations typical of different CTCL subtypes, as well as to find shared aberrations common to many subtypes

    II.   identify genes important to CTCL pathogenesis, develop novel diagnostic tools and identify candidate molecules for the development of targeted therapy

    III.   study CTCL-associated secondary cancers and reveal genomic changes behind them in relation to primary CTCL or primary cancers occurring at the site of the secondary malignancies

3. MATERIAL AND METHODS

All studies included in this thesis have been approved by the Ethical Review Boards of the Skin and Allergy Hospital, Helsinki University Hospital, and of Internal Medicine, The Joint authority of Helsinki and Uusimaa as indicated in the original publications. All patient material was collected with the patients' (or parents' in case of children) written informed consent.

3.1 Patient samples and preparation of research / study material (I-IV)

Altogether 48 CTCL samples (Table 3), 5 CTCL-associated lung cancer samples, and 29 reference samples of healthy volunteers (studies I and II) or diseased individuals with either non-malignant inflammatory skin disease (studies I and II) or primary lung cancer (study IV) were obtained.

Table 3. CTCL patients studied in the thesis (Studies I-III)

Patient	Diagnosis, sex and age	Applied methods and tissue / cell type	Study
1	SS (M65)	CGH (PBMC), MFISH (PBMC), FISH (PBMC, ln)	I
2	SS (M63)	MFISH (PBMC), FISH (skin)	I
3	SS (M53)	CGH (PBMC), MFISH (PBMC), FISH (PBMC, skin)	I
4	SS (F64)	MFISH (PBMC)	I
5	SS (M68)	MFISH (PBMC)	I
6	SS (M50)	MFISH (PBMC)	I
7	SS (M54)	MFISH (PBMC)	I
8	SS (M52)	CGH (PBMC, skin), MFISH (PBMC), Array (PBMC), QPCR (PBMC), IHC (skin)	II
9	SS (M63)	CGH (PBMC, skin), MFISH (PBMC), Array (PBMC, CD4+), QPCR (PBMC), IHC (skin)	II
10	SS (M72)	CGH (PBMC, skin), MFISH (PBMC), Array (PBMC), QPCR (PBMC)	II
11	SS (M72)	Array (PBMC), QPCR (PBMC)	II
12	SS (M58)	QPCR (PBMC)	II
13	SS (M74)	QPCR (PBMC)	II
14	SS (M59)	IHC (skin)	II
15	SS (M62)	IHC (skin)	II
16	SS (F56)	CGH (PBMC), FISH (skin)	I
17	MF IA (M42)	MFISH (PBMC), FISH (skin), IHC (skin)	I, II
18	MF IA (F79)	CGH (skin), MFISH (PBMC), Array (PBMC, CD4+, skin), QPCR (PBMC, CD4+, skin)	II
19	MF IA/B (M53)	FISH (skin)	I
20	MF IB (M58)	CGH (skin), MFISH (PBMC), FISH (skin), Array (PBMC, CD4+, skin), QPCR (PBMC, CD4+, skin), IHC (skin)	I, II
21	MF IB (M44)	FISH (skin)	I
22	MF IB (F48)	FISH (skin)	I
23	MF IB (F72)	FISH (skin)	I
24	MF IB (M76)	FISH (skin), IHC (skin)	I, II
25	MF IB (M20)	MFISH (PBMC), FISH (skin)	I
26	MF IB (F69)	Array (PBMC), QPCR (PBMC)	II
27	MF IB, CD30+ (M71)	CGH (skin), MFISH (PBMC), Array (PBMC, CD4+), QPCR (PBMC, CD4+), IHC (skin)	II
28	MF IIB (M62)	MFISH (PBMC), FISH (skin), IHC (skin)	I, II
29	MF IIB (M48)	MFISH (PBMC), FISH (skin)	I
30	MF IIB (F56)	MFISH (PBMC), FISH (skin), IHC (skin)	I, II
31	MF IIB (F45)	FISH (skin)	I
32	MF IIB (M52)	FISH (skin)	I
33	MF IIB (F49)	FISH (skin)	I
34	MF III (M69)	FISH (skin)	I
35	MF IVA (M55)	CGH (skin), FISH (skin), IHC (skin)	I, II
36	MF IVA (M83)	CGH (skin), FISH (skin)	I
37	MF IVB (F45)	Array (PBMC), QPCR (PBMC)	II
38	MF (F73)	QPCR (skin)	II
39	MF (F59)	QPCR (PBMC)	II
40	SPTL (F18)	CGH (skin)	III
41	SPTL (M16)	CGH (skin), FISH (skin), LOH (skin)	III
42	SPTL (M13)	CGH (skin), MFISH (PBMC), FISH (skin), LOH (skin)	III
43	SPTL (M18)	CGH (skin), MFISH (PBMC)	III
44	SPTL (M23)	CGH (skin), LOH (skin)	III
45	SPTL (F48)	CGH (skin), MFISH (PBMC), LOH (skin)	III
46	SPTL (F59)		III
47	SPTL (M27)	FISH (skin)	III
48	SPTL (F15)		III

SS = Sezary syndrome, MF = mycosis fungoides, SPTL = subcutaneous panniculitis-like T-cell lymphoma, CGH = comparative genomic hybridization, MFISH = multicolor fluorescent in situ hybridization, FISH = fluorescent in situ hybridization, Array = Affymetrix gene expression array, QPCR = real-time quantitative PCR, IHC = immunohistochemistry, LOH = loss of heterozygosity analysis, PBMC = peripheral blood mononuclear cell, CD4+ = CD4 positive lymphocyte, ln = lymph node

3.1.1 Microdissection of tumor cells (III-IV)

Areas or single morphologically malignant cells (lymphocytes for Study III and lung carcinoma cells for Study IV) were laser capture microdissected using the P.A.L.M. Laser-Microbeam system (P.A.L.M. Microlaser Technologies). Sections of 5-µm were cut from the samples using a microtome and mounted onto a 1,35 µm thin polyethylene membrane (P.A.L.M. Microlaser Technologies, Bernried, Germany) attached to a glass slide. After deparaffinization and hematoxylin staining, single or small areas (50000-200000 µm^2) of morphologically malignant cells were laser capture microdissected using the P.A.L.M. Laser-Microbeam system (P.A.L.M. Microlaser Technologies). Hematoxylin-eosin staining with standard protocol served as morphological control for microdissection. After proteinase K digestion, the DNA was amplified with single cell comparative genomic hybridization (SCOMP) as previously described (Klein et al., 1999, Stoecklein et al., 2002; see 3.1.2.2).

3.1.2 Nucleic acid isolation (I-IV)

3.1.2.1 DNA isolation using standard techniques
DNA was isolated from Ficoll-enriched blood samples, fresh-frozen or paraffin-embedded skin samples from CTCL patients and from control individuals diagnosed with an inflammatory skin disease using standard procedures with phenol and chloroform extraction (Sambrook, 1989).

3.1.2.2 DNA isolation after laser microdissection
After laser capture microdissection of morphologically malignant T lymphocytes (Study III) or lung carcinoma cells (Study IV), microdissected and proteinase K - digested DNA was further digested with Mse I restriction enzyme (New England BioLabs, Ipswich, MA), adaptors were ligated to the 5' overhangs, and DNA fragments were amplified by polymerase chain reaction. The success of the amplification was PCR-tested with microsatellite markers D5S500 and D17S1161, as previously described (Stoecklein et al., 2002). The oldest paraffin blocks with successful DNA isolation and amplification dated back to the 1970's.

3.1.2.3 RNA isolation from PBMC, CD4+ cells and skin
PBMC from patients and healthy controls were isolated with density gradient centrifugation (Ficoll-Paque PLUS, Amersham Biosciences, Uppsala, Sweden), and CD4+ cells were enriched with magnetic beads (CD4+ T-cell isolation kit or CD4+ microbeads, Miltenyi Biotech, Bergisch Gladbach, Germany). Total RNA was isolated with Trizol reagent (Invitrogen Life Technologies, Grand Island, NY). Fresh skin biopsies were immediately placed in RNA Later buffer (Ambion, Austin, TX) and homogenized in Trizol reagent and RNA was isolated.

3.1.3 Cell preparates for FISH, MFISH and CGH (I-IV)

Touch preparates for FISH analysis (see 3.2.2) were prepared from snap-frozen skin or lymph node biopsies of 23 patients with MF, SS, or SPTL, and 10 control samples of inflammatory skin lesions. Briefly, freshly cut tissue surface of snap-frozen (stored in liquid nitrogen or mounted in Tissue-Tek) tissue biopsies were gently pressed against a SuperFrost plus slide, air-dried and stored frozen until analyzed. Normal or patient-derived PHA-stimulated peripheral blood lymphocytes were cultured for 3 days under standard conditions to yield high quality metaphases for CGH or MFISH analysis.

3.2 Molecular cytogenetics

3.2.1 Comparative genomic hybridization (I-IV)

Since the development of comparative genomic hybridization (CGH) in the early 1990's (Kallioniemi et al., 1992), it has become one of the most widely used techniques in cancer research. CGH is based on the competitive hybridization of DNA isolated from the tumor and reference DNA obtained from healthy individuals to normal metaphase chromosome slides. There is no requirement for metaphase preparations of malignant cells, which makes the technology especially feasible for studies of solid tumors, often difficult to cultivate. CGH enables identification and localization of DNA copy number aberrations of the whole genome in a single experiment. The main disadvantage of the technology is its limited resolution being 10-20 Mb for deletions and 1 Mb for amplifications (Kallioniemi et al., 1994; Bentz et al., 1998, Forozan et al., 1997). The sensitivity for deletions can be improved to 3 Mb by using standard reference intervals, based on a series of normal samples (Kirchoff et al., 1999). The length of amplified sequences that can be detected depends on the copy number so that the copy number times the amplicon size is at least 2 Mb (Kallioniemi et al., 1994). Polyploidy weakens the sensitivity. Some chromosomal areas cannot be analysed reliably, e.g. repeat sequences in pericentromeric or heterochromatic regions, and weakly staining telomeres. Additionally, indirect labelling technique may cause false deletions in chromosomal areas 1p32-pter, 16p, 19, and 22 (Kallioniemi et al., 1994). Since CGH analysis is often complicated by normal cell contamination resulting in more normal DNA in the tumor sample, analysis of single cells by single cell comparative genomic hybridization (SCOMP) has become useful in cancer research (Klein et al., 1999, Stoecklein et al., 2002). Also microarray techniques based on the comparative genomic hybridization (Pollack et al., 1999) have revolutioned the tumor biology research (see 3.3.1).

