

# Aberrant Expression of the Tyrosine Kinase Receptor EphA4 and the Transcription Factor Twist in Sézary Syndrome Identified by Gene Expression Analysis

Remco van Doorn, Remco Dijkman, Maarten H. Vermeer, Jacoba J. Out-Luiting, Elisabeth M. H. van der Raaij-Helmer, Rein Willemze, and Cornelis P. Tensen

Department of Dermatology, Leiden University Medical Center, Leiden, the Netherlands

## ABSTRACT

Sézary syndrome (Sz) is a malignancy of CD4+ memory skin-homing T cells and presents with erythroderma, lymphadenopathy, and peripheral blood involvement. To gain more insight into the molecular features of Sz, oligonucleotide array analysis was performed comparing gene expression patterns of CD4+ T cells from peripheral blood of patients with Sz with those of patients with erythroderma secondary to dermatitis and healthy controls. Using unsupervised hierarchical clustering gene expression patterns of T cells from patients with Sz were classified separately from those of benign T cells. One hundred twenty-three genes were identified as significantly differentially expressed and had an average fold change exceeding 2. T cells from patients with Sz demonstrated decreased expression of the following hematopoietic malignancy-linked tumor suppressor genes: TGF- $\beta$  receptor II, Mxi1, Riz1, CREB-binding protein, BCL11a, STAT4, and Forkhead Box O1A. Moreover, the tyrosine kinase receptor EphA4 and the potentially oncogenic transcription factor Twist were highly and selectively expressed in T cells of patients with Sz. High expression of EphA4 and Twist was also observed in lesional skin biopsy specimens of a subset of patients with cutaneous T cell lymphomas related to Sz, whereas their expression was nearly undetectable in benign T cells or in skin lesions of patients with inflammatory dermatoses. Detection of EphA4 and Twist may be used in the molecular diagnosis of Sz and related cutaneous T-cell lymphomas. Furthermore, the membrane-bound EphA4 receptor may serve as a target for directed therapeutic intervention.

## INTRODUCTION

Sézary syndrome (Sz) is a leukemic variant of cutaneous T cell lymphoma (CTCL) characterized by erythroderma, generalized lymphadenopathy, and the presence of neoplastic CD4+ skin-homing memory T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood (1, 2). In the early phases of the disease, differentiation between Sz and benign forms of erythroderma secondary to atopic dermatitis, chronic dermatitis, or adverse drug reactions may be very difficult. Because of the similarity of their histopathological and immunophenotypical features, Sz is generally considered to be a leukemic variant of the more common CTCL mycosis fungoides (3). Severe pruritus, ectropion, alopecia, palmoplantar keratoderma, and generalized immunosuppression are common associated features. The results of various treatments for Sz are generally disappointing. Subjects with Sz have an unfavorable prognosis with an estimated 5-year survival of 15% (4).

Previous studies on the pathogenesis of Sz have pointed to aberrations of signaling by the STAT family of transcription factors (5–8), overexpression of JunB (9), diminished expression of the tumor suppressor genes TGF- $\beta$  receptor II (10), p15, p16 (11), and Fas (12). Other studies have pointed to the presence of chromosomal alterations, indicating genomic instability (13, 14). Nonetheless, compre-

hension of the pathogenic mechanisms implicated in the development and progression of this T-cell malignancy is still limited.

To expand the understanding of the pathogenesis and to facilitate the molecular diagnosis of Sz, we performed oligonucleotide array analysis on T cells isolated from the peripheral blood of patients with Sz. Gene expression patterns were compared with those of CD4+ T cells isolated from the blood of patients with erythroderma secondary to atopic or chronic dermatitis and of healthy volunteers.

The gene expression patterns of malignant T cells from patients with Sz demonstrated consistent differences with those of benign T cells. The transcriptional program distinctive for the malignant phenotype revealed evidence for the dysregulation of multiple growth-regulatory signal transduction pathways, such as the TGF- $\beta$  receptor and c-myc pathway. We identified high and selective expression in Sz and related CTCLs of the tyrosine kinase receptor EphA4 and the transcription factor Twist. These genes, which may also be implicated in the pathogenesis of Sz, could serve as molecular markers in the diagnosis or as therapeutic targets in the treatment of this malignancy.

## MATERIALS AND METHODS

**Selection of Patients.** Cryopreserved blood samples from 10 patients with Sz (seven males, three females; median age 62 years) were available for inclusion in this study. Sz was defined by the criteria of the European Organization for Research and Treatment of Cancer classification (4). All patients showed highly elevated CD4/CD8 ratios and clonal T cells in the peripheral blood, as described previously (15). Follow-up data revealed that all patients had died of Sz; the median survival time was 26 months.

For comparative analysis, blood samples were obtained from a group of eight control patients that included five patients with a benign form of erythroderma (BE; three males, two females; median age, 57 years) and three healthy volunteers. The BE group included three patients with atopic dermatitis and two with idiopathic chronic dermatitis. Examination of the peripheral blood in these five BE patients showed an absence of atypical T cells, normal CD4/CD8 ratios, and no evidence of a T cell clone. Follow-up (median duration 29 months) was unremarkable. From all Sz patients and controls, peripheral blood samples were collected at the time of diagnosis before systemic or phototherapeutic treatment had been given. In addition, for real-time quantitative PCR experiments, lesional skin biopsy specimens from patients with plaque-stage ( $T_2N_0M_0$ ) and tumor-stage ( $T_3N_0M_0$ ) mycosis fungoides as well as primary cutaneous CD30-negative large T cell lymphoma were obtained. As controls for these experiments phytohemagglutinin-activated and interleukin 2-expanded peripheral blood T cells as well as biopsies of benign cutaneous lymphocytic infiltrates from patients with inflammatory skin diseases (M. Jessner, chronic discoid lupus erythematosus, graft-versus-host disease, benign erythroderma secondary to dermatitis, lichen planus, and cutaneous vasculitis) were used.

**T-Cell Isolation and RNA Isolation.** Peripheral blood mononuclear cells were obtained by Ficoll density centrifugation. The percentage of CD4+ Sz cells in the peripheral blood mononuclear cells samples of Sz patients ranged from 90 to 97%, as verified by fluorescence-activated cell sorter analysis. Mononuclear cells of the patients with BE and from healthy volunteers were subjected to further purification by negative selection using magnetic beads (CD4+ T-cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). The purification of control samples yielded >95% CD4+ T cells, similar to the percentage of CD4+ T cells in peripheral blood mononuclear cell samples from Sz patients. RNA was extracted from T-cell samples and homogenized cryopreserved skin biopsy samples using the RNeasy kit (Qiagen, Hilden, Germany).

