Th1 Response and Cytotoxicity Genes Are Down-Regulated in Cutaneous T-Cell Lymphoma

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Abstract

Purpose: Increased production of Th2 cytokines characterizes Sezary syndrome, the leukemic form of cutaneous T-cell lymphomas (CTCL). To identify the molecular background and to study whether shared by the most common CTCL subtype, mycosis fungoides, we analyzed the gene expression profiles in both subtypes.

Experimental Design: Freshly isolated cells from 30 samples, representing skin, blood, and enriched $CD4^+$ cell populations of mycosis fungoides and Sezary syndrome, were analyzed with Affymetrix (Santa Clara, CA) oligonucleotide microarrays, quantitative PCR, or immunohistochemistry. The gene expression profiles were combined with findings of comparative genomic hybridization of the same samples to identify chromosomal changes affecting the aberrant gene expression.

Results: We identified a set of Th1-specific genes [e.g., *TBX21* (*T-bet*), *NKG7*, and *SCYA5* (*RANTES*)] to be down-regulated in Sezary syndrome as well as in a proportion of mycosis fungoides samples. In both Sezary syndrome and mycosis fungoides blood samples, the *S100P* and *LIR9* gene expression was up-regulated. In lesional skin, *IL7R* and *CD52* were up-regulated. Integration of comparative genomic hybridization and transcriptomic data identified chromosome arms 1q, 3p, 3q, 4q, 12q, 16p, and 16q as likely targets for new CTCL-associated gene aberrations.

Conclusions: Our findings revealed several new genes involved in CTCL pathogenesis and potential therapeutic targets. Down-regulation of a set of genes involved in Th1 polarization, including the major Th1-polarizing factor, *TBX21*, was for the first time associated with CTCL. In addition, a plausible explanation for the proliferative response of CTCL cells to locally produced interleukin-7 was revealed.

Primary cutaneous T-cell lymphomas (CTCL) represent a group of malignancies of mature T lymphocytes, which show a homing preference for skin. The most common type of CTCL is mycosis fungoides, which presents with skin lesions showing epidermotrophic clonal T lymphocytes. CTCL may also present in a leukemic form with erythrodermic skin involvement and lymphadenopathy (Sezary syndrome), and 10% to 20% of mycosis fungoides cases transform to large T-cell lymphoma with time (1). The molecular mechanisms leading to CTCL are still largely unknown. Previously done microarray studies have found no uniform gene expression signatures (2–4) most likely due to a wide range of different experimental designs and microarray platforms used. In addition, the very low concordance in array profiles obtained with the same samples on different devices has been clearly shown (5). To get a better comprehension of the genes important to CTCL pathogenesis and to identify diagnostic and therapeutic target molecules, we analyzed fresh cells from various tissues of both Sezary syndrome and mycosis fungoides and compared their expression profiles with DNA copy number data.

Materials and Methods

Patient and control samples. Altogether, 30 samples obtained from 18 patients volunteering to the study were analyzed (Supplementary Table S1). Peripheral blood mononuclear cell (PBMC) samples were obtained from 12 Sezary syndrome and mycosis fungoides patients

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† Deceased.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/). S. Hahtola, S. Tuomela, R. Lahesmaa, and A. Ranki contributed equally to this work.

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and lesional skin biopsies from 9 mycosis fungoides patients (stage IA-IVB; defined according to the WHO-European Organization for Research and Treatment of Cancer classification for cutaneous lymphomas; ref. 1). More precisely, Affymetrix (Santa Clara, CA) analysis was done for 6 PBMC or $CD4^+$-enriched cell samples of four Sezary syndrome patients and for 11 PBMC, $CD4^+$, or skin lesion samples of five mycosis fungoides patients (Supplementary Table S1). The percentage of Sezary cells (medium-sized lymphoid cell with a highly cleaved "cerebriform" nucleus and darkly clumped chromatin) among peripheral blood lymphocytes of Sezary syndrome patients ranged from 16% to 70%. None of the Sezary syndrome patients had received any anticancer therapy before sampling. Real-time quantitative PCR (qPCR) analysis was done for PBMC samples of six Sezary syndrome patients and for 12 PBMC, $CD4^+$, or lesional skin samples of seven mycosis fungoides patients. In addition, skin lesion samples of two Sezary syndrome and seven mycosis fungoides patients were studied immunohistologically (Supplementary Table S1). For reference material, blood samples were obtained from 5 healthy volunteers and skin biopsies were obtained from 10 voluntary patients with nonmalignant, lymphoid skin infiltrates (Supplementary Table S1). The study was approved by the Ethical Review Board of the Skin and Allergy Hospital, Helsinki University Hospital.

Cell enrichment and RNA isolation. 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.