For studies I and II, CGH was performed as described in Karenko et al., 1999. Briefly, 1 µg of tumor DNA labeled with fluorescein isothiocyanate (FITC) in a standard nick translation reaction was cohybridized with 1 µg of control DNA labeled with

Texas Red (both Research Genetics). For studies III and IV, SCOMP was performed as described in Klein et al., 1999 and Stoecklein et al., 2002. Briefly, microdissected and proteinase K-digested DNA obtained from single malignant cells was digested with Mse I restriction enzyme (New England BioLabs, Ipswich, MA), adaptors were ligated to the 5' overhangs, and DNA fragments were amplified by polymerase chain reaction. The amplified DNA was then labeled with digoxigenin-dUTP (Roche, Mannheim, Germany) and similarly processed aliquots of reference DNA obtained from peripheral blood mononuclear cells of healthy volunteers with biotin-dUTP (Roche). For all studies, the labeled probes were cohybridized with human Cot-1-DNA (Invitrogen, Carlsbad, CA) on normal metaphase slides in a humid chamber at 37 degrees for 2 to 3 nights. After posthybridization washes, the preparates were counterstained with 4,6-diamidino-2-phenylindole (DAPI), and metaphases were viewed under a fluorescence microscope. Three-color digital images were captured using an epifluorescence microscope (Axioplan imagining 2, Carl Zeiss AG, Oberkochen, Germany) equipped with a CCD camera. Images were analyzed with ISIS digital image analysis system (MetaSystems GmbH, Altlussheim, Germany) using statistical limits for green to red ratios to determine DNA copy number gains and losses. Eight to twelve metaphases were included in the analysis for each case. As an internal control, normal male and female DNA were cohybridized and only differences in sex chromosomes were identified.

3.2.2 Fluorescence in situ hybridization, FISH (I, III–IV)

The term "in situ hybridization" generally refers to hybridization of a nucleic acid probe to a nucleic acid target, e.g. chromosomes in a patient tissue -derived sample (Gall and Pardue, 1969). Centromere-specific probes, representing repetitive sequences, are used for the detection of gains and losses of a specific whole chromosome. Gene deletions, amplifications and specific translocations can be studied with locus-specifc probes. Locus-specific probes, i.e. probes that recognize unique sequences normally present in only one copy in the haploid genome, are produced by cloning them in different vectors, such as cosmids, plasmids, phages, YACs (yeast artificial chromosome) and BACs (bacterial artificial chromosomes). Nowadays, commercial probes are available for several specific genes. FISH can be applied for metaphase chromosomes (metaphase-FISH) and interphase cells (interphase-FISH). The advantage of interphase-FISH is its usefulness for stored tissue material (Cuneo et al., 1997; Wolfe and Herrington, 1997), its suitability for retrospective studies as well as diagnostics and follow-up of clinical material. The resolution of interphase-FISH varies from 50-200 kb to 1-2 Mb depending on the condensation level of the target chromatin (Lawrence et al., 1990; Trask et al., 1991).

In studies I and II, touch preparates were used to produce preparates of whole cells. In study IV, also whole nuclei isolated from paraffin-embedded tissue sections (Hyytinen et al., 1994) were used. For detection of *NAV3* gene, digoxigenin-labeled

BACs 136F16 and 36P3 were cohybridized together with a centromere 12 -specific probe labeled with biotin. The *NAV3* translocation was detected with digoxigenin-labeled BACs 136F16 and P36P3 with biotin-labeled BACs 786A1 and 494K17. For detection of genes in chromosome 4q12, chromosome 4 copy number was determined with centromere-specific probe CEP4 (Spectrum Green, Vysis Inc., Downers Drive, IL), and *KIT* (clone RP11-586A2), *PDGFRα* (RP11-231C18), and *VEGFR2* (RP11-662M13) copy numbers using BAC probes (Invitrogen Ltd., Paisley, U.K.) (Joensuu et al., 2005). All probes were labeled with nick translation, hybridized in a humid chamber for 2–3 nights and detected with anti-digoxigenin rhodamine or avidin-FITC depending on the primary fluorochrome. After post-hybridization washes, slides were counterstained with 0.1 µmol/L 4,6-diaminido-2-phehylindole in an antifade solution and viewed under a fluorescence microscope equipped with an ISIS digital image analysis system (MetaSystems, Altlussheim, Germany). Signal copy numbers were counted from 50 to 100 randomly chosen non-overlapping interphase nuclei. A nucleus with an equal number of fluorescence signals from the centromere probe and the BAC probes was considered normal. Amplification is manifested as a nucleus with a higher number of signals from the BAC than from the centromere areas, whereas in a deletion the number of centromere signals exceeds the number of BAC signals. The analyses were done blinded to the diagnosis or sample identity. The highest percentage of cells with aberrant signal patterns observed in reference samples was considered as cutoff level.

3.2.3 Multicolor fluorescence in situ hybridization (I–II, unpublished)

Multicolor FISH by staining each chromosome with a specific color combination reveals the origins of marker chromosomes detected by less sensitive methods, and more importantly reveals structural chromosomal aberrations, even the balanced translocations, not demonstrable with other hybridization techniques (Nederlof et al., 1990, Dauwerse et al., 1992, Ried et al., 1992). However, intrachromosomal aberrations, like inversions, deletions and amplifications of small chromosomal areas are difficult to observe. Multicolor-FISH utilizes two different technologies in fluorescent detection: multifluor FISH (MFISH), where the imaging is performed separately for each six colors (Speicher et al., 1996) and spectral karyotyping (SKY), where only one image is necessary and the analysis utilizes Fourier transformation (Schröck et al., 1996). In this study patient-derived PHA-stimulated peripheral blood lymphocytes were cultured for three days for conventional metaphase spreads. The 24-color probe mixture (24XCyte-MetaSystems' 24 color kit, MetaSystems GmbH, Altlussheim, Germany) and the metaphase slides were denatured, and hybridization was performed in a humid chamber for 4-6 days. After posthybridization washes, the biotin labeled probes were detected with streptavidin-Cy5 and the preparations were mounted in antifade

and 4,6-diamidino-2-phenylindole (DAPI, B-tect kit MetaSystems GmbH) according to the manufacturer's instructions. Digital images of the metaphases were taken with an epifluorescence microscope (Axioplan imaging 2, Zeiss, Germany) equipped with a CCD camera, and analyzed with ISIS digital image analysis system (MetaSystems). The number of metaphases analyzed for each case ranged from 30 to 75 depending on the availability of representative metaphases.

3.3 Microarrays

Gene-level aberrations characterizing CTCL and providing new pathomechanistic insights, diagnostic, therapeutic and follow-up tools, are studied by microarray technology. The main purposes for microarray technology are gene expression analysis and gene copy number analysis. Additionally, other microarray technologies, e.g. single nucleotide polymorphism (SNP) arrays, and chromatin immunoprecipitation-on-a-chip (ChIP-on-chip) arrays exist. To date, several microarray platforms are available. In this thesis, gene expression analysis on oligonucleotide microarrays (Affymetrix) and gene copy number analysis on custom-made cDNA arrays was performed.

3.3.1 Array-based comparative genomic hybridization (unpublished data)

The array-CGH method is based on simultaneous hybridization of differentially labelled tumor and normal DNA on microarrays that contain short fragments of DNA as probes. CGH microarrays can be composed of genomic clones (e.g. BACs), cDNA clones, or short oligonucleotide sequences spotted on a glass slide by a robotic arrayer or synthesized in situ. Compared to chromosomal CGH (see 3.2.1), the main advantage of the array technology is the significantly improved resolution allowing the analysis of single gene copy number aberrations across the whole genome. Another advantage is that one can study both the expression and copy number on an identical array.

In this study, CGH microarray was performed using custom-made arrays containing 32,000 cDNA clones representing approximately 16,000 genes in duplicates. Hybridizations were performed as previously described (Pollack et al., 1999; Monni et al., 2001). Briefly, 2 µg of tumour cell DNA was labelled with Cy5-dUTP (Amersham Pharmacia Biotech) and similarly processed gender-matched reference DNA with Cy3-dUTP (Amersham Pharmacia Biotech) by random priming using a BioPrime DNA labeling system kit (Invitrogen). After hybridization over night at 65 °C and post-hybridization washes, the slides were scanned using Agilent laser confocal scanner (Agilent Technologies, Palo Alto, CA), and the results were analyzed using Agilent Feature Extraction Software 8.1. Additionally, two normal versus normal hybridiza-

tions were performed (one self versus self and one sex-mismatch) in order to identify variation that is due to technical aspects. DNA copy number ratios were calculated by dividing the background corrected red fluorescence intensity representing the test hybridization signal by the background corrected green fluorescence intensity representing the control hybridization signal. Logarithms of the fluorescent ratios (log2 values) were used for the analysis. Within slide normalization for each array was performed using Lowess method. After within slide normalization, the data was filtered to eliminate low quality measurements. Filtering was performed in GeneSpring by eliminating features that were flagged as saturated or for which the distribution of signal and background pixels were flagged as non-uniform or as population outliers by the flag outliers algorithm adopted in the Feature Extraction software. Mapping of genes to the clones present on the arrays was performed using software developed at the Biomedicum Bioinformatics Unit (University of Helsinki, Finland). In brief, the software followed a procedure in which nucleotide sequences for all the clones spotted onto the array were retrieved from GenBank and blasted against the most recent version of the human genome assembly. The blasting algorithm employed was optimised with regards to window size and how to handle multiple hits, allowing the flagging of clones whose physical location was ambiguous so that they could be excluded from further analysis. Unambiguous information was available for 13,383 clones representing an average resolution of 187.5 kilobases throughout the genome. The CGH copy number data were ordered according to the location of clones along chromosomes. Genes with copy number ratio exceeding the value 1.32 (log2 0.4) were regarded as amplified, whereas genes with copy number ratio less than 0.76 (log2 –0.4) were considered deleted. The limits were defined based on the variation of copy number signals in normal versus normal hybridizations. In self versus self hybridization, 95.7% of the clones represented on the array, are inside these limits. Based on the fact that amplicons usually span larger regions of the genome rather than a single gene, four or more adjacent clones were required to show a copy number ratio exceeding 1.32. The same criteria were used to determine the deleted areas.

3.3.2 Gene expression microarrays (II)

Gene expression analysis can be performed using either cDNA or oligonucleotide microarrays. The principle of the technique is to compare the relative abundance of expressed sequences in two RNA samples (test and similarly processed reference) (Golub et al., 1999; Singh et al., 2002). The expression of tens of thousands of genes can be measured in a single experiment (Lockhart et al., 1996; Wodicka et al., 1997). Various commercial array platforms are available at the moment, e.g. from Affymerix, Agilent, or Illumina bead array chips. Each Affymetrix array contains several thousand genes or ESTs (expressed sequence tag) with 8-16 oligonucleotide sequences chosen to represent each gene. With specific optimized protocols for small samples,

the amount of starting material may be as little as 100 ng of purified RNA. The bead array technology of Illumina is based on molecules in the sample binding to their matching molecules on the coated bead.