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**Note:** R. van Doorn and R. Dijkman contributed equally to this study.

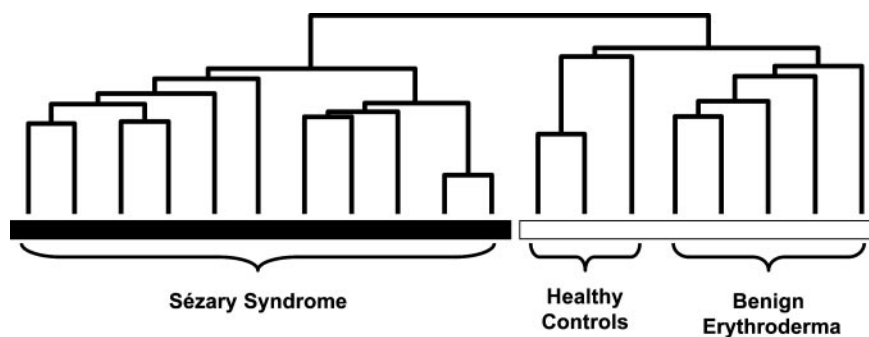
**Requests for reprints:** Cornelis P. Tensen, Department of Dermatology, Sylvius Building Room 3042, Wassenaarseweg 72, 2333 AL Leiden, the Netherlands. Phone: 31-71-5271900; Fax: 31-71-5271910; E-mail address: c.p.tensen@lumc.nl.

Table 1 Sequences of primers used for amplification of selected transcripts by qPCR

Name	Accession	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
EphA4	L36645	ggaaggcgtggtcactaaat	tctgccatcatttttctctga	96
Twist	BC036704	cactgaaaggaaaggcatca	ggccagtttgatcccagtat	107
STAT4	L78440	cagaaagggtgacaaagggt	atgggaagaaggctctgatgg	102
TNFSF7	L08096	atcacacaggacctcagcag	atacgtagctgcccttgctc	105
LCK	AF228313	ctacgggacattcccatca	gttggtcatcctgggtaag	99
CREBBP	AJ251843	cacaagtcatttggacagc	gttgaccatgctctgtttgc	99
TGF-β R2	M85079	atctcgctgtaatgcagtg	cacgttgctcctcatgcttt	99
SATB1	NM_002971	gacttttagcccagcagctcc	gtggtggtcgaaacctgttg	102
CCR4	NM_005508	actgctgcttaatcccatc	ccacagtattggcagagcac	111
KIR3DL2	AF263617	gggacctcagtggtcatctt	gctcttggtccattacagca	99

NOTE. Also indicated are sizes of the PCR products and accession numbers of the respective genes. Abbreviation: bp, base pair(s).

Fig. 1. Unsupervised hierarchical clustering of gene expression profiles generated from CD4+ T cells from peripheral blood of 10 patients with Sz, CD4+ T cells from three healthy volunteers, and five patients with BE. The dendrograms are generated using a hierarchical clustering algorithm on 12,625 transcripts based on the average-linkage method.



**Preparation and Hybridization of Fluorescent-Labeled antisense RNA.**

Samples and microarrays were processed according to the manufacturer’s protocol (available from Affymetrix, Santa Clara, CA). In brief, using the MessageAmp antisense RNA kit (Ambion, Huntingdon, United Kingdom), total RNA was reverse transcribed using an oligodeoxythymidylic acid-T7 promoter primer to prime first-strand synthesis. After second-strand synthesis, the purified cDNA product was *in vitro* transcribed using T7 RNA polymerase, biotin-UTP, and biotin-CTP to generate fragmented biotinylated antisense RNA.

Fragmented antisense RNA (5 μg) was hybridized to a Human Genome U95Av2 Array (Affymetrix), interrogating 12,625 human transcripts, for 16 h at 45°C with constant rotation at 60 rpm. After hybridization, the microarray was washed, stained on an Affymetrix fluidics station, and scanned with an argon-ion confocal laser with 488-nm excitation and 570-nm detection wavelengths.

**Microarray Experimental Design and Data Analysis.** The array images were quantified using MicroArray Suite v5.0 software (Affymetrix, Santa Clara, CA). The average fluorescence intensity was determined for each microarray, and then the output of each experiment was globally scaled to 200. Normalization was performed using variant stability and normalization (16) part of the R statistical software package.<sup>3</sup> Significance analysis of microarrays (SAM; ref. 17) was applied to compare gene expression patterns of T cells from 10 Sz patients with those of the eight controls. A false discovery rate of <1 was chosen to select genes significantly up- or down-regulated. Gene expression patterns were further analyzed, and output was visualized using Spotfire DecisionSite (Spotfire, Göteborg, Sweden) and MicroArray Suite v5.0 software.

**Real-Time Quantitative PCR.** cDNA synthesis was performed on 1 μg of total RNA after treatment with RQ1 DNase I (Promega, Madison, WI) using Superscript III reverse transcriptase (Invitrogen, Breda, the Netherlands) and an oligo(dT)<sub>12-18</sub> primer (Invitrogen, Breda, the Netherlands) in a final volume of 20 μl. Real-time quantitative PCR (qPCR) was performed using SYBR Green PCR Master Mix in an ABI-Prism 7700 sequence detection system (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). The primer sequences (Invitrogen) of selected transcripts are given in Table 1. The cycle parameters for these transcripts and for the housekeeping genes U1A and RPS11 used for normalization were as follows: denaturing for 15 s at 95°C; annealing and extension for 60 s at 60°C, for 40 cycles. Specificity of the PCR product was confirmed by agarose gel electrophoresis and subsequent DNA

sequence analysis of test samples and melting curve analysis in the case of patient material. The crossover point (Ct) values, defined as the cycle number at which each curve intersects the threshold detection value, were used to calculate the gene-specific input mRNA amount for each tissue according to the calibration curve method. Data were evaluated using the SDS software version 1.9.1 (Applied Biosystems) and the second derivative maximum algorithm.