Analysis of gene expression microarray data. Purified RNA (100 ng; RNeasy Mini, Qiagen, Valencia, CA) was prepared for hybridization according to Affymetrix small sample protocol (Affymetrix Technical Note, GeneChip Eukaryotic Small Sample Target Labeling Assay version II). cDNA was hybridized against Affymetrix HGU133A chip (Affymetrix). Gene expression estimates were calculated using the GC-RMA procedure (ref. 6; http://www.bioconductor.org). In each two-group comparison, the statistical significance of the difference in gene expression levels between the groups was assessed with a modified *t* test (7). A gene was considered changed if $P < 0.05$ and there was at least a 2-fold change in the mean expression levels. The statistical analyses were carried out with R packages Affy and Limma (http://cran.rproject.org).

Identification of regional biases in gene expression. Patient-specific gene expression profiles were constructed by calculating gene expression ratios between each patient and the average of the matched controls. To assess regional biases in the expression profiles, the microarray probe sets were mapped along the chromosomes using the Bioconductor annotation package HGU133A. To determine whether the set of expression ratios that map to a particular chromosomal arm exhibit upward or downward bias, a sign test was applied (8). The algorithm scores a gene as up-regulated or down-regulated if the expression change is at least 1.8-fold, and the sign test determines whether the corresponding chromosomal arm contains a statistically significant number of genes that change in the same relative direction. An expression bias was considered significant if the $P < 0.05$. Of the acrocentric chromosomes, only q arms were included in the analysis.

Real-time qPCR and immunohistochemistry. The key findings were confirmed with real-time qPCR. Samples were treated with DNase I, amplification grade (Invitrogen Life Technologies, Carlsbad, CA), and cDNA was prepared with SuperScript II kit (Life Technologies, Paisley, United Kingdom). FAM-TAMRA dual-labeled and ProbeLibrary probes (Exiqon A/S, Vedbaek, Denmark) were used in the analysis (Supplementary Table S2). The results were normalized against EF1a detection value (9). Immunostainings were done with CD52, interleukin (IL)-7 receptor, IL-7, KLK10, and matrix metalloproteinase-9 (MMP-9) specific antibodies as described in Supplementary Data.

Comparative genomic hybridization and multifluor fluorescent* in situ *hybridization. Comparative genomic hybridization (CGH) was done as reported previously (10) from the DNA of three Sezary syndrome and three mycosis fungoides patients (Supplementary Table S1). Nine to 12 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. Multifluor fluorescent *in situ* hybridization of metaphase preparations from cases 1 to 3, 5, 7, and 8 was done as described previously (11). At least 50 metaphases were analyzed for each case.