In this study, Affymetrix oligonucleotide array HG-U133A was used. The quality of RNA samples was confirmed with gel electrophoresis and samples were prepared for hybridization according to Affymetrix small sample protocol (Affymetrix Technical note, GeneChip Eucaryotic Small Sample Target Labelling Assay Version II). Starting material for the synthesis was 100 ng of total purified RNA (RNeasy Mini, Qiagen, Valencia, CA). Labeled and fragmented cDNA was hybridized against Affymetrix HG-U133A chip (Affymetrix, Santa Clara, CA) containing probes for over 22 000 transcripts.

Gene expression data were GC-RMA –normalized and log-transformed (Wu et al., 2004; www.bioconductor.org). Gene expression levels of each patient were compared to the average of the controls to obtain a patient-specific expression profile. Genes that were differentially expressed between the patient and the control groups were identified by the modified t-test (Smyth, 2004). A gene was considered changed if the p-value of the test was less than 0.05 and there was at least a 2-fold change in the mean expression levels. The statistical analyses were carried out with R (http://cran.r-project.org) packages Affy and Limma. The expression results were visualized with the TreeView software (Eisen et al., 1998).

3.3.3 Correlating gene expression microarray data with CGH data (II)

Patient-specific gene expression profiles were constructed by calculating gene expression ratios between each patient and the average of the matched controls. Microarray probe sets were mapped along the chromosomes using the Bioconductor annotation package hgu133a to assess regional biases in the expression profiles. If multiple probe sets mapped to the same locus, the average of their expression values was used in the analysis. To identify chromosomal regions with upward or downward expression bias, the sign test proposed by Crawley and Furge (2002) was applied to each cytoband. The sign test scores a gene as up- or down-regulated if the expression change is at least 1.8-fold, and determines whether the corresponding chromosomal arm contains a statistically significant number of genes that change in the same relative direction.

The DNA copy number gain and loss regions were identified from the chromosomal CGH data by using MetaSystems ISIS software (see 3.2.1). When comparing the CGH data with the chromosomal regions (arms) showing gene expression biases by sign test, chromosomal arms with gene copy number and gene expression changing to the same direction, were identified.

3.4 Confirmation of gene expression in tissue samples

In addition to the microarray technology, expression levels of certain specific genes or proteins, were analysed by real-time quantitative PCR, and immunohistochemistry.

3.4.1 Real-time quantitative PCR (I–II)

Real-time quantitative PCR is a method to monitor the progress of the PCR as it occurs (i.e., in real time). At first, complementary DNA (cDNA) is produced from total RNA extracted from the samples. Then, a PCR is performed during which the accumulation of the PCR product is assessed in real time. PCR reactions are performed by using specific probes or dyes, and the amount of the PCR products of the gene of interest in relation to the reference gene are measured by standard curve method or comparative C_t method (see below).

In SYBR Green I Dye technology, SYBR Green I dye detects PCR products by binding to all double-stranded DNA formed during PCR, and no probe is required. The TaqMan technology, instead, is based on the usage of a fluorogenic probe, which consists of an oligonucleotide with a reporter and quencher fluorescent dye attached. During PCR, if the target of interest is present, the probe anneals specifically between the forward and reverse primer sites. Taq DNA polymerase cleaves the probe during the PCR with its 5'-3' exonuclease activity, if the probe hybridizes with the target sequence (Holland et al., 1991). The cleavage separates the reporter dye from the quencher and therefore increases the reporter dye fluorescence signal (Lee et al., 1993). The progress of the PCR can be monitored in real time as the accumulating intensity of the reporter dye (Mullah et al., 1998). The parameter C_t (threshold cycle) is used to measure the number of amplification cycles required to detect a signal, and it is defined as the number of PCR cycles required for the detection of fluorescent signal to exceed a fixed threshold

To analyse NAV3 expression (Study I) or to confirm the microarray gene expression data (Study II), real-time quantitative PCR was performed. The cDNA was prepared with Revert Aid ™ First Strand cDNA Synthesis Kit (Fermentas, St.Leon-Rot, Germany; Study I) or with Superscript II kit (Gibco BRL, Life Technologies, Paisley, Scotland; Study II). PCR reactions were performed in LightCycler ™ apparatus using an LC Fast Start DNA SYBR Green 1 kit (Roche Diagnostics, Mannheim, Germany) followed by melting curve analysis according to the manufacturer's guidelines (Wittwer et al., 1997; Study I) or the PCR reactions were carried out using ABsolute QPCR ROX mix (ABgene, Epsom, UK) with 300 nM primers and 200 nM probe followed by detection Applied Biosystems' ABI Prism 7700 sequence detector (Study II). Primer and probe sequences are listed in Table 4. Linearity of amplification was confirmed by running standard curves for each amplicon and specificity of the reagents was verified by gel electrophoresis. The results were normalized against TBP (Study I, Linja et al., 2004) or EF1α (Study II, Hämäläinen et al., 2001) detection value.

Table 4. Real-time quantitative PCR reagents

Probe ID	Target gene	1) 5' -F primer - 3' 2) 5' -R primer - 3' 3) 5' -Probe - 3'
	NAV3	1) ATCCATGGAGCTCAGCAACTC 2) TGGCTGCTTCTTGGAGTTT
201681_s_at	DLG5	1) GGGGTAGGGGCTGTTTTCTA 2) TGTGCACACTGTACCATCTCAG 3) ProbeLibrary Human#13
	EF1a	1) CTGAACCATCCAGGCCAAAT 2) GCCGTGTGGCAATCCAAT 3) AGCGCCGGCTATGCCCCTG
205291_at	IL2Rb	1) CCCAATACAAAAATACCTACTGCTG 2) TTTGGATATAAAGGCAACAGGAA 3) ProbeLibrary Human#66
207314_x_at	KIR3DL2_1	1) CTGAGCCCAGATCCAAAGTT 2) AACCCCCTCAAGACCTGACT 3) ProbeLibrary Human#51
	KIR3DL2_2	1) CAGTGACCCCCTGGACAT 2) GAGCTACAGGACAAGGTCACG 3) ProbeLibrary Human#51
215838_at	LIR9	1) TCCTGCAGGTATGGTCAGAA 2) ACTGAGGTTATCAGCTGCTCCT 3) ProbLlibrary Human#79
203936_s_at	MMP-9	1) GTGCCATGTAAATCCCCACT 2) TTTGTATCCGGCAAACTGG 3) ProbeLibrary Human#60
213915_at	NKG7	1) TCCCTGGGCCTGATGTTCT 2) TGGGACCCACAGCCTCAA 3) CCTGATTGCTTTGAGCACCGATTTCTG
204351_at	S100P	1) CCGTGGATAAATTGCTCAAGGA 2) CATTTGAGTCCTGCCTTCTCAAA 3) TTCGTGGCTGCAATCACGTCTGC
204655_at	SCYA5	1) TCCCGAACCCATTTCTTCTCT 2) CCCAGCAGTCGTCTTTGTCA 3) TTGGCACACACTTGGCGGTTCTTTC
204466_s_at	SNCA	1) TGTTCCATCCTGTACAAGTGCT 2) CGAGATACACTGTAAAAACTTTGAGAA 3) ProbeLibrary Mouse#80
220684_at	TBX21	1) ACAGCTATGAGGCTGAGTTTCGA 2) GGCCTCGGTAGTAGGACATGGT 3) TCAGCATGAAGCCTGCATTCTTGCC
218807_at	VAV3	1) CGTCAGCCGAACTTTGTTATG 2) TCCACAGGAGTGTTTCTGCTT 3) ProbeLibrary Human#32

3.4.2 Immunohistochemistry (II–IV)

Immunostainings for CD52 (source and dilution for all antibodies are given in Table 5), IL7R, IL7, and KLK10 were performed with Vectastain Elite Mouse kit (Vector Laboratories, Burlingame, California) on frozen tissue sections, according to manufacturer's instructions. MMP-9 immunostaining was performed on formalin-fixed paraffin-embedded tissue sections using a mouse monoclonal antihuman MMP-9 antibody as previously described (Saarialho-Kere et al., 1993). For study III, sections cut from the paraffin-embedded biopsies were immunostained for membrane antigens CD3, CD4, CD5, CD7, CD8, CD30, CD56, Granzyme B, TIA1, Ki-67, and TCR alpha/beta according to manufacturers' instructions and visualized with DakoEnvision (Glostrup, Denmark). KIT expression was demonstrated with a rabbit polyclonal anti-CD117 antibody, as described in detail elsewhere (Sihto et al., 2007). The binding of the primary antibody was detected with a Powervision+ Poly-HRP histostaining kit (DPVB+110DAB, Immunovision Technologies Co, Daly City, CA). All tissue sections were counterstained with hematoxylin. The stainings were graded as negative (-), weakly positive (+; less than 10% of tumor cells expressed the protein), moderately positive (++; 10 to 50% of tumor cells were positive) or strongly positive (+++; over 50% of the tumor cells expressed the protein).

| Table 5. Antibodies used in immunohistochemistry			
Antibody	**Source**	**Dilution**	**Study**
CD52	Abcam, Cambridge, UK	1:100	II
IL7R	R&D Systems, MN, USA	1:10	II
IL7	R&D Systems, MN, USA	1:20	II
KLK10	R&D Systems, MN, USA	1:30	II
MMP-9	Diagnostics Inc, Flanders, New Jersey	1:50	II
CD3	Novocastra, New Castle, UK	1:100	III
CD4	Novocastra, New Castle, UK	1:150	III
CD5	Novocastra, New Castle, UK	1:25	III
CD7	Novocastra, New Castle, UK	1:100	III
CD8	Novocastra, New Castle, UK	1:25	III
CD30	Dako, Glostrup, Denmark	1:25	III
CD56	Zymed, South San Fransisco, CA	1:50	III
Granzyme B	Monosan, Uden, The Netherlands	1:100	III
TIA-1	Biocare, Birmingham, UK	1:200	III
Ki-67 / MIBI	Dako, Glostrup, Denmark	1:50	III
TCR alpha / beta	GeneTex, TX, USA	1:100	III
KIT = CD117	Dako, Glostrup, Denmark	1:300	IV

3.5 Other methods

3.5.1 Loss of heterozygosity analysis (III–IV)

Loss of heterozygosity (LOH), also called allelic imbalance, can be measured by comparing the amount of PCR products amplified from the two constitutional chromosomes. This is possible when the sequence amplified contains a polymorphism that distinguishes between the two alleles. Typically, the polymorphic sequences that are utilised for LOH mapping are microsatellite repeats. Microsatellites are short tandem repeats of very simple DNA sequences, usually 1–4 bp long, for example $(GT)_n$, that are widely dispersed throughout the genome.