**RESULTS**

**Distribution of Gene Expression.** Initial analysis of the hybridization signal of the 12,625 transcripts represented on the oligonucleotide array revealed that malignant T cells of Sz patients on average expressed 6,100 of the interrogated genes; in CD4+ T cells from peripheral blood of BE patients and healthy volunteers 5,771 respectively 5,975 of the genes were expressed.

Analysis of the entire set of expressed genes using an unsupervised hierarchical clustering analysis algorithm, grouping the samples on the basis of similarity of their expression profiles, showed that the Sz samples display a relatively homogeneous gene expression pattern that is classified separately from that of benign CD4+ T cells (see Fig. 1 for dendrogram representing cluster analysis of the entire set of expressed genes). The transcript profiles of T cells from patients with BE were markedly different from those of Sz patients and were more related to transcript profiles of T cells from healthy volunteers.

**Sézary Syndrome-Specific Gene Expression Pattern.** Comparative analysis of the 10 Sz samples with the 8 control samples implementing the SAM algorithm demonstrated that 176 genes were statistically significantly differentially expressed at *P* < 0.01. Of these genes significant in the separation of Sz CD4+ T cells and benign CD4+ T cells, 69 were relatively overexpressed, with fold changes ranging from 1.7 to 19.8. One hundred and seven genes were down-regulated in the malignant T cells, with fold changes ranging from 1.4 to 13. Of the 176 significantly differentially expressed genes, 123 genes were up- or down-regulated with fold changes exceeding 2. Comparative gene expression profiles of these 59 overexpressed and 64 underexpressed genes are shown in Fig. 2.

<sup>3</sup> www.bioconductor.org.

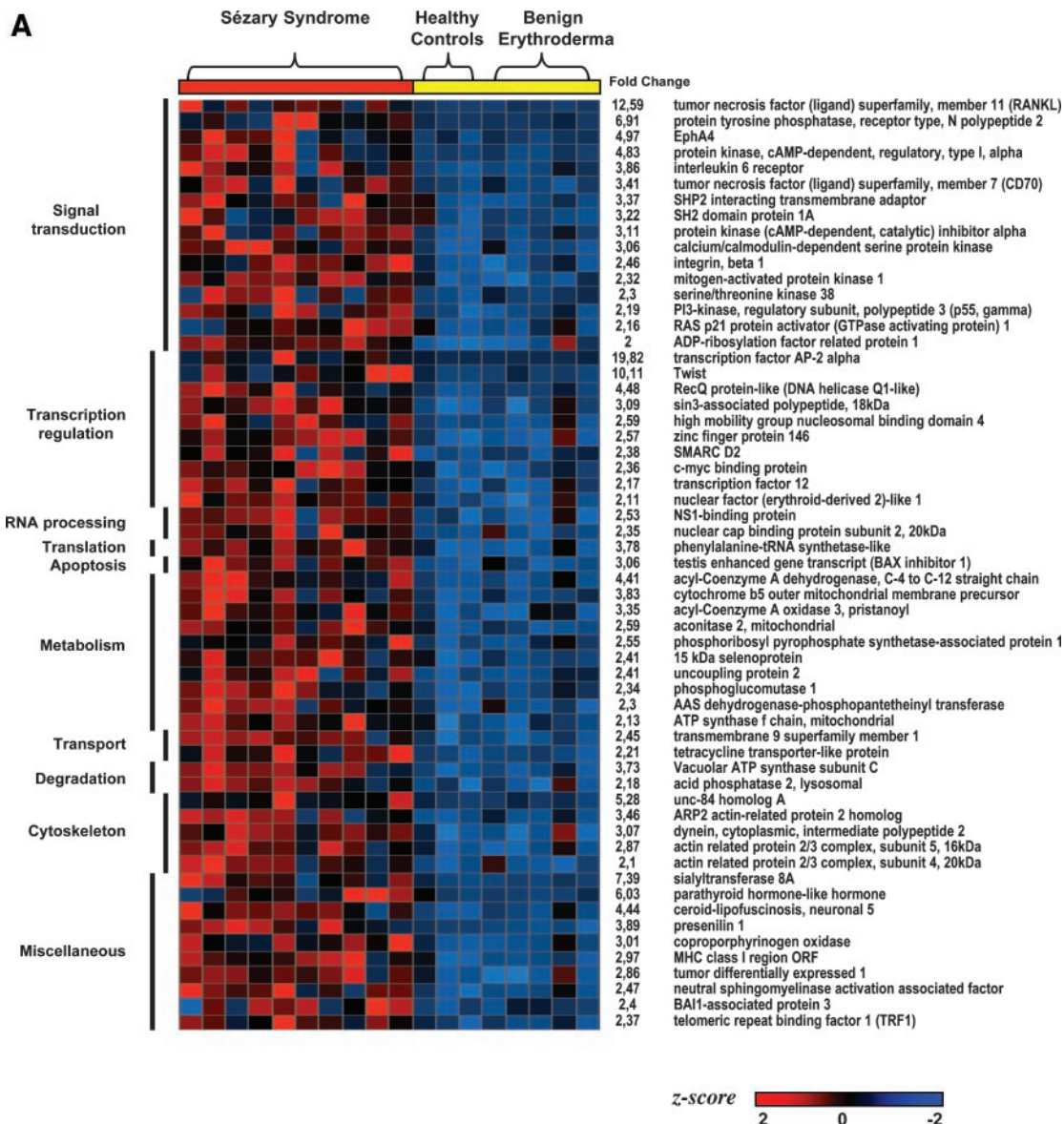


Fig. 2. Supervised analysis performed on 10 tumor samples of CD4+ T cells from peripheral blood of patients with Sz versus eight control samples, consisting of CD4+ T cells from three healthy volunteers and five patients with BE. Represented genes are selected by the SAM algorithm and have an average fold change exceeding 2. Values are visualized according to the scale bar that represents the difference in the z-score (expression difference/SD) relative to the mean. A, the analysis identified 69 genes that are up-regulated in CD4+ T cells from peripheral blood of patients with Sz compared with the controls. Fold change denotes the ratio of average normalized expression level in the Sz and the control group of samples. B, 107 genes are down-regulated in CD4+ T cells from peripheral blood of patients with Sz compared with the controls. Fold change denotes the ratio of average normalized expression level in the control and the Sz group of samples.