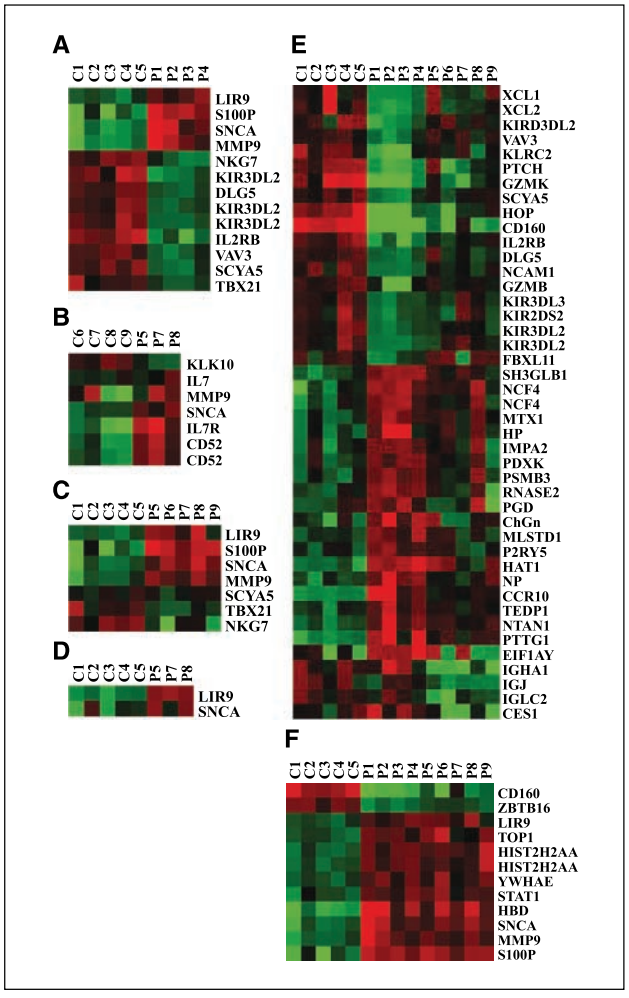

Fig. 1. Substantial differences in gene expression profiles were found in Sezary syndrome PBMC, mycosis fungoides PBMC, $CD4^+$, and skin biopsy samples compared with corresponding control samples. The expression of the genes selected for further analysis from each sample category is displayed: Sezary syndrome PBMC (*A*), mycosis fungoides skin biopsies (*B*), mycosis fungoides $CD4^+$ cells (*C*), and mycosis fungoides PBMC (*D*). Gene expression profiling revealed a gene expression pattern distinguishing between Sezary syndrome and mycosis fungoides PBMC samples but also a subset of changes common to these samples (*E* and *F*). *E,* set of subtype-specific genes identified by comparing the Sezary syndrome and mycosis fungoides samples together and selecting the genes distinguishing these two subtypes. The genes that were also differentially regulated between control samples and either Sezary syndrome or mycosis fungoides samples were included in the final data. *F,* panel of genes found to be CTCL specific (i.e., differentially expressed between controls and both Sezary syndrome and mycosis fungoides PBMC samples).

Table 1. Genes showing similar expression profiles in all CTCL subtypes and in different tissue samples (fold change of at least 2; $P < 0.05$)

Gene	Gene Ontology molecular function	Probe ID	UniGene ID	Locus
ZBTB16	Nucleic acid binding	205883_at	Hs.171299	11q23.1
CD160	Receptor activity	207840_at	Hs.488237	1q21.1
HIST2H2AA	—	218280_x_at	Hs.530461	1q21.2
HIST2H2AA	—	214290_s_at	Hs.530461	1q21.2
HBD	Oxygen transporter activity	206834_at	Hs.36977	11p15.5
MMP-9	Gelatinase B activity	203936_s_at	Hs.297413	20q11.2-q13.1
SNCA	Protein binding	204466_s_at	Hs.271771	4q21
YWHAE	Protein domain-specific binding	213655_at	Hs.513851	17p13.3
LIR9	—	215838_at	Hs.512233	19q13.4
TOP1	DNA topoisomerase type I activity	208900_s_at	Hs.472737	20q12-q13.1
S100P	Calcium ion binding	204351_at	Hs.2962	4p16
STAT1	Transcription factor activity	209969_s_at	Hs.470943	2q32.2
TRIB1	Protein kinase activity	202241_at	Hs.444947	8q24.13
GLIPR1	—	204221_x_at	Hs.553516	12q21.1
GLIPR1	—	214085_x_at	Hs.553516	12q21.1
GLUL	Glutamate-ammonia ligase activity	217202_s_at	Hs.518525	1q31
MS4A4A	Receptor activity	219607_s_at	Hs.325960	11q12
PSMB3	Threonine endopeptidase activity	201400_at	Hs.82793	17q12
ITM2A	—	202747_s_at	Hs.17109	Xq13.3-Xq21.2
ETHE1	—	204034_at	Hs.7486	19q13.31
BARD1	Ubiquitin protein ligase activity	205345_at	Hs.54089	2q34-q35
RECQL	Nucleotide binding	212918_at	Hs.235069	12p12
HIST2H2BE	DNA binding	202708_s_at	Hs.2178	1q21-q23
CDC42	Nucleotide binding	208727_s_at	Hs.467637	1p36.1
RPL31	Structural constituent of ribosome	200962_at	Hs.469473	2q11.2
LOC55831	—	217882_at	Hs.475392	3p25.3
G0S2	—	213524_s_at	Hs.432132	1q32.2q41
ARPC1A	—	200950_at	Hs.124126	7q22.1
C1QBP	—	208910_s_at	Hs.553487	17p13.3
C1QBP	—	214214_s_at	Hs.553487	17p13.3

*Statistically significant change to the same direction.
†Statistically significant change to the opposite direction.

Results

Genes expressed differentially among CTCL patients and controls. We identified altogether 168 probe sets (fold change > 2; $P < 0.05$) to be differentially regulated in Sezary syndrome PBMC samples compared with control PBMC samples and substantial variation of gene expression between control and mycosis fungoides skin samples. (Supplementary Tables S3-S6 show differentially regulated genes in all studied cell populations; the raw data of all hybridizations is available on request.) Figure 1A to D shows the expression of genes selected for further analysis. 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 (Fig. 1E). However, a subset of genes was found to change in a similar manner in both Sezary syndrome and mycosis fungoides PBMC samples (Fig. 1F). To mask the effect of reactive T cells, commonly present in the samples of CTCL (12), comparison of microarray data from different cell populations was done, and changes common to different cell sources of mycosis fungoides patients (PBMC, $CD4^+$ lymphocytes, and lesional skin) and Sezary syndrome PBMC samples were identified (Table 1).

In Sezary syndrome samples, two Th1-specific genes (*SCYA5* and *NKG7*) and *IL-2Rβ*, *VAV3*, *DLG5*, and *KIR3DL2* were found to be >2-fold down-regulated (Fig. 1A). Genes up-regulated 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 (Table 1). In addition, *TBX21* (*T-bet*) was selected for further analysis based on its crucial role in Th1 differentiation (13).