LOH analysis by microsatellite markers was performed to reveal the allelic loss of *NAV3* or confirm the *NAV3* deletion detected by interphase-FISH. For *NAV3* LOH analysis we chose three microsatellite markers spanning the *NAV3* gene locus at 12q21.2–q21.31 and surrounding the gene from both directions (Figure 3). The PCR amplification primers are shown in Table 6. The forward primers were fluorescently labeled with FAM and PCR fragments were run on the ABI3730 sequencer/genotyper and the results were analysed using GeneMapper v3 software (Applied Biosystems, Europe). A sample was scored as showing LOH, if one of the alleles in the tumor sample had decreased 40% or more compared to its matching normal and decrease of 21–39% of one allele relative to the other allele (in tumor tissue compared to matching normal tissue) is termed putative LOH. (Canzian et al., 1996).

Table 6. Microsatellite markers used for NAV3 LOH analysis		
Marker	Distance from NAV3	1) 5' - F primer - 3'
		2) 5' - R primer - 3'
D12S1684	1.0 Mb	1) CCTGCATGCCTCAGTTATGA
		2) AACAAGCCATACCAGTCAGG
D12S326	0.2 Mb	1) ACCAGGCTCCCCTAAAAGTG
		2) AGAATGACCAGACCCACAGG
D12S1708	3.8 Mb	1) GGGAACTTATGTCAAGGCTAGGA
		2) GATCTAGTGCTCAAGAGGTTTTCAA
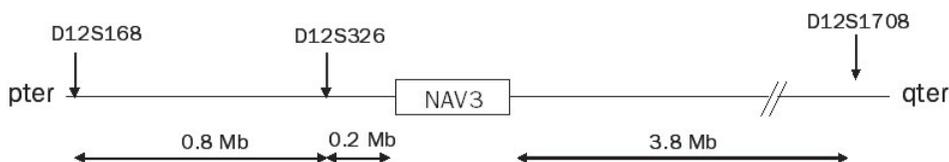
Figure 3. NAV3 gene in relation to the microsatellite markers used in studies III–IV. Drawn according to ideas of Dr. Ulla Aapola, Dermagene Oy.

3.5.2 Gene silencing by RNA interference technology (I)

RNA interference (RNAi) is a mechanism for RNA-guided regulation of gene expression in which double-stranded ribonucleic acid inhibits the expression of genes with complementary nucleotide sequences. RNAi technique was used to silence the gene producing NAV3-protein in human lymphoid cell lines and thus, mimicking the situation in vivo in CTCL. *NAV3* gene silencing was performed with small interfering RNA-expressing lentivirus constructs. Several DNA sequences encoding small interfering RNA (siRNA) precursors based on the NAV3 sequence were cloned in the lentiviral vector pLL3.7 for expression under the U6 promoter, including GFP expression, and the most specific inhibition was observed with a 23-nucleotide sequence from the *NAV3* exon 19. This derivative was then used for production of infectious short hairpin RNA–expressing lentiviruses as described in more detail in Study I. The number of infected cells (GFP-positive) were estimated by fluorescence-activated cell sorting (FACS). The NAV3 expression of FACS-sorted (BD FACSAria; BD Biosciences) GFP-positive cells was studied with quantitative RT-PCR by Light Cycler device (Roche Diagnostics, Mannheim, Germany), as described in 4.4.1.

3.5.3 Additional methods

The most important methods for this thesis are presented above. Other methods that have been used, include immunofluorescence, Western blot, DNA sequencing, denaturing high-performance liquid chromatography (DHPLC), reverse transcriptase PCR / conventional PCR, and flow cytometry analyses. These methods are described in more detail in Study I.

4. RESULTS AND DISCUSSION

4.1 Chromosomal aberrations characterizing different CTCL subtypes (I–IV)

4.1.1 Combination of molecular cytogenetics and gene expression profiling reveals chromosomal regions with both gene expression and DNA copy number changes (II)

It has been shown previously, that at least 12% of the differences seen at transcriptional level are due to the variation in gene copy number (Pollack et al., 2002). To reveal the impact of gene copy number on gene expression among CTCL patient series, an integrated analysis of CGH and microarray-based gene expression profiling on 4 SS PBMC and 3 MF skin samples was performed. By gene expression analysis, a significant, consistent upward bias in gene expression levels was found in at least four of the seven patient samples in the following five chromosome arms: 1q, 3p, 3q, 16p, and 16q. In addition, chromosome arms 4q and 12q, exhibited both an upward and a downward bias (Study II, Figure 4a). To integrate the chromosomal and transcriptomic data, these chromosomal regions were compared with the CGH data. In three of the above seven chromosomal arms, 1q, 4q, and 16q, with an upward bias, a significant gain was detected also in CGH in at least four of the seven MF skin or SS blood samples. A significant gain was detected in at least two of the samples in chromosomes 3p, 3q, and 12q and in one sample in chromosome 16p. In chromosomes 4q and 12q, where also downward expression bias was identified, a loss was detected by CGH in one and two of the samples, respectively. (Study II, Figure 4c.)

In CTCL, gains of chromosome arms 1q, 3p, 3q, 4q, and 16q have been observed previously by CGH (Karenko and Kähkönen, unpublished observation) and diverse aberrations of these chromosomes are common by other cytogenetic or molecular cytogenetic methods (Karenko et al., 1997; Mao et al., 2002). These chromosome arms contained altogether 23 and 30 probe sets found to be upregulated in SS patients' PBMC samples and MF patients' skin samples, respectively, which represent 0.7 and 1.0 percent of all array probe sets included in these chromosome arms. For example, the upregulated SNCA gene (see 5.3.2) locates to cytoband 4q21. The above chromosome arms, thus, are potential targets for searching for further recurring gene aberrations in CTCL.

4.1.2 Chromosomal aberrations characterizing SPTL and differentiating SPTL from the other CTCL subtypes (III, unpublished)

Of the rare subtypes of CTCL, i.e. other than MF and SS, SPTL was studied because of its different clinical picture (see 1.2.1.3), and more importantly because of its difficult clinical diagnosis. Chromosomal and genomic analysis was performed in order to achieve some more knowledge about the poorly known mechanisms of this disease entity.

To ensure the best possible representativeness of the sample material where malignant cells are sparse and scattered among other normal cells of the skin, the morphologically malignant cells were collected by single-cell laser microdissection. The pure malignant cell population thus obtained, showed several novel DNA copy number abnormalities by CGH. The DNA copy number changes were very uniform between individual patients with a large number of changes occurring in the vast majority of the cases. The most common CGH findings were DNA copy number losses in chromosomes 1p (6/6 cases), 2p (5/6), 2q (4/6), 5p (5/6), 7p (4/6), 9q (5/6), 10q (5/6), 11q (5/6), 12q (5/6), 16 (5/6), 17q (4/6), 19 (4/6), 20 (6/6), 22 (6/6), and gains in chromosomes 2q (5/6), 4q (5/6), 5q (4/6), 6q (4/6), and 13q (4/6) (Study III, Figure 2).

Previous CGH studies on MF and SS have demonstrated gains in chromosomes 2q (Mao et al., 2002; Karenko et al., 1999) and 4q (Mao et al., 2002; Study II). As described above (4.1.1), chromosome arm 4q is also among the areas with the highest frequency of upregulated genes among the more common CTCL subtypes (Study II). Chromosomal losses of 1p, 17p, 10q, and 19 were the most common CGH changes in a British study of 34 CTCL patients (Mao et al., 2002), and other groups have demonstrated losses of 10q and 17p to be the key aberrations in CTCL (Karenko et al., 1999; Fischer et al., 2004). On the other hand, losses of chromosomes 5q and 13q occur in the most common forms of CTCL (Karenko et al., 1999; Fischer et al., 2004), but in SPTL these areas showed DNA copy number gains by CGH. The current finding of losses in chromosome 12q in most cases of SPTL is in concordance with our previous identification of both DNA copy number loss and a downward gene expression bias in chromosome 12q observed in Study II. It may be speculated that aberrations in chromosome arms common to both SPTL and MF/SS, may not influence the prognosis, since the prognosis of SPTL is favourable and that of SS less favourable. It has previously been observed that 17p loss and 7 gain do not influence the prognosis of CTCL (MF and SS) patients (Fischer et al., 2004). Instead, 13q loss has been found to associate with shorter survival among MF and SS patients (Fischer et al., 2004), and interestingly, SPTL patients did not show this abnormality, but instead, frequently had a gain at 13q.

Although the malignant cells of the subcutaneous SPTL lesions showed a large number of genomic changes, clonal aberrations in blood lymphocytes could be identified by MFISH in only one case. The neoplastic cell clones, thus, seem mainly to

be restricted to the subcutaneous tissue. This finding is in line with the recently revealed favourable clinical prognosis of SPTL with TCR α/β positive T-cell phenotype (Willemze et al., 2005; Willemze et al., 2008), and further encourages introduction of less potentially hazardous treatment modalities than multi-agent chemotherapy. Interestingly, one case had three different clones in blood by MFISH [dup(16)(q?q?), del(11)(q?), and del(13)(q?)], one of which was also identified in the subcutaneous tissue by CGH, whereas the two others were not. This suggests the presence of different clones or subclones in the skin and blood, which has been observed in other CTCL forms as well (Muche et al., 2004).

The array-CGH performed on microdissected skin samples amplified with the whole genome amplification method resulting in DNA fragments of 256 bp of length in average, was not technically successful. On the contrary, array-CGH of one case (the one with changes in MFISH) on peripheral blood DNA sample isolated with standard methods demonstrated frequent DNA copy number aberrations in chromosomes 1, 2, 5, 7, 11, 17, and 19. These aberrations were only partly overlapping with those characteristic for the malignant subcutaneous T-lymphocytes as defined by CGH, further pointing to the existence of different subclones in skin and blood.