Table 2 displays the most discriminating genes, according to fold change between average expression levels in the malignant T cells compared with the benign T cells. The most differentially expressed genes, with fold changes exceeding 10, were the transcription factor activator protein 2- $\alpha$ ; dual specificity phosphatase 8; the tumor necrosis factor (TNF) receptor family ligand, receptor activator of nuclear factor- $\kappa$ B ligand (RANKL; TNFSF11); and the transcription factor Twist. Among the genes highly overexpressed in T cells of Sz patients, especially up-regulation of the genes Twist and EphA4 was consistently seen. Transcripts of Twist and EphA4 were present in 9 respectively 8 of 10 Sz samples.

Expression of the following genes was undetectable in any of the control samples but present in Sz T cells: EphA4 (expressed in 8 of 10 Sz samples), phenylalanine-tRNA synthetase-like (expressed in 7 of 10 Sz samples), RANKL (expressed in 7 of 10 Sz samples), transcription factor activator protein 2- $\alpha$  (expressed in 3 of 10 Sz samples), and peroxisomal

acyl-CoA oxidase 3 (expressed in 3 of 10 Sz samples). Although the level of RANKL transcript level was undetectable in benign T cells in this array analysis study, it is normally expressed by activated T cells (18). Interestingly, phenylalanine-tRNA synthetase-like transcript has been demonstrated previously to be expressed in a human acute-phase chronic myeloid leukemia cell line but not in its non-tumorigenic variant, suggesting that selective expression of this member of the tRNA synthetase family is more common in hematopoietic neoplasms (19).

**Real-Time Quantitative PCR of Selected Genes, Including EphA4 and Twist.** To validate the results of microarray analysis data, qPCR was applied on a panel of 10-selected genes. As Fig. 3A shows, qPCR results were all in agreement with gene-profiling data. In general, differences in transcript levels between malignant and benign T cells appeared to be more pronounced when measured by qPCR compared with oligonucleotide microarray analysis.

Next we evaluated the expression of EphA4 and Twist, the two

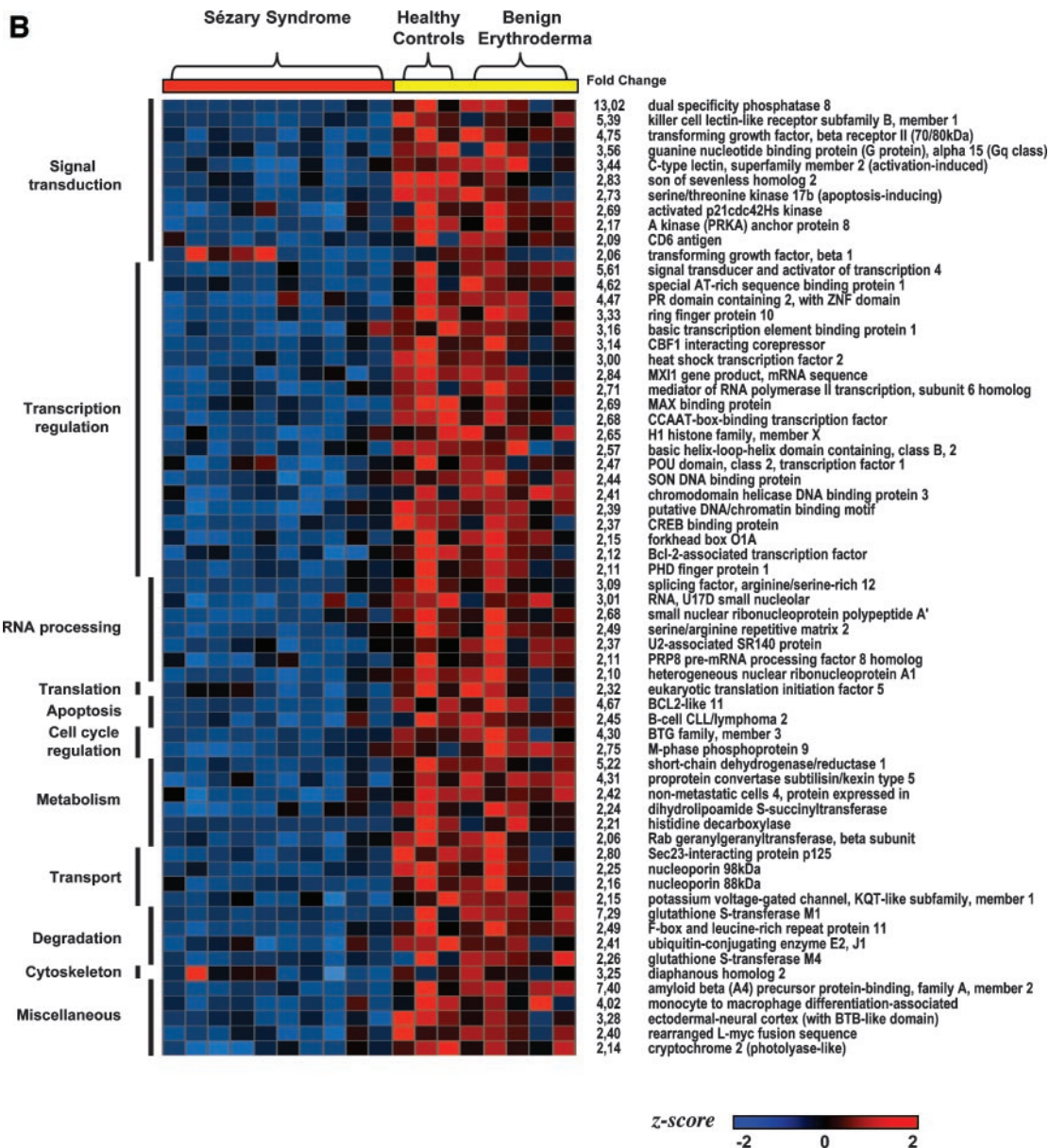


Fig. 2 Continued

most selectively and consistently up-regulated genes in the malignant T cells of Sz patients, using qPCR in a larger set of CTCL and control samples. To assess whether EphA4 and Twist were also expressed by malignant T cells of patients with mycosis fungoides and CD30-negative primary cutaneous large T-cell lymphoma, transcript levels were analyzed in lesional skin biopsy samples of patients with these

Sz-related CTCLs. Included as additional controls were T cells *in vitro* activated with phytohemagglutinin and expanded with interleukin-2 as well as lesional skin biopsy samples from patients with T cell-rich inflammatory dermatoses. EphA4 was highly expressed in lesional skin of three of nine patients with mycosis fungoides, whereas its expression was nearly undetectable in any of the