Real-time qPCR and immunohistochemistry confirmed the microarray results. qPCR done on the aforementioned 10 genes (Fig. 2) and immunohistochemistry done on 4 gene products (CD52, IL-7R, IL-7, and MMP-9; Fig. 3) validated the microarray data. Especially, we wanted to make sure that our array results on *KIR3DL2*, reported previously to be a marker gene of Sezary syndrome (14) and now found down-regulated in our Sezary syndrome patients, were not due to differences in target sequence. Therefore, the *KIR3DL2* result was confirmed by using qPCR reagents detecting the same sequence as the Affymetrix probe set (207314_x_at) and region reported previously (15). Both sets of reagents for *KIR3DL2* inevitably showed that the expression of this gene was down-regulated in our sample set. Interestingly, we also show the down-regulation of *TBX21* gene in PBMC samples of both Sezary syndrome and mycosis fungoides patients (Fig. 2B). Down-regulation in the expression of *SCYA5* (*RANTES*)

Table 1. Genes showing similar expression profiles in all CTCL subtypes and in different tissue samples (fold change of at least 2; $P < 0.05$) (Cont'd)

Sezary syndrome PBMC		Mycosis fungoides PBMC		Mycosis fungoides $CD4^+$		Mycosis fungoides skin	
M	P	M	P	M	P	M	P
−1.84	0.0001	−1.07	0.0014	—	—	—	—
−3.51	0.0000	−1.64	0.0290	—	—	—	—
1.32	0.0021	1.43	0.0063	2.09	0.0180	—	—
1.39	0.0008	1.19	0.0250	2.36	0.0220	—	—
3.45	0.0057	1.86	0.0065	—	—	—	—
1.98	0.0083	1.17	0.0070	—	—	—	—
2.08	0.0120	1.48	0.0079	*	*	1.19	0.0250
1.25	0.0033	1.06	0.0340	—	—	—	—
1.10	0.0065	1.09	0.0360	1.91	0.0001	—	—
1.18	0.0026	1.29	0.0480	—	—	†	†
2.21	0.0230	2.12	0.0220	—	—	—	—
1.24	0.0270	1.10	0.0410	—	—	—	—
1.07	0.0390	—	—	3.36	0.0240	—	—
1.14	0.0180	—	—	1.01	0.0083	—	—
1.20	0.0270	—	—	1.05	0.0090	—	—
1.54	0.0078	—	—	1.57	0.0260	—	—
1.96	0.0140	—	—	1.52	0.0370	—	—
1.15	0.0059	—	—	—	—	1.26	0.0047
1.17	0.0350	—	—	—	—	1.75	0.0200
1.29	0.0260	—	—	—	—	1.40	0.0340
1.03	0.0220	—	—	—	—	1.28	0.0320
1.06	0.0085	—	—	—	—	1.00	0.0440
—	—	1.73	0.0270	2.46	0.0490	—	—
—	—	−1.00	0.0058	−1.36	0.0220	*	*
—	—	−1.14	0.0470	−2.27	0.0008	—	—
—	—	1.06	0.0091	1.13	0.0440	—	—
—	—	1.55	0.0360	3.73	0.0200	—	—
—	—	—	—	1.06	0.0230	1.28	0.0072
—	—	—	—	−1.39	0.0008	−1.20	0.0420
—	—	—	—	−1.22	0.0012	−1.30	0.0110

and *NKG7* were observed also among some of the mycosis fungoides PBMC samples (Fig. 2A). In immunohistochemistry, CD52 protein was expressed by the majority (in average, 3 of 4) of skin-infiltrating lymphocytes of all 9 CTCL patients when compared with inflammatory dermatoses with sparse expression (Fig. 3A and B). *IL-7R* was expressed in basal keratinocytes (focally) but also in skin-infiltrating lymphocytes of all CTCL biopsies. The number of lymphocytes or keratinocytes expressing *IL-7R* was, in average, thrice higher than in control samples (Fig. 3C and D). No difference in the expression levels of IL-7 protein was found between CTCL patients and controls, however. MMP-9 protein was demonstrable in 25% to 50% of infiltrating lymphocytes in Sezary syndrome samples, whereas MMP-9 expression in mycosis fungoides samples was variable (Fig. 3E and F). In inflammatory dermatoses, the lymphocytes did not express MMP-9 (Fig. 3G). With the available antibody, no expression of *KLK10* was found on frozen sections.