4.1.3 CTCL-associated lung cancers show chromosomal aberrations differing from primary lung cancer and resembling aberrations observed in CTCL primary lesions (skin/blood) (IV)

Since CTCL and lung cancer share some chromosomal aberrations (see 1.2.4.1), eventual chromosomal and genomic similarities and differences in lung cancers developed in CTCL patients and in solitary lung cancers were searched. Comparative genomic hybridization on five CTCL-associated secondary lung cancer cases and on five reference cases with primary lung cancer was performed. The most common DNA copy number aberrations in both groups are illustrated in Figure 4. The reliability of the microdissection-based method was confirmed by the fact that DNA copy number aberrations in reference lung cancer without CTCL-association were in concordance with those reported in the literature. These results revealed that CTCL-associated lung cancer differs from primary lung cancer for the DNA copy number changes. CTCL-associated lung cancers showed some of the typical lung cancer aberrations, e.g. losses of 5q, 10q, and 16q, as well as gains of 3q, and 5p, but also aberrations that are not frequent primary lung cancer, like gains of 4q gain 9p. Additionally, some of the DNA copy number changes found in CTCL-associated lung cancer (both SCLC and NSCLC) are frequent in CTCL, e.g. losses of 1p and 10q, and gains of 4q, 7, and 8q, which were present in at least 3/5 of the CTCL-associated lung cancer samples and losses of 13q, 17p, and 19, as well as 18 gain, which were present in fewer samples. The changes common to both CTCL and lung cancer were loss of 10q, 13q and 17p, and gains of 7 and 8q (Coe et al., 2006; Garnis et al., 2006). Additionally, in CTCL-associated lung

cancer, some aberrations frequent in primary / reference lung cancer, were rare, if any (detected in maximum one of the five cases), especially 4q loss, 8p loss, and 19q gain. (Study IV, Figure 1a.) Typically, deletions of 4q have been considered as hallmark of lung cancer, especially for small cell lung cancer (Balsara and Testa, 2002), but in CTCL 4q belongs to the most frequently amplified (Mao et al., 2002) as well as overexpressed chromosomal regions (Study II). Three of the five CTCL-associated secondary lung cancer cases showed gains in 4q, and only one showed losses. Taken together, CGH revealed CTCL-associated lung cancers to harbour chromosomal aberrations differing from primary lung cancer, especially losses of 1p and 19, and gains of 4q and 7.
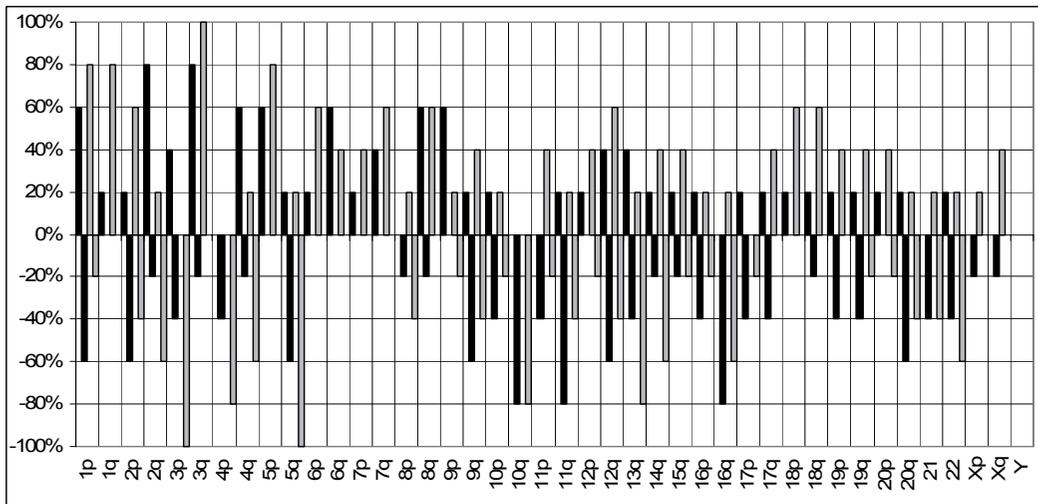
Figure 4. The main differences between CTCL-associated and reference lung cancer.
X-axis illustrates the chromosome arms, whereas Y-axis depicts the percentages of gains (positive percentages, above the central line) and losses (negative percentages, below the central line) identified for CTCL-associated lung cancer (black bars) and reference lung cancer (grey bars). Original figure from Study IV, supplementary data.

4.2 Gene expression profiling of CTCL (II)

A total of 17 samples from 9 patients and 14 samples from 9 controls were successfully hybridized on Affymetrix gene expression microarrays. To mask the effect of infiltrating non-malignant, reactive T cells on CTCL-associated gene expression profiling, commonly present in the samples of CTCL (Wood et al., 1994; Bagot et al., 1998; Berger et al., 2005), comparison of microarray data from different cell populations of CTCL patients was performed, and changes common to different cell sources of MF patients (PBMC, CD4+ lymphocytes, and lesional skin) and SS PBMC samples were identified.

Altogether 168 probe sets (fold change > 2, p-value < 0.05; representing approximately 0.8 percent of all the probe sets included in the array) were differentially regulated in Sezary syndrome PBMC samples compared to control PBMC samples. Of these genes, the majority was upregulated. Comparable amount of differences was also found between MF blood CD4+ and control blood CD4+ samples (205 probe sets, 0.9%). As expected, fewer differences, only 40 differentially regulated probe sets (0.4%), were found in MF PBMC samples. Instead, there were substantial differences in gene expression when MF skin samples were compared to control skin samples from patients with inflammatory dermatoses (223 probe sets, 1.0%), and most of the differences were due to the increased gene expression. The gene expression changes found in different cell types are shown in Figure 5. Because the number of malignant cells in Sezary syndrome patient blood samples is considerably greater than that in mycosis fungoides patient blood samples, the gene expression profiles varied remarkably between Sezary syndrome and mycosis fungoides PBMC samples (Study II, Figure 1E). However, a subset of genes was found to change in a similar manner in both Sezary syndrome and mycosis fungoides PBMC samples (Study II, Figure 1F and Table 1). Among these similarly regulated genes, are e.g. *CD160* downregulation, and upregulation of *LIR9*, *STAT1*, *SNCA*, *MMP-9*, and *S100P*. *SNCA* and *LIR9* genes were upregulated both in SS and MF patients' PBMC samples, and MF skin biopsies and MF CD4+ cells, respectively. Commonly upregulated genes in the cell populations representing the primary or most frequent site of malignant cells (SS PBMC and MF skin) included e.g. *ITM2A*, *ETHE1*, and *BARD1*.

In Sezary syndrome samples, two Th1-specific genes (*SCYA5* and *NKG7*) and *IL-2R*β, *VAV3*, *DLG5*, and *KIR3DL2* were found to be >2-fold down-regulated. Genes upregulated in both mycosis fungoides and Sezary syndrome blood samples included *S100P* and *MMP-9*. In lesional mycosis fungoides skin samples compared with inflammatory dermatoses, *IL-7R* and *CD52* were up-regulated. *SNCA* and *LIR9* genes were up-regulated in several cell populations of Sezary syndrome and mycosis fungoides patients. These genes were selected for further analysis by quantitative PCR and immunohistochemistry, in addition to the *TBX21* (T-bet) gene, based on its crucial role in Th1 differentiation (Szabo et al., 2000). The selection of the genes for further analysis was not only based on fulfilling the statistical and biological criteria for different regulation in microarray analysis (p < 0.05 and fold change > 2) but also on the presumed impact of a certain gene on CTCL pathogenesis. (Study II, Figures 1A-D)

QPCR done on 11 selected genes (*T-bet*, *SCYA5*, *NKG7*, *IL-2R*β, *VAV3*, *DLG5*, *KIR3DL2*, *S100P*, *MMP-9*, *SNCA*, and *LIR9*; Study II, Figure 2) and immunohistochemistry performed on 4 gene products (CD52, IL-7R, IL-7, and MMP-9; Study II, Figure 3) validated the microarray data. In general, the changes in the expression levels of the selected genes, demonstrated even more pronounced when measured with QPCR compared to microarrays. Eleven additional CTCL patient samples and 7 additional control samples were included in the QPCR and IHC analyses.

To conclude, these findings provide the basis for the previously observed Th2-type cytokine-profile for Sezary syndrome, because a set of Th1-specific genes was downregulated in Sezary syndrome samples. Moreover, the different biology behind MF and SS was demonstrated by different gene expression signatures of these common CTCL subtypes (Study II, Figure 1E).

Figure 5. Substantial differences in gene expression profiles were found in SS PBMC, MF CD4+ and skin biopsy samples, compared to corresponding control samples. The most differentially (p<0.05 and fold change>3) expressed genes between patient and control samples are shown: SS PBMC (A), MF skin biopsies (B), MF CD4+ cells (C) and MF PBMC (D). Original picture in Study II, supplementary data.

4.3 Novel target genes characterizing different CTCL subtypes (I–IV)

4.3.1 NAV3 gene deletion is frequent in many CTCL sybtypes and happens already at the early stages (I, III, IV)

In search for recurrent chromosomal aberrations in the most common forms of CTCL, structural chromosome aberrations, as defined by MFISH, were found most frequently in chromosome 12 (in 6/7 SS patients). In one of these patients, a balanced, clonal translocation t(12;18)(q21;q21) was identified and lead to the identification of the gene involved in the translocation. When minimizing the common area of deletion in the above cases, signal division in FISH analyses of metaphase chromosomes indicated that the translocation break point lies within BAC probes RP11-494K17 and 36F16, which both contain parts of the *NAV3* gene, thus disrupted by the translocation (Study I, Figure 2). The *NAV3* gene, also named *POMFIL1*, locates to chromosome band 12q21, and losses of one copy of its distal part in relation to chromosome 12 centromere was found in 4/8 early stage (MF IA-IIA) and 11/13 advanced stage CTCL patients (MF IIB-IV or SS) as studied with locus-specific FISH on touch preparates of skin lesions (Study I, Figure 4). Allelic loss of *NAV3* was also demonstrated in more rare CTCL subtypes, namely SPTL, in which it was found in 2 of 3 patients with FISH and in 4 of 4 patients by the LOH technique (Study III). Of the blood lymphocyte DNA from the seven SS cases, six with a cytogenetic aberration of 12q, studied with DNA sequencing or DHPLC, only one showed a missense mutation in the microscopically intact allele of NAV3.

Allelic loss of *NAV3* was also studied in CTCL-associated lung cancer by LOH technique. However, the CTCL-associated lung cancer cases were uninformative due to constitutional homozygosity and/or suboptimal performance in the LOH assays. Instead, all five reference lung cancer cases were successfully analyzed, and loss of heterozygosity for *NAV3* was found in two of the four informative cases. One sample was uninformative for all three LOH markers used. (Study IV, Figure 4)

NAV3 is a large gene, consisting of 40 exons and spanning around 400 kb of genomic sequence. The function of NAV3 is largely unknown. It is one of three human homologues of *UNC-53*, involved in axon guidance in C. Elegans, and it has predictive properties of a signal transduction protein and actin interactions. (Coy et al., 2002; Ishiguro et al., 2002; Maes et al., 2002; Merrill et al., 2002; Stringham et al., 2002). By structure it is also a helicase and an exonuclease with similarity to proteins causing Werner's and Bloom's syndromes and with the role of maintaining chromosomal stability (Coy et al., 2002; Maes et al.; 2002; Ishiguro et al., 2002; Nakayama, 2002). By its location in the nuclear membrane, NAV3 might also affect nuclear transport or kinetochore formation and cell cycle control (Maes et al., 2002; Coy et al., 2002; Fahrenkrog and Aebi 2003). Deletions or lowered expression of *NAV3* has been found

in neuroblastoma, too (Coy et al., 2002). By RT-PCR, NAV3 expression was found only in PHA-stimulated but not in resting human lymphocytes (Study I). Moreover, NAV3-siRNA silenced Jurkat cells and peripheral blood lymphocytes showed increased IL-2 production (Study I, Figure 6). Based on these functional consequences of *NAV3* allelic loss, and the fact that point mutations in the remaining *NAV3* allele were only rarely observed, *NAV3* is believed to function as a haploinsufficient tumor suppressor gene. Further characterization on the effects of *NAV3* loss in different human cells and tissues as well as in an animal model is in progress.