Table 2 List of 10 transcripts including fold change that are most significantly up- or down-regulated in CD4+ T cells from patients with Sz when comparing average expression levels to those in CD4+ T cells from controls

Up-regulated in Sézary syndrome		Down-regulated in Sézary syndrome	
Gene description	Fold change	Gene description	Fold change
Transcription factor AP2- $\alpha$	19.82	Dual specificity phosphatase 8	13.02
Twist	12.59	Amyloid beta (A4) precursor protein-binding, family A, member 2	7.4
TNFSF11 (RANKL)	10.11	Glutathione S-transferase M1	7.29
Protein tyrosine phosphatase, receptor type, N polypeptide 2	7.39	STAT4	5.61
Parathyroid hormone-like hormone	6.91	Killer cell lectin-like receptor subfamily B, member 1	5.39
Sialyltransferase 8A	6.03	Short-chain dehydrogenase/reductase 1	5.22
Unc-84 homolog A	5.28	TGF- $\beta$ receptor II	4.75
EphA4	4.97	BCL2-like 11 (Bcl-11a)	4.67
Protein kinase, cAMP-dependent, regulatory, type I, $\alpha$	4.83	Special AT-rich sequence binding protein 1 (SATB1)	4.62
RecQ protein-like (DNA helicase Q1-like)	4.48	PR domain containing 2 (PRDM2, Riz1)	4.47

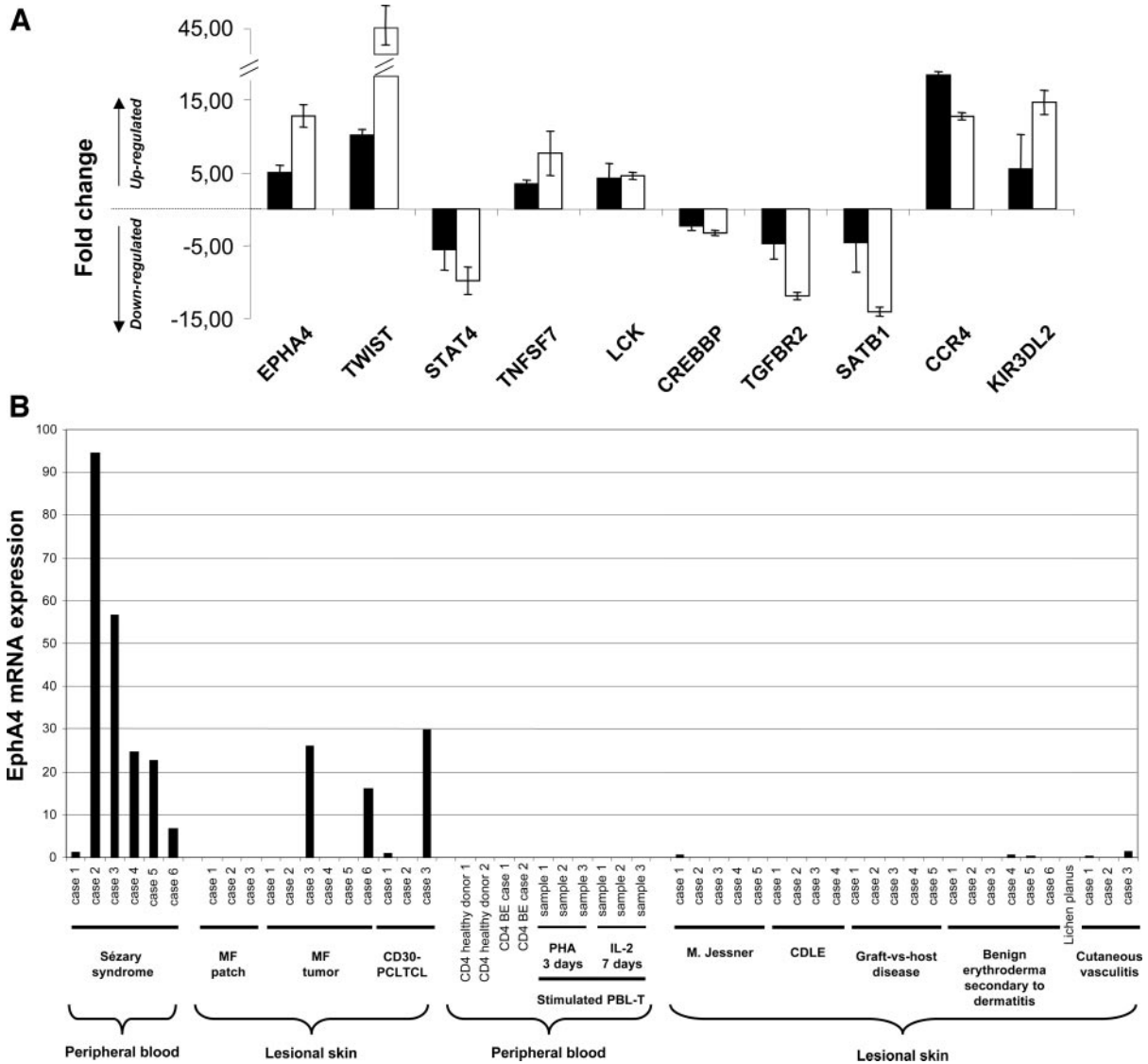


Fig. 3. Results of real-time quantitative PCR analysis. A, histogram showing expression levels of 10-selected genes as measured by oligonucleotide array analysis (■) and qPCR (□). Fold change of oligonucleotide and qPCR experiments denote average expression level in patients with Sz relative to average expression level in controls. For qPCR experiments, expression levels of the gene of interest were normalized to those of two housekeeping genes. B, histogram showing expression level of the EphA4 gene as measured by qPCR in peripheral blood T cells of Sz patients, BE patients, healthy volunteers, *in vitro*-stimulated peripheral blood T cells, as well as in lesional skin biopsy samples from patients with cutaneous T-cell lymphoma and various inflammatory skin diseases. C, histogram showing expression level of the Twist gene as measured by qPCR in these samples. Fold change denotes the expression of EphA4 or Twist relative to expression of the housekeeping gene U1A. MF, mycosis fungoides; CD30-PCLTCL, CD30-negative primary cutaneous large T-cell lymphoma; PHA, phytohemagglutinin; PBL-T, peripheral blood T cells; CDLE, chronic discoid lupus erythematosus.

isolated or *in vitro*-activated T cell samples (Fig. 3B). In the benign lesional skin biopsy samples, occasionally weak expression of EphA4 was observed, which might be attributable to the presence of endothelial cells in skin that have been reported to be capable of expressing the EphA4 receptor. The Twist gene was overexpressed in four of nine mycosis fungoides skin biopsy samples including one patient with patch-stage disease, whereas its expression in control samples was only weak or absent (Fig. 3C).