Chromosomal regions showing both gene expression and DNA copy number changes. Finally, we examined the gene expression profiles by chromosome arms in four Sezary syndrome PBMC and three mycosis fungoides skin samples. Five chromosomal arms showed consistently significant upward bias in at least four of the seven patient samples: 1q, 3p, 3q, 16p, and 16q. In addition, in chromosomes 4q and 12q, both an upward and a downward bias were detected (Fig. 4A). As an example, clusters of differentially expressed genes could be visualized especially in the areas 12q13 and 12q23-q24 (Fig. 4B).

To integrate the chromosomal and transcriptomic data, the chromosomal regions identified with the sign test 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 mycosis fungoides skin or Sezary syndrome blood samples. For example, the *SNCA* gene, which was found to be up-regulated, locates to cytoband 4q21. 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 (Fig. 4C).

Discussion

The aim of this study was to obtain information on the poorly understood pathogenesis of CTCL by microarray gene expression analysis of both mycosis fungoides and Sezary syndrome cells with Affymetrix oligonucleotide array containing >22,000 transcripts and to combine the data with results obtained with CGH. To identify shared expression profile

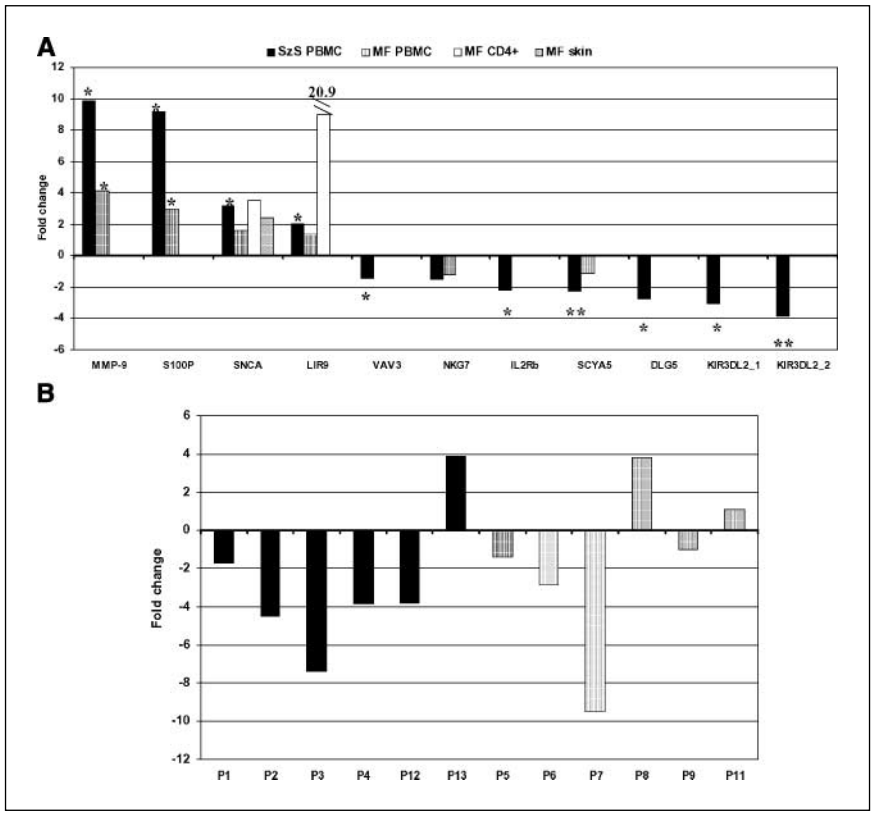

Fig. 2. Taqman real-time qPCR analysis validated the Affymetrix findings. *A,* average fold change of patient gene expression compared with average expression of control samples. *, $P < 0.05$; **, $P < 0.005$, statistical significance (*t* test). *KIR3DL2* expression was measured from two parts of the transcript as described in Results. *B, TBX21* expression was down-regulated in all Sezary syndrome PBMC samples hybridized to Affymetrix array (*P1-P4*; Supplementary Table S1) and also in most of the mycosis fungoides PBMC samples when compared with the average expression in control samples. *Black columns,* Sezary syndrome patients (*SzS*); *shaded columns,* mycosis fungoides (*MF*) patients.