4.3.2 Th1 response and cytotoxicity genes are downregulated in SS and MF (II)

Of the Th1-specific genes down-regulated in Sezary syndrome, *TBX21* (T-bet) and *TXK* represent transcription factors essential for T helper cell commitment to Th1 phenotype. They belong to a positive feedback loop promoting Th1 cytokine secretion, leading to Th1 development (Kashiwakura et al., 1999; Szabo et al., 2000). *TBX21* downregulation in Sezary syndrome would explain the previous observation of the loss of the chemokine ligand CXCR3 expression along the progression of mycosis fungoides (Lu et al., 2001), because *TBX21* regulates the CD4+ cell trafficking via CXCR3 (Lord et al., 2005).

In SS PBMC samples, the expression of *SCYA5* (*RANTES*) and *NKG7* was downregulated. These genes have also been linked to Th cell differentiation and shown to be more abundantly expressed in cells polarized to Th1 than Th2 direction (Nagai et al., 2001; Lund et al., 2005). SCYA5 is a chemokine that mediates the trafficking and homing of T-cells (Appay et al., 2001). Very little is known about the functional role of NKG7 in the Th cell differentiation. NKG7 is expressed in activated T cells in peripheral blood (Turman et al., 1993). It is induced by IL-4 in the early stages of differentiation process, but later on its expression is upregulated also by IL-12. After one week of polarization, NKG7 is preferentially expressed by Th1 cells (Lund et al., 2005).

Among the upregulated genes, several genes important for the early polarization of T helper cells into Th2 direction (Lund et al., 2005) were identified, e.g. *S100P* and *LIR9* genes. Increased expression of *S100P* was observed in both SS and MF PBMC samples. S100P has a role in cell cycle progression and differentiation, and its upregulation has been found in various malignancies (Guerreiro Da Silva et al., 2000; Diederichs et al., 2004; Sato et al., 2004; Hammacher et al., 2005). Because an expression bias for *S100P* was found in mycosis fungoides blood samples also, S100P may have a role in the early oncogenesis of CTCL. In addition, membrane-bound *LIR9* (215838_at) was overexpressed in SS PBMC, MF PBMC, and MF CD4+ samples. LIR9 is a member of leukocyte immunoglobulin-like receptor family mostly expressed on monocytes and neutrophils but not on normal T cells. In monocytes, activation of LIR9 induces secretion of IL-1β, tumor necrosis factor-α, and IL-6 (Borges

et al., 2003). Deregulation of tumor necrosis factor signalling pathway has been linked to both Sezary syndrome and mycosis fungoides pathogenesis (Tracey et al., 2003; French et al., 2005), and IL-6 has been shown to be a marker of SS tumor burden and to correlate with clinical stage in non-leukemic CTCL (Hassel et al., 2004). Moreover, IL-6 is an important cytokine for Th2 cell differentiation (Diehl et al., 2002) and also induces S100P (Hammacher et al., 2005).

Of the cytotoxicity-associated genes, *XCL1* (lymphotactin), *GZMB* (granzyme B), and *VAV3* were downregulated. XCL1 is known to augment antitumor responses (Cairns et al., 2001), and VAV3, like the other two VAV proteins, functions specifically in signaling pathways that trigger natural killer cell cytotoxicity (Cella et al., 2004). KIR3DL2 (CD158K), a member of the killer cell immunoglobulin-like receptors, has been suggested previously as a phenotypic marker for Sezary cells (Poszepczynska-Guigne et al., 2004) and has been found upregulated in Sezary syndrome (van Doorn et al., 2004). Contradictory, in this study the *KIR3DL2* gene was downregulated in Sezary syndrome samples. The discrepant observations of *KIR3DL2* may be due to the considerable polymorphism of the *KIR3DL2* gene (Dohring et al., 1996; Gardiner et al., 2001), or may reflect the fact that only about half of the expanded T-cell clones express CD158K and that CD158K expression is heterogeneous even within the malignant clones (Ortonne et al., 2006).

*IL-2R*β was downregulated in Sezary syndrome blood samples, which is of interest because IL-2 is the major cytokine for T-cell activation and proliferation. IL-2R consists of three subunits, of which IL-2Rβ and IL-2Rγ are expressed on resting T cells and upregulated by IL-2. The high/intermediate affinity IL-2R is expressed on ca. 50% of CTCL cells (Jones et al., 2004). Consequently, IL-2-targeted therapy has been used for CTCL, most recently with a fusion protein denileukin diftitox (ONTAK; Duvic et al., 1998). Interestingly, the retinoid X receptor retinoid, bexarotene, a new therapeutic agent for mycosis fungoides (Duvic et al., 2000), upregulates both the α and β subunits of IL-2R. This, in turn, enhances the susceptibility of the malignant cells to denileukin diftitox, resulting in overall response rates of 67% in relapsed CTCL patients (Foss et al., 2005). These findings indicate that changes observed by gene expression profiling may be applied for the development of novel targeted therapy.

The expression levels of *IL-7R*, but not *IL-7*, were upregulated in the lesional skin biopsies of mycosis fungoides patients, and immunohistology revealed that the origin of the increased IL-7R was in the basal keratinocytes and infiltrating lymphocytes in CTCL lesions. IL-7 is used as growth factor for CTCL cell lines that are extremely difficult to propagate (Dalloul et al., 1992; Möller et al., 1996; Döbbeling et al., 1998). IL-7 has also been shown to increase IL-7R expression on Sezary cells (Foss et al., 1994). Knockout studies in mice suggest that IL-7R has an important role in differentiation and activation of T lymphocytes as well as in blocking apoptosis (Maraskovsky et al., 1996; Akashi et al., 1997). In CTCL, inhibition of apoptosis might also provide a growth advantage to the malignant T-cells in the skin. Since IL-7 has a role in traffick-

ing of T lymphocytes to and from the skin (Williams et al., 1997; Jiang et al., 2005), the IL-7/IL-7R balance might influence the homing of malignant lymphocytes to the epidermis in CTCL. Thus, a plausible molecular explanation for the proliferative response of CTCL cells to skin-derived IL-7 was revealed (Foss et al., 1994).

CD52 upregulation was found at both transcriptional and protein level. Immunohistochemistry revealed CD52 protein expression of skin-infiltrating lymphocytes of early stages mycosis fungoides (stages IA-IB). CD52 membrane antigen is normally expressed on all lymphocytes, but its actual function is unclear. Nevertheless, humanized anti-CD52 monoclonal antibody (Campath-1H, alemtuzumab) has been used to treat advanced forms of CTCL (Sezary syndrome and late-stage mycosis fungoides; Ravandi et al., 2005). This finding might warrant the use of alemtuzumab also for earlier stages of mycosis fungoides, in case its adverse effects allow.

MMP-9 upregulation was observed in SS and MF PBMC samples, whereas MMP-9 protein was demonstrable in skin infiltrating lymphocytes in SS but not in MF skin biopsies. This might indicate that the original malignant clone arises in blood and thereafter homes to the skin. In inflammatory dermatoses, the lymphocytes did not express MMP-9. In addition to their role in facilitating tumor cell invasion and metastasis (Overall et al., 2002; Klein et al., 2004), MMPs may be involved in cancer initiation possibly by activating intracellular mediators that are inducers of genomic damage and may cause genomic instability (Egeblad et al., 2002). Concordantly, MMP-9 overexpression has been previously observed in MF in relation to an advancing stage (Vacca et al., 1997).

Dysregulation of genes located in chromosomal areas showing DNA copy number aberrations were of special interest. The *SNCA* gene, mapping to chromosome 4q21 frequently amplified in CTCL (Mao et al., 2002), was upregulated in all studied tissues or cell types of MF and SS patients. SNCA is a major component of protein aggregates present in Parkinson's disease (Kim et al., 2004). Although some genes linked to Parkinson's disease may also have aberrant expression levels in cancers (West et al., 2005), such a connection has not been found for SNCA so far. However, overexpression of SNCA has been shown to increase cell proliferation (Lee et al., 2003). The *DLG5* tumor suppressor gene (Woods et al., 1989; Nakamura et al., 1998), found to be downregulated in SS samples, is located in 10q23, a chromosomal area frequently deleted in CTCL (Scarisbrick et al., 2000; Mao et al., 2002). This gene has a role in cell growth control and maintenance of cell adhesion and cell polarity (Woods et al., 1993 and 1996). Downregulation of *DLG5* has previously been observed in several neoplasms (Cavatorta et al., 2004; Taniuchi et al., 2005), and our finding reinforces its function as tumor suppressor.

In this data set, there were differences compared to previously reported findings by Kari and coworkers and by van Doorn and coworkers (Kari et al., 2003; van Doorn et al., 2004). The two significantly overexpressed genes in the Dutch sample set, *EPHA4* and *TWIST1* (van Doorn et al., 2004), were not differentially regulated in these SS and MF blood cells compared to control samples. Increased expression of *PLS3* (T-plastin) or *JUNB* in SS as has previously been reported (Su et al., 2003;

Mao et al., 2003), was neither observed. Instead, a statistically non-significant *STAT4* downregulation of average expression level was observed in SS and MF PBMC samples and MF CD4+ samples as previously reported (Showe et al., 1999; van Doorn et al., 2004). The causes for these discordances are discussed in 5.1.

Identification of single, aberrantly expressed genes not only allows the better understanding of the pathomechanisms of the disease, but may also provide diagnostic aid. The usage of microarray results in clinical diagnosis has recently been demonstrated with a 5-gene QPCR assay including *STAT4*, *GATA-3*, *PLS3, CD1D* and *TRAIL* developed to aid in the diagnosis of SS (Nebozhyn et al., 2006). Assays including only a single gene would be easier to implement in the SS diagnostics, but the variation in expression patterns among patients has been too great to allow single gene diagnostics. For instance the expression of T-plastin alone was informative only to 50 percent of the patients in the study of Nebozhyn and coworkers (2006), has been even lower (30 percent) in previous array studies (Kari et al., 2003), and unreported in some studies (Ortonne et al., 2007; Study II).

Table 7 (presented in Study II) summarizes the presumed consequences of the gene expression changes observed in this study. These findings provide the basis for future development of similar diagnostic or prognostic assays. Before the clinical use of such assays, the markers need to be validated among a larger patient material.

Table 7. Genes found to be differentially expressed and relevant to CTCL pathogenesis.