**Expression Patterns of Genes Reported to Be Differentially Expressed in Sézary Syndrome.** Subsequently we evaluated the transcript levels of selected genes reported in the literature to be specifically or differentially expressed by Sz T cells. Presented in Fig. 4 are Sz-associated genes reported in previous publications accompanied by a heat map indicating the relative expression levels of each gene in Sz and control samples resulting from our microarray analysis. It should be mentioned that for some of the selected genes, different expression has been described on the protein level but not on

the mRNA level. As Fig. 3 shows, consistent with reports from the literature, we found high expression of JunB (9), versican (20), TRAIL (20), T-plastin (22), Kir3DL2 (23), integrin  $\beta$ 1 (26), as well as low expression of STAT4 (6), TGF- $\beta$  receptor II (10), Fas (12) and CD26 (33) in Sz T cells. In addition to the reported selective expression of the killer cell immunoglobulin-like receptor KIR3DL2, we found expression of KIR2DL4 transcript by Sz cells. We did not observe aberrant expression of MAGE1 (34), P-glycoprotein (35), or SOCS3 (36). Contradictory to previous reports we found increased rather than decreased levels of TIA1 (30) and SHP1 (31) transcripts.

**DISCUSSION**

The purpose of this study was to apply gene expression analysis using oligonucleotide microarrays to gain more insight into the pathogenesis of Sz and to identify tumor-associated markers for potential use in the diagnosis and therapy of this malignancy. We compared

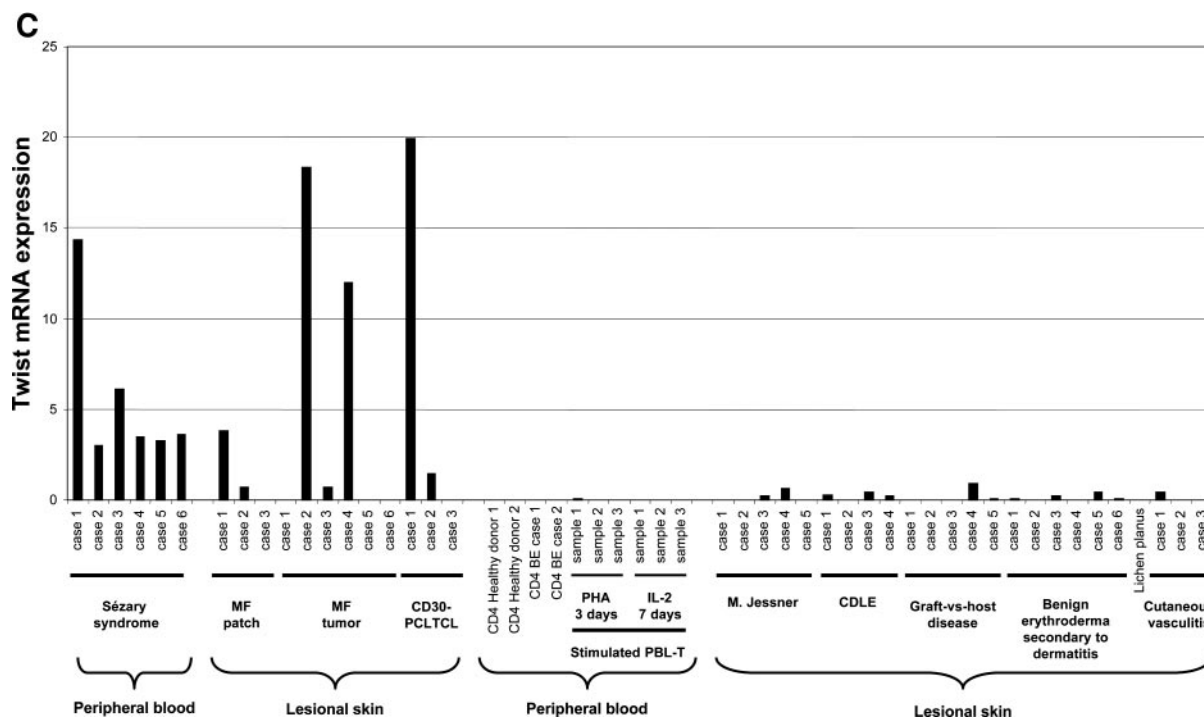


Fig. 3 Continued

gene expression patterns of malignant T cells from peripheral blood of patients with Sz with expression of CD4+ T cells from healthy volunteers and from patients with benign forms of erythroderma. Microarray results were validated by real-time quantitative PCR on a subset of genes.

Transcriptional profiles of Sz T cells demonstrated to be relatively homogeneous and unsupervised hierarchical clustering revealed that malignant T-cell profiles were classified separately from those of benign T cells used as controls. The expression patterns of T cells from patients with BE were more related to those of T cells from healthy volunteers than to those of T cells from Sz patients.

By implementing the SAM algorithm, 176 genes were identified that were significantly differentially expressed in the 10 Sz samples compared with the group of eight control samples. Fifty-nine of these differentially expressed genes were up-regulated, and 64 genes were down-regulated >2-fold (Fig. 2). Approximately half of the genes encode proteins that function in cell signaling and transcription regulation.

First, we examined these differentially expressed genes to identify genes specifically expressed in Sz that might serve as tumor-associated markers. Second, we attempted to discern oncogenic pathways in this transcriptional pattern that separates malignant T cells of Sz patients from benign T cells. Among the most highly overexpressed genes in Sz, two genes (Twist and EphA4) were very consistently up-regulated, whereas transcripts were nearly undetectable in any of the control samples.