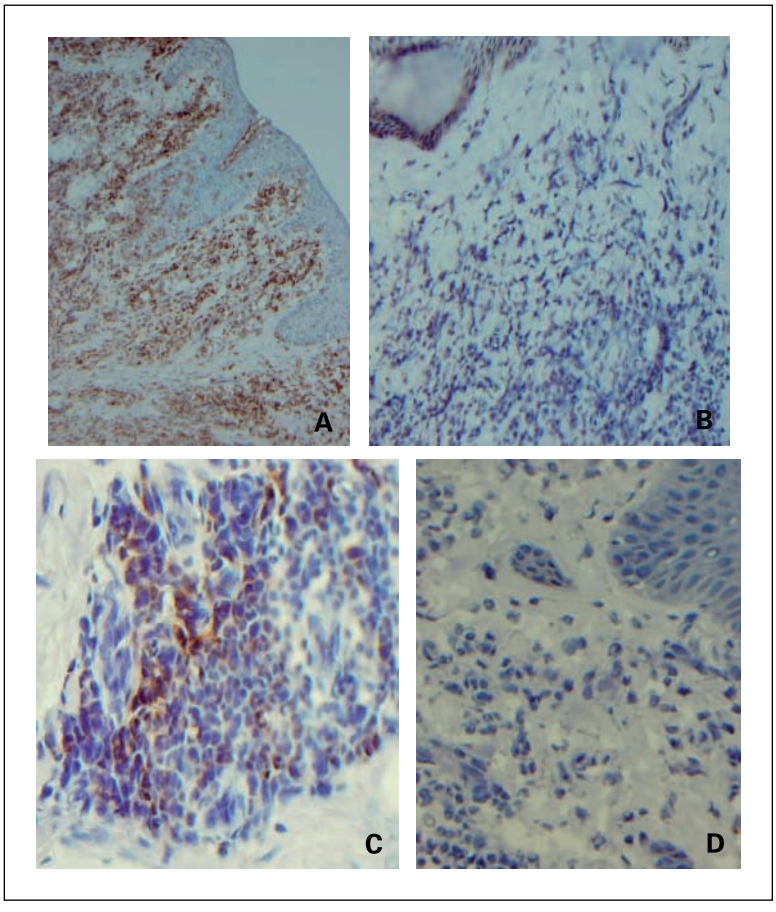

Fig. 3. Example of immunohistochemical detection of *CD52, IL-7R*, and *MMP-9*, found up-regulated at RNA level, in lesional mycosis fungoides and Sezary syndrome skin samples before therapy. *A,* abundant expression of CD52 protein in the skin-infiltrating lymphocytes of CTCL (patient P2). Magnification, ×15). *B,* only few $CD52^+$ lymphocytes were found in inflammatory lesions (case C14). Magnification, ×40. *C,* IL-7R is expressed by ~30% of lesional lymphocytes in CTCL (patient P15). Magnification, ×60. *D,* only few cells are positive for IL-7R in the inflammatory control samples.

for the most common forms of CTCL and to mask the effect of reactive T cells, likely to influence the results, we selected a sample material representing several cell subpopulations of both Sezary syndrome and mycosis fungoides patients. Despite the difference in the quantity of malignant T cells in Sezary syndrome and mycosis fungoides blood, we identified a common gene expression pattern. More precisely, our findings provide basis for previous findings of a preferential Th2-type cytokine profile in Sezary syndrome, because we identified a panel of Th1-specific genes [refs. 13, 16–18; e.g., *TBX21*, *SCYA5*, *NKG7*, *XCL1* (*lymphotactin*), *TXK*, and *GZMB* (*granzyme B*)] to be down-regulated in Sezary syndrome samples. Furthermore, for the first time for CTCL, we identified chromosomal arms where both DNA copy number and gene expression levels were changed.

Of the Th1-specific genes down-regulated in Sezary syndrome, *TBX21* and *TXK* represent transcription factors essential for Th commitment to Th1 phenotype. They both regulate IFN-γ expression, which we have shown previously to be absent from the chromosomally clonal (i.e., true malignant) cells in Sezary syndrome (19). In addition, they belong to a positive feedback loop promoting Th1 cytokine secretion, leading to Th1 development (13, 18). The expression of *TBX21* was very low also in one mycosis fungoides patient, but the overall variation among mycosis fungoides patients (various tumor-node-metastasis stages) was greater than among the leukemic Sezary syndrome patients. Our finding of *TBX21* down-regulation in Sezary syndrome would explain the previous observation of the loss of the chemokine ligand CXCR3 expression along the progression of mycosis fungoides (20), because *TBX21* regulates the $CD4^+$ cell trafficking via CXCR3 (21). Furthermore, the expression of *SCYA5*, a chemokine mediating the trafficking and homing of T cells (22), and *NKG7* was down-regulated in our Sezary syndrome PBMC samples. These genes have also been linked to Th cell differentiation and are more abundantly expressed in cells polarized to Th1 than to Th2 direction (16, 17).

The genes up-regulated during the early polarization of T helper cells into Th2 direction (16) and up-regulated in both our Sezary syndrome and mycosis fungoides PBMC samples included the *S100P* gene. S100P has a role in cell cycle progression and differentiation, and its up-regulation has been found in various malignancies (23). 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, we found membrane-bound *LIR9* (215838_at) to be overexpressed in Sezary syndrome PBMC, mycosis fungoides PBMC, and mycosis fungoides $CD4^+$ samples. LIR9 is a member of leukocyte immunoglobulin-like receptor family, which induces secretion of IL-1β, tumor necrosis factor-α, and IL-6 in monocytes (24). Deregulation of tumor necrosis factor signaling pathway has been linked to both Sezary syndrome and mycosis fungoides pathogenesis (4, 25). IL-6 is an important cytokine for Th2 cell differentiation (26) and also induces S100P (23). A third membrane protein up-regulated in Sezary syndrome cells was *MS4A4A*, a member of the MS4A superfamily (27). Another member of this superfamily, CD20, has been the target of monoclonal antibody-mediated therapy in large B-cell lymphomas.