Gene designation	Finding	Molecular function	Presumed function or expected functional consequence in CTCL
TBX21 (T-bet)	Downregulated	Transcription factor	Th1 down
SCYA5 (Rantes)	Downregulated	Chemokine	Th1 down
NKG7	Downregulated	Th differentiation	Th1 down
XCL1 (lymphotactin)	Downregulated	Chemokine, T-cell cytotoxicity (CTL)	Th1 down, lack of CTL activity
TXK	Downregulated	Transcription factor	Th1 down
GZMB (granzymeB)	Downregulated	T-cell cytotoxicity	Th1 down, lack of CTL activity
S100P	Upregulated	Th2 polarisation, cell cycle and differentition	Th2 up
LIR9	Upregulated	Membrane receptor, induces IL-1β, TNF-α and IL-6	Th2 up
KIR3DL2	Downregulated	Membrane receptor	Lack of CTL activity
IL2Rβ	Downregulated	Cytokine receptor	Impaired immune response
VAV3	Downregulated	Signal transduction, activates e.g. Rho family	Disturbed T cell activation
DLG5	Downregulated	Tumor suppressor	Increased cell proliferation
MMP-9	Upregulated	Matrix metalloproteinase, regulates angiogenesis	Carcinogenesis and tumour spread
IL7R	Upregulated (basal keratinocytes)	Cytokine receptor	Lymphocyte activation and homing to epidermis
CD52	Upregulated	Membrane antigen	Target molecule of alemtuzumab
MS4A4A	Upregulated	Membrane antigen	Signal transduction in haematopoietic cells

4.3.3 Copy number and expression of genes encoding receptor tyrosine kinases (KIT, PDGFRα, VEGFR2) is different between the CTCL-associated and primary lung cancers (IV)

Inspired by the finding that CTCL-associated lung cancers demonstrated DNA copy number gains in chromosome arm 4q, whereas reference lung cancers showed losses in that arm, three receptor tyrosine kinase genes located adjacent at 4q12 were selected, and their copy numbers were studied by FISH and protein expression by IHC. FISH showed increased copy number of *KIT* gene in three CTCL-associated lung cancer samples and in one reference lung cancer sample. *PDGFRα* gene was coamplified with KIT in all of the above cases, and *VEGFR2* coamplification was evident in secondary lung cancers but not observed in the reference lung cancer sample with *KIT* and *PDGFRα* amplifications. Both of the CTCL-associated SCCs showed increased copy numbers of *KIT*, *PDGFRα*, and *VEGFR2*, whereas none of the reference SCC samples did. The KIT protein expression was also demonstrated in these CTCL-associated SCC samples, whereas it was absent from the reference SCC samples. In the reference lung cancer samples, *KIT* deletions were neither observed, but chromosome 4 monosomy was evident in approximately one third of the nuclei thus explaining the DNA copy number loss in chromosome 4q observed by CGH in two samples. (Study IV, Table 3 and Figure 3.)

KIT, *PDGFRα* and *VEGFR2* genes, encoding receptor tyrosine kinases, are of major clinical interest, since they are the targets for tyrosine kinase inhibitor therapy (e.g. imatinib mesylate). In CTCL, the KIT expression is reported to be very rare (Brauns et al., 2004), but in lung cancers, KIT amplification or overexpression has been observed in small cell lung cancer (Sihto et al., 2005) and pulmonary large-cell neuroendocrine carcinoma (LCNEC) resembling SCLC (Rossi et al., 2005), but not in NSCLC (Sekido et al., 1991). Interestingly, both CTCL-associated NSCLC cases but none of the reference NSCLC cases demonstrated increased *KIT* gene copy numbers together with KIT protein expression. To date, *PDGFRα* and *VEGFR2* amplifications have not been reported in NSCLC, which is characterized by deregulation of mainly EGFR family tyrosine kinases (Bunn et al., 2006). Thus, the finding of *PDGFRα* and *VEGFR2* gene copy number increase in CTCL-associated SCC samples, but not in primary SCC samples, further underlines the differences of CTCL-associated and primary lung cancer.

5. GENERAL DISCUSSION

5.1 Validity of the methods

Molecular genetic studies on CTCL have been hampered by the difficulty of obtaining sufficient numbers of the truly malignant, clonal cells. They are often are sparse in the early CTCL stages, and their propagation in vitro has proven difficult. Additionally, in the skin, the cancer cells are interspersed within the reactive lymphocytes and other normal cells of the skin. The focus of this thesis has been to allocate studies to the true malignant cells. This was achieved by laser microdissection of picking the morphologically malignant cells (Studies III and IV), or by combining the data from different tissues and cell populations of the same individual patients (Study II). Through this approach, the genetic changes characteristic to the cancer cells (microdissection-based method) or characteristic to the whole tumor microenvironment (combination of different cell types) were assessed.

Due to the aforementioned, the availability of research material from CTCL patient -derived tissues was problematic during the course of this study. The amount of genomic material achieved by microdissection of single cells was too sparse to allow any further studies without whole genome amplification. The method of Stoecklein and coworkers (2001) was chosen to reliably amplify the microdissected DNA. The whole genome amplification succeeded but the amplified DNA was not suitable for a reliable genomic microarray analysis.

Especially, the usage of microarrays has brought a lot of data, but the results are not uniform due to the different experimental designs, practically varying probe and sample sets as well as the variability in the analysis methods (Marshall et al., 2004). Interestingly, Kari and coworkers discovered that panels of fewer than 10 genes can be used to classify CTCL cases and separate them from controls even when the Sézary cell count is as few as 5% (Kari et al., 2003). This might indicate that even a small proportion of morphologically malignant cells is able to produce factors that induce CTCL-specific expression pattern (Wood, 2005) or that the actual malignant transformation of the cell occurs before the cell turns morphologically malignant (Karenko et al., 2001). In the future, methods selectively picking up the morphologically and/or immunohistologically/karyotypically malignant cells should be used also in the microarray analyses. Combination of DNA and RNA copy number changes (as performed in Study II) detected by microarray techniques would provide further knowledge of the poorly known aetiology of CTCL, as well as provide new diagnostic and therapeutic tools.

5.2 The pathomechanisms of CTCL

5.2.1 NAV3 in the pathogenesis of CTCL

A new gene was identified to be recurrently deleted or translocated in association with CTCL. This gene, *NAV3*, is hypothesized to be a haploinsufficient tumor suppressor increasing IL-2 production in CTCL cells. IL-2 induces T cell proliferation by activating STAT5 (Moriggl et al., 1999), and the sustained expression of an IL2-inducible truncated STAT5 protein may allow Sezary cells to escape IL-2 activated cell death (Mitchell et al., 2003). Thus, the malignant T-cells would have a defect in activation induced cell death which forms the basis for the survival of the early malignant T-cell. Whether also allelic *NAV3* loss contributes to the STAT imbalance, remains to be seen. At the moment, the function of NAV3 is studied by gene expression profiles in NAV3-silenced target cells (neural, epithelial and lymphoid cells) and animal models are being generated. According to preliminary findings, *NAV3* would have a role in the mitotic process and, thus, its deletion could be linked to aneuploidy. Moreover, in the glioblastoma cells, preliminary results indicate co-localization of the embryonic stem cell transcription factor NANOG and NAV3 (Carlsson et al., abstract, 2006). NANOG is a transcription factor, essential for the pluripotent phenotype and the self-renewal of human embryonic stem cells. It is not expressed in adult tissues, except for various cancers, and has been shown to accelerate cell proliferation. Further characterization and identification of NAV3-regulated genes and signalling pathways in different human cells and tissues is in progress by using targeted NAV3 silencing with oligonucleotide and lentiviral siRNA techniques. Also, the possible association of NAV3 with other stem cell -related proteins warrants further studies.

NAV3 is also aberrated in other cancers than CTCL (Study I), lung cancer (Study IV), and neuroblastoma (Coy et al., 2002), the majority of which are of epithelial origin (Krohn et al., unpublished). LOH of some microsatellite markers at the chromosomal band 12q21, but not specific for the *NAV3* region, has been shown to occur in pancreatic carcinoma (Kimura et al., 1998). Thus, *NAV3* might be important for early carcinogenesis functioning in cancers of different origins and, thus, the ongoing studies focusing on the identification of NAV3-associated signalling pathways will be important.

5.2.2 T helper cell balance and CTCL

This study identified down-regulation of a panel of Th1-specific genes, e.g. *TBX21*, *SCYA5*, *NKG7*, *XCL1* (lymphotactin), *TXK*, and *GZMB* (granzyme B) in Sezary syndrome samples by gene expression analysis. These findings provide basis for previous observations of a preferential Th2-type cytokine profile in SS. Moreover, downregulation of the major Th1 polarizing transcription factor *TBX21* was for the first time as-

sociated with CTCL. Its expression was lowered in PBMC samples of both Sezary syndrome and mycosis fungoides patients. Down-regulation in the expression of *SCYA5* (*RANTES*) and *NKG7* were observed also among some of the MF PBMC samples. TBX21, TXK, and XCL1 regulate the key cytokine secreted by the Th1 cells, IFN-γ, which has previously shown to be absent from the chromosomally clonal (i.e. true malignant) cells in Sezary syndrome (Karenko et al., 2001). GZMB promotes Th1 differentiation by influencing IL-12 secretion, and interestingly, GZMB is reported to be absent from the chromosomally clonal cells in skin and lymph node samples of Sezary syndrome (Karenko et al., 2001). The genes upregulated during the early polarization of T helper cells into Th2 direction (Lund et al., 2005) and upregulated in both Sezary syndrome and mycosis fungoides PBMC samples included the *S100P* and *LIR9* genes. They both act via IL-6 to promote Th2 type immune response.

These findings not only confirm that Th1/Th2 balance regulates CTCL pathogenesis (Vowels et al., 1992 and 1994; Dummer et al., 1996), but also suggests that bias towards Th2 type immune response takes place already in the mycosis fungoides stage, prior to progression to the leukaemic phase. This kind of a skewing is likely to influence the progressive immune dysregulation in CTCL and would thus provide a growth advantage for the malignant cell clone(s) (Cosmi et al., 2004).

5.2.3 Histogenesis of SPTL

This study clarified the classification of cutaneous T-cell lymphomas. When the study was begun, subcutaneous panniculitis-like T-cell lymphomas were classified among aggressive CTCL subtypes (Willemze et al., 1997) and were often treated with heavy chemotherapy. Later, two different T-cell subgroups were distinguished with different clinical features and prognosis (Willemze et al., 2005 and 2008). This study reinforced that the current classification is relevant also at molecular cytogenetic level.