The potential tumor-associated gene Twist was expressed in 9 of 10 Sz patient T-cell samples and only weakly in one of the control samples; the average fold change was 12.6. The Twist gene encodes a transcription factor that functions as a regulator of mesodermal differentiation and is normally not expressed in lymphoid cells (37, 38). Twist belongs to the basic helix-loop-helix family of transcription factors, several members of which are known to be T-cell oncoproteins (39). Twist has been shown to have oncogenic properties because it can prevent c-myc-induced apoptosis by antagonizing the p53 pathway (40). The antiapoptotic properties of Twist have additionally

been suggested by its interaction with components of the nuclear factor- $\kappa$ B pathway regulating susceptibility to TNF- $\alpha$ -induced apoptosis (41). Interestingly the nuclear factor- $\kappa$ B pathway, which both regulates and is regulated by Twist activity in mammalian cells, has been reported to be constitutively activated in CTCL cells (42). In human rhabdomyosarcoma and experimental avian nephroblastoma, increased expression of the Twist gene was noted (40, 43). It was shown recently that overexpression of Twist in cancer cell lines is associated with acquisition of resistance to the anticancer drugs taxol and vincristine (44). However, it remains to be established whether increased expression of Twist also has an important role in the pathogenesis of Sz.

EphA4, another potential tumor-associated marker that was highly expressed in 8 of 10 Sz T-cell samples and none of the control samples, belongs to the Eph-receptor subfamily of transmembrane protein-tyrosine kinases (45–47). The expression of Eph receptors has been found most consistently in brain where they are implicated in regulation of neuronal migration and angiogenesis (48, 49). Recently, EphA4 and active signaling by this receptor has been demonstrated in human T cells (50). However, its function in T cells is not clear; nor is it clear whether expression of EphA4 is confined to a specific subset of T cells. Expression of Eph receptors has been linked to malignant transformation. The EphA2 receptor, closely related to EphA4, is highly expressed in several human cancers such as breast, colon, and prostate carcinoma where it has been demonstrated to function as an oncoprotein (51, 52). A possible functional role of EphA4 tyrosine kinase activity in the pathogenesis of Sz may be suspected because components of its downstream signal transduction pathway such as Fyn, Grb2, and Abl were also found to be up-regulated in Sz T cells. Recently activation of EphA4 was described to activate Jak/STAT signaling and induce phosphorylation of STAT3, a transcriptional activator reported to be constitutively phosphorylated in malignant T cells of Sz patients (5, 7, 53). If EphA4 expression would also be functionally significant in Sz T cells, this membrane-bound receptor would constitute an attractive target for therapeutic intervention using

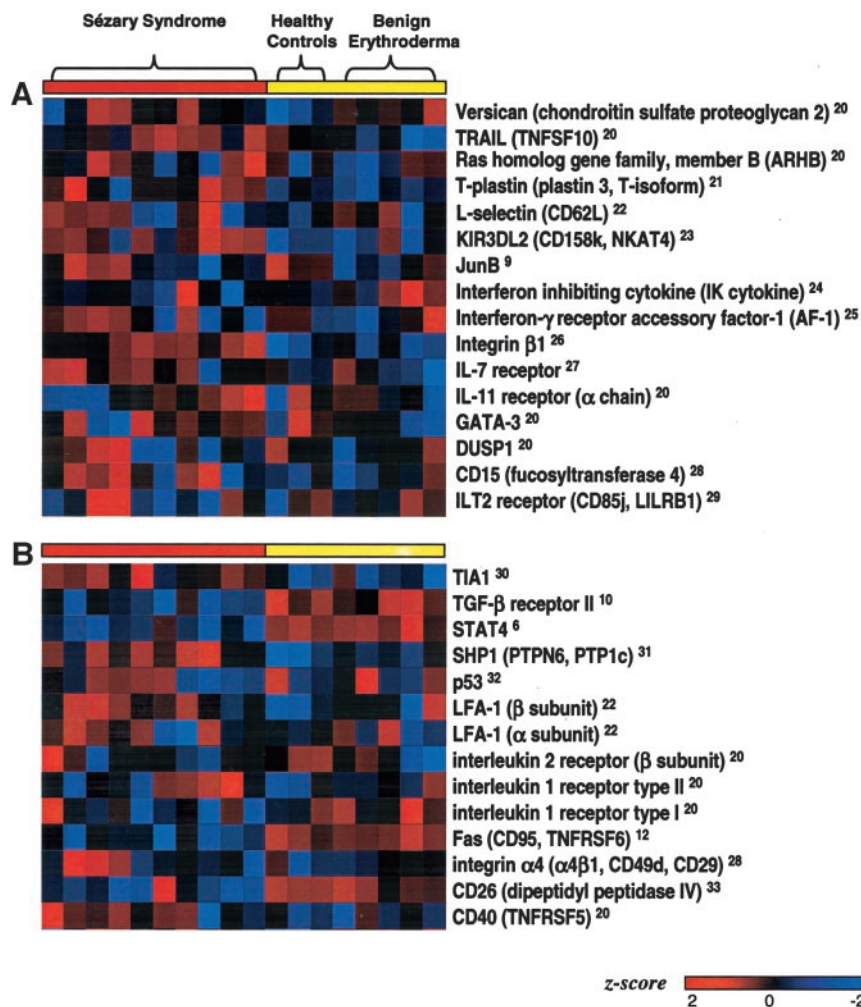


Fig. 4. Expression patterns of selected genes reported in the literature to be differentially expressed in T cells of patients with Sz. Values are visualized according to the *scale bar* that represents the difference in the z-score (expression difference/SD) relative to the mean, similar to Fig. 2. *A*, relative normalized expression levels of genes reported to be overexpressed in Sz. *B*, relative normalized expression levels of genes reported to be underexpressed in Sz.

monoclonal antibodies or small molecular inhibitors of its kinase domain.

Both EphA4 and Twist appeared to be expressed in lesional skin biopsy samples of a subset of patients with mycosis fungoides, a more common CTCL related to Sz, and CD30-negative primary cutaneous T cell lymphoma, but not or only weakly in skin lesions of patients with inflammatory dermatoses. This observation indicates that aberrant expression of these two genes is not limited to malignant T cells of Sz patients and might be a feature of CTCL other than Sz.