Summarizing the above knowledge (Table 2), our findings seem to explain the functional bias toward Th2 in Sezary syndrome (28). Our findings also indicate that such a bias takes place already in the mycosis fungoides stage, before progression to the leukemic phase. This kind of a skewing is likely to influence the progressive immune dysregulation in CTCL and

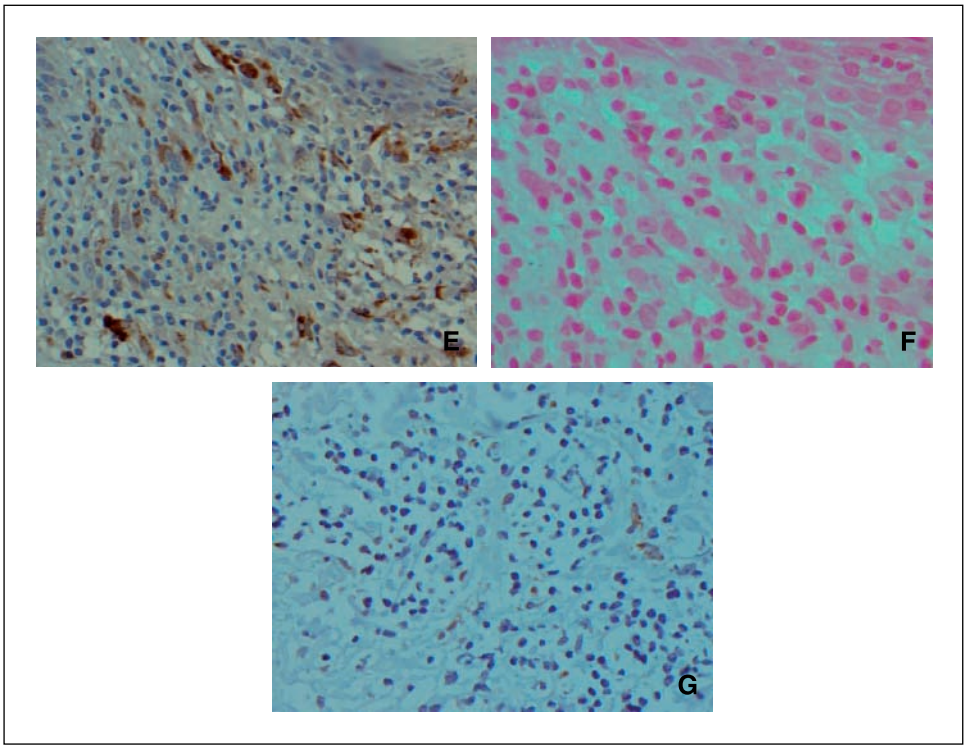

Fig. 3 *Continued. E,* MMP-9 protein is expressed in the skin-infiltrating lymphocytes of Sezary syndrome patients (patient P2). Magnification, ×40. *F,* Fontana staining of parallel sections was done to exclude melanin pigment, frequent in Sezary syndrome skin, as the colorigenic substrate. *G,* only occasional infiltrating cells expressed MMP-9 in eczema lesions. Magnification, ×40.