There are speculations about the true nature of SPTL. According to one theory, it is a continuum of lupus erythematosus profundus, rather than a cutaneous lymphoma. Since both SPTL and lupus panniculitis are characterized by a lobular panniculitis, the histological differentiation among these is usually difficult. Also, the constitutional symptoms of many SPTL patients are similar as in systemic lupus erythematosus. Several similarities in the histopathology of SPTL and lupus panniculitis (LEP, lupus erythematosus profundus) have been observed, namely lymphoid atypia with pleomorphic cells, erythrophagocytosis, histiocytes, eosinophilic necrosis of the fat lobule, loss of certain pan T-cell markers (e.g. CD3, CD5, CD7, CD8), a reduced CD4/8 ratio in the skin and clonal TCR rearrangement (Magro et al., 2001). In contrast, the most useful histopathologic criteria for distinguishing LEP from SPTL are the presence of involvement of the epidermis, lymphoid follicles with reactive germinal centers, mixed cell infiltrate with prominent plasma cells, clusters of B lymphocytes, and polyclonal TCR-gamma gene rearrangement in LEP (Massone et al., 2005). Because

of the difficult differential diagnosis of these two panniculitis forms, it is suggested that patients with lupus panniculitis and atypic cell infiltrates should be prospectively followed (Cassis et al., 2004). In this study, a large number of CTCL-specific chromosomal aberrations was identified, discouraging the usage of the term "continuum of LEP" for SPTL. Future studies on a larger sample material of SPTL suitable for aCGH or expression array studies are likely to even identify single target genes or molecules applicable for e.g. clinical diagnostics.

These current findings also provide new information on the usage of systemic steroids as a first line therapy for SPTL. While it took almost ten years to reach the definite SPTL diagnosis for three of the first cases, they were all treated with oral steroids for unclassified panniculitis and long-lasting remissions, even for 10 years, were achieved. Moreover, three of the more recent patients have responded completely to long-term systemic steroid therapy. The factors determining response to sole high-dose systemic corticosteroid therapy and the requirement of multi-agent chemotherapy need to be defined in a larger prospective study, however.

Autoimmune phenomena occur frequently among SPTL patients (Willemze et al., 2008). Five of the nine cases had an autoimmune disease or presence of laboratory abnormalities suggestive of an autoimmune disorder. It is noteworthy that the autoimmune phenomena of four of the five cases completely disappeared after the successful treatment of SPTL with multiagent chemotherapy. This observation supports a link between the autoimmune disorder and the development of SPTL. However, such association still needs to be confirmed in a multinational prospective study.

5.2.4 The development of CTCL-associated lung cancer

Chromosomal aberrations in CTCL-associated lung cancers were identified to resemble CTCL-specific chromosome changes rather than those typical of lung cancer. The fact that there are so many molecular cytogenetic similarities between CTCL and CTCL-associated lung cancer supports the hypothesis of an existing precursor cell that may lead to both cancer types (Figure 6). An alternative mechanism explaining the association of lung cancer with CTCL could be related to an interaction between the epithelial cells and surrounding lymphocytes, the landscaping effect, leading to malignancy, as has been recently described in colon cancer (Kim et al., 2006).
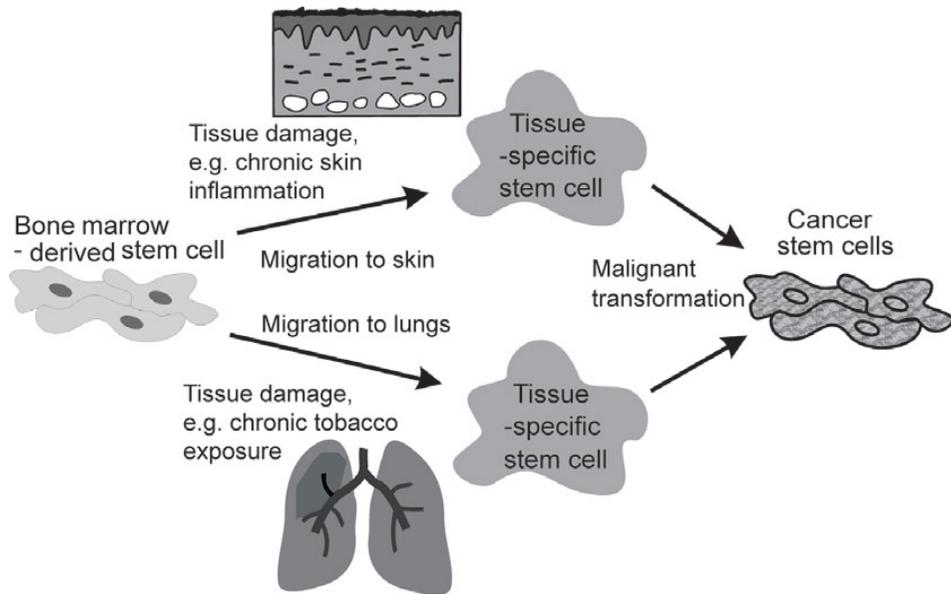
Figure 6. Hypothesis of the development of CTCL-associated lung cancer from a bone-marrow-derived cancer stem cell. Bonemarrow -derived stem cells migrate to the sites of tissue damage in skin and lungs where they become tissue-specific stem cells and are prone to malignant transformation.

5.3 Clinical relevance of the novel CTCL target genes

The diagnosis of CTCL is difficult. Especially the lack of clearly defined criteria to differentiate early MF from benign inflammatory disorders is by far the most difficult, debated and yet crucial issue. The current diagnosis is based on clinical, histopathological and molecular genetic criteria but when additional genetic and molecular information in CTCL patients become available and their prognostic implications determined, further revisions to the diagnostic criteria will be needed (Olsen et al., 2007).

Current CTCL therapies are based on a variety of unspecific mechanisms (Trautinger et al., 2006). Most therapies, e.g. PUVA, bexarotene and extracorporeal photopheresis, induce apoptosis of CTCL cells. Successful therapies with bexarotene and ONTAK lead to a Th1 favoured balance. Monoclonal antibodies against CD52 (alemtuzumab) and IL2R (ONTAK), as well as interferon induce cytotoxic immune response against the cancer cells. None of these therapies is curative, because they are not able to target the pure malignant cells.

5.3.1 NAV3 in CTCL diagnosis

This study demonstrated a novel FISH-based diagnostic test for CTCL (Study I). Deletion of *NAV3* can be demonstrated on interphase nuclei from archival patient derived material either fresh frozen or stored in liquid nitrogen, or more easily, from paraffin-embedded tissue blocks by FISH technology. Alternatively, LOH methods may be used when DNA from both affected and unaffected tissue is available. Besides for diagnostic purposes, demonstration of *NAV3* aberration would be used as a biomarker to monitor the response to therapy and to detect early relapses of the disease. Follow-up studies to reveal the role of *NAV3* aberrations as such biomarker are ongoing.

5.3.2 Membrane antigens as targets for therapy

The few monoclonal antibodies available at the moment target either CD52 (alemtuzumab), IL2R (Ontak), or CD4 molecules (zanolimumab). The usage of antibody-based therapy requires the malignant cells to express the certain membrane antigen. Monoclonal antibodies are easy to prepare (David et al., 1990; Nikolova et al., 2001), and thus suitable therapy means targeted against membrane proteins. In the gene expression analysis (Study II), upregulated genes included *MS4A4A* and *LIR9*, which would become novel targets for antibody-based therapy. MS4A4A belongs to the MS4A superfamily (Liang et al., 2001), another member of which (CD20) is already now the target of monocloncal antibody -mediated therapy in large B-cell lymphoma (Press et al., 1987).

5.3.3 Chromosomal areas likely to harbor new target genes

In this study, CTCL gene expression profiling was for the first time combined with the moleculocytogenetic findings reflecting DNA copy number aberrations. Through this approach, chromosome arms were identified where both gene expression and DNA copy number was changed to the same direction (i.e., 1q, 3p, 3q, 4q, 12q, 16p, and 16q). Thus, the above chromosome arms are potential targets for searching for further recurrent gene aberrations in CTCL.

Regarding CTCL, chromosome 4 is interesting. It belongs to the frequently amplified (Mao et al., 2002) and overexpressed (Study II) chromosome arms. Interestingly, CTCL-associated lung cancers showed DNA copy number gain in 4q, and amplification and protein expression of genes located in 4q12, even though primary lung cancers are characterized by DNA copy number loss in chromosome 4q (Study IV). *KIT*, *PDGFRα*, and *VEGFR2* genes, receptor tyrosine kinases, located adjacent in chromosome band 4q12 were expressed differentially among CTCL-associated and reference lung cancer. Additionally, *SNCA* gene, mapping to 4q21, was up-regulated in all stud-

ied tissues or cell types of mycosis fungoides and Sezary syndrome patients (Study II). Thus, further studies, identifying novel target genes are encouraged to concentrate on chromosome 4.

6. CONCLUSIONS

This study was initiated in the early 2000's at the golden time of cancer genetics inspired by the completion of the Human Genome Project (Lander et al., 2001; Venter et al., 2001).

The first two substudies aimed at characterizing the genes involved in the pathogenesis of the most common CTCL subtypes, MF and SS. The purpose of substudies III and IV was to characterize the more rare subtypes of CTCL, including SPTL and CTCL-associated lung cancers.

NAV3 gene aberrations occur in many CTCL subtypes and a diagnostic test for the demonstration of allelic *NAV3* loss in patient-derived tissue samples was developed. Since then, *NAV3* aberrations have been observed in human malignancies of different origin suggesting its crucial function in oncogenesis.

Gene expression profiling revealed several novel genes involved in CTCL pathogenesis and potential therapeutic targets. Downregulation of a set of genes involved in Th1 polarization, including the major Th1-polarizing factor, *TBX21*, was for the first time associated with CTCL. Molecules relevant for skin homing were revealed by observing increased expression of IL-7R and CD52 in MF but not in SS samples. Furthermore, chromosomal arms with both gene copy number and gene expression level changes were identified as potential targets for searching for further novel target genes.

Aberrated chromosomal regions in morphologically malignant SPTL cells were identified differentiating this subtype from the commonest subtypes. Aberrations in chromosome regions 10q, 17p and 19 are common to many CTCL subtypes (MF, SS, and SPTL), and are likely to harbour genes common for the molecular pathogenesis of several subtypes of CTCL. Instead, 5q and 13q gains characterize SPTL.

CTCL-associated lung cancers show chromosomal aberrations different from primary lung cancer, especially amplifications of 4q, a chromosome arm frequently deleted in the latter tumor type. Moreover, copy numbers and expression of selected genes in chromosome 4 differed between CTCL-associated and reference lung cancers. These observations suggest common underlying factors between CTCL and CTCL-associated lung cancer and warrant further prospective studies to identify them.

Taken together, several novel candidate molecules for more accurate diagnosis of CTCL as well as targeted therapy were identified. To evaluate the suitability of these molecules for clinical practise is the mission for the next few years.

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