The transcriptional profile of Sz T cells demonstrated dysregulation of several other potentially oncogenic signal transduction pathways. We observed a general increased expression of growth-promoting tyrosine kinases including several mitogen-activated protein kinases and decreased expression of inactivating phosphatases such as dual specificity phosphatase 8. In T cells from Sz patients, the expression of a number of tumor suppressor genes that are known to be implicated in the pathogenesis of hematopoietic neoplasms was diminished, including the histone methyltransferase PRDM2 (Riz1; 54), the proapoptotic protein Bcl-11a (55), CREB-binding protein (56), TGF- $\beta$  receptor II (10), Mxi1 (57), and Forkhead box O1A (FOXO1A, FKHR; 58).

A recurrent feature of Sz cells in our study was the decreased expression of the tumor suppressor gene TGF- $\beta$  receptor II and of its ligand TGF- $\beta$ 1. Also its downstream signaling components SMAD3 and SMAD7 were significantly down-regulated in T cells from Sz patients, with fold changes of 1.8 and 1.7, respectively. The resulting disruption of the TGF- $\beta$  receptor signaling pathway is associated with

loss of growth inhibition by TGF- $\beta$ , an antiproliferative and proapoptotic cytokine for lymphoid cells (59). Diminished cell surface expression of the TGF- $\beta$  receptor II protein on CD4+ T cells from Sz patients and loss of sensitivity to TGF- $\beta$  in the progression of CTCL have been described previously (60, 61). The consistent down-regulation of multiple components of the TGF- $\beta$  receptor pathway in patients with Sz supports the notion that abrogation of this signaling pathway is a critical event in the pathogenesis of this malignancy.

The tumor suppressor genes Mxi1 and Mnt, which both antagonize the activity of the potentially oncogenic transcription factor c-myc (62), were consistently down-regulated in the malignant T cells. In addition, malignant T cells in Sz demonstrate high expression of MycBP (Amy1) that encodes a protein that stimulates transcriptional activity of c-myc (63). In mice, targeted deletion of the Mxi1 gene results in a phenotype that shows concordance with that of c-myc-overexpressing mice, including predisposition to the development of lymphomas, implying that Mxi1 functions as a tumor suppressor gene in lymphoid cells (57). Interestingly, the Mxi1 gene is located at chromosome 10q24–26 and the Mnt gene at 17p13.3, chromosomal regions that are frequently lost in Sz cells as shown by cytogenetic studies (14). Although the c-myc gene itself was not consistently overexpressed in the malignant T cells, potentially oncogenic activation of the c-myc pathway could result from altered expression of three of its regulatory proteins, Mxi1, Mnt, and MycBP (62).

Another tumor suppressor gene, which may be relevant in the pathogenesis of Sz and which was down-regulated in T-cell samples from each Sz patient with an average fold change of 2.2, is the

transcription factor FOXO1A. FOXO1A is a downstream target of the phosphatidylinositol 3'-kinase-PTEN-AKT signal transduction pathway (64). Dysregulation of this pathway is a critical event in several hematopoietic neoplasms such as T-cell chronic lymphocytic leukemia (65). In addition, T cell-specific deletion of PTEN in mice has been shown to result in the development of CD4+ T-cell lymphomas (66). An essential role of FOXO1A in the lymphomagenic properties of this signaling pathway is suggested by the recent observation that in PTEN-deficient cells tumorigenicity is reversed by restoration of FOXO1A activity (67). It therefore seems likely that the loss of FOXO1A expression has pro-oncogenic consequences, comparable with those conferred by the loss of PTEN.

Another noticeable feature of the expression pattern particular to Sz T cells is increased expression of the TNF receptor ligands TNFSF7 (CD70) and TNFSF11 (RANKL) in 7 respectively 8 of 10 Sz patients. Deregulated expression of several other members of the TNF receptor family CD40, CD40L and TRAIL has been reported previously in related CTCLs (20, 68, 69). In Sz T cells, overexpression of the TNFSF7 gene encoding the transmembrane protein CD70 is paralleled by high expression of its receptor CD27. CD70 is normally only transiently expressed on antigen-activated T cells and interaction with its receptor CD27 promotes T-cell proliferation and effector T-cell functions (70, 71). In transgenic mice, constitutive expression of CD70 leads to a state of chronic immune activation with excessive formation of effector T cells and progressive depletion of naive T cells resulting in lethal T-cell immunodeficiency (72). Also RANKL can induce inappropriate immune activation through enhancing the co-stimulatory properties of dendritic cells (18, 73). Sz patients can also be severely immunocompromised and susceptible to opportunistic infection, one of the main causes of mortality in these patients. It has been proposed that the immunodeficiency observed in advanced CTCL is caused primarily by T-cell depletion (74). Indicative of T-cell depletion, also observed in CD70-transgenic mice, peripheral blood of patients with Sz displays a marked decrease of absolute normal CD4+ T cells as well as a reduction of the complexity of the T-cell receptor repertoire (75). The coordinate expression of CD70 and CD27 can thus be expected to stimulate activation and proliferation of the malignant T-cell pool in Sz patients. An additional consequence of high expression of CD70 as well as RANKL in Sz patients may be detrimental persistent immune activation and depletion of the naive T-cell pool, resulting in immunodeficiency.

As shown in Fig. 4, many of our findings on gene expression in malignant T cells from Sz patients are consistent with previous findings reported in the literature. We identified the proposed tumor-associated markers T-plastin (21), Kir3DL2 (23), and JunB (9) as up-regulated in Sz T cells, but in the included Sz patient samples their expression patterns were not significantly discriminating according to the SAM algorithm. Recently Kari *et al.* (20) performed cDNA microarray analysis on T cells from patients with erythrodermic CTCL. Although the results of their study show similarities with the present study such as decreased expression of STAT 4 and high expression of transcription factor activator protein 2- $\alpha$  in malignant T cells, differences predominate. The discrepancies between the results are likely to be attributable to the characteristics of the patient group they studied that included erythrodermic CTCL other than Sz, their choice of cultured peripheral blood mononuclear cells skewed to the Th2 phenotype as control, the use of cDNA microarrays that analyze a different and smaller set of genes, as well as the data analysis methods applied.

Our studies indicate that malignant T cells of Sz patients display a gene expression pattern that separates them from benign T cells of patients with BE and healthy controls. Among the most highly and consistently expressed genes in T cells of Sz patients are the

membrane-bound tyrosine kinase receptor EphA4 and Twist, a potentially oncogenic transcription factor. Additional studies are necessary to evaluate whether these tumor-associated proteins are involved in the development and progression of Sz and if they can be used as targets for therapeutic intervention.

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