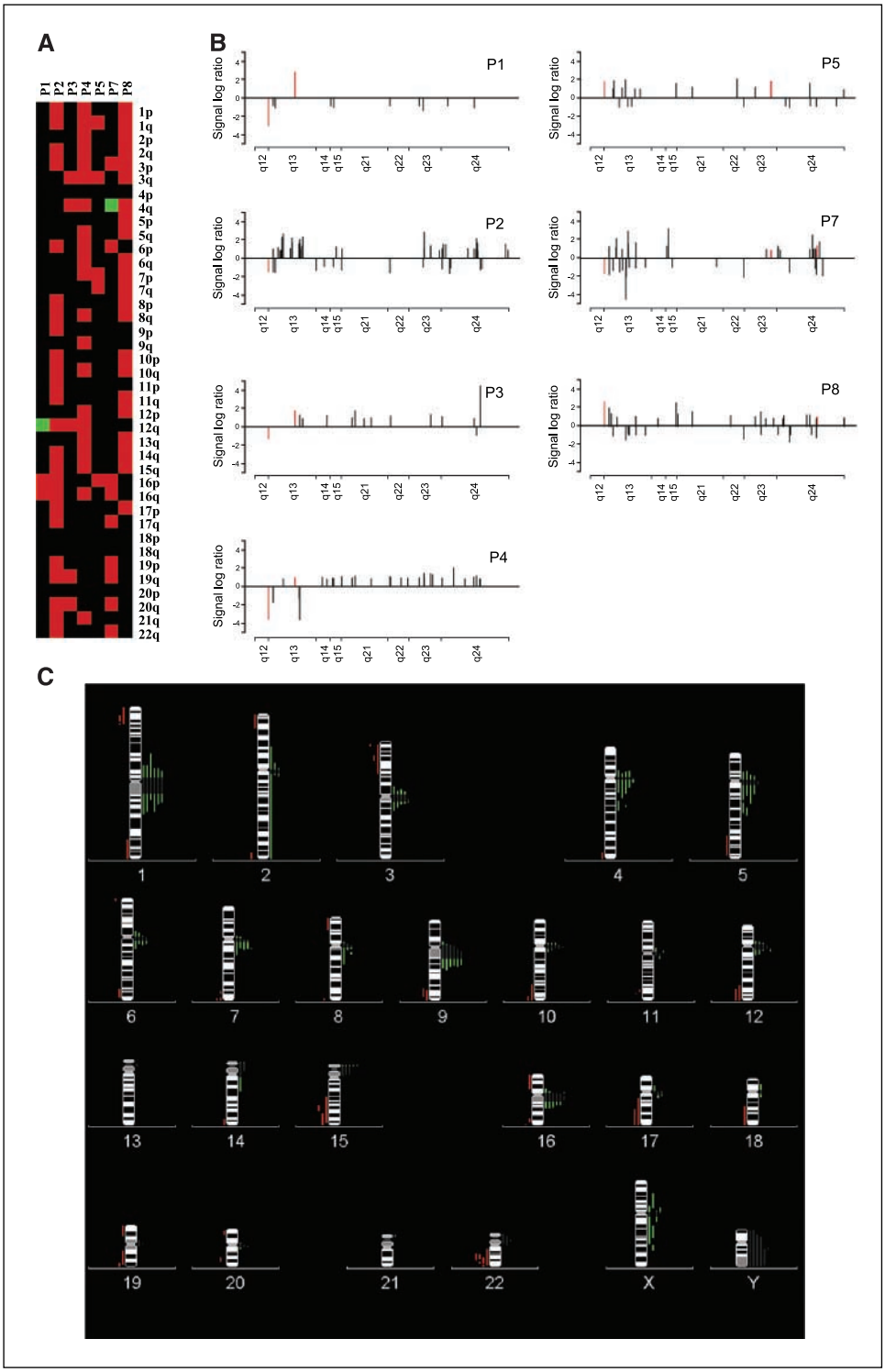

Fig. 4. In chromosomal arms 1q, 3p, 3q, 4q, 12q, 16p, and 16q, both gene expression and DNA copy number were changed to the same direction. Identified regions with expression bias for the four Sezary syndrome PBMC and the three mycosis fungoides skin samples. *A, red,* chromosomal arms that show a significant upward bias; *green,* arms that show a significant downward bias. $P < 0.05$ (sign test). *B,* gene expression ratios mapped to chromosome 12q (*black* and *red columns*). For each patient, only ratios >1.8-fold were included in the analysis. The expression values are organized along the chromosome. *Red,* genes identified as differentially expressed in the comparison between Sezary syndrome patients and controls (PBMC samples) or in the comparison between mycosis fungoides patients and controls (skin samples); *black,* individual expression levels of chromosome 12q genes for each patient. $P < 0.05$ (modified *t* test). *C,* combined CGH profiles of three Sezary syndrome (blood samples) and three mycosis fungoides (skin samples) patients. *Green,* copy number gains; *red,* copy number losses. Pericentromeric gains were excluded from the analysis if the amplified areas were small and rather due to technical aspects. Additionally, of the acrocentric chromosomes, only q arms were included in the analysis.

would thus provide a growth advantage for the malignant cell clone(s) (29).

Of the cytotoxicity-associated genes, we found down-regulation of *XCL1*, *GZMB*, and *VAV3*. We have shown previously that GZMB is absent from the chromosomally clonal cells in skin and lymph node samples of Sezary syndrome (19). VAV3, like the other two VAV proteins, functions specifically in signaling pathways that trigger natural killer cell cytotoxicity (30). KIR3DL2, a member of the killer cell immunoglobulin-like receptors, has been suggested previously as a phenotypic marker for Sezary cells (14) and has been found up-regulated in Sezary syndrome (3). Contradictory, we found the *KIR3DL2* gene to be down-regulated in Sezary syndrome. However, the *LIR9* gene, located in the same leukocyte receptor cluster as *KIR3DL2*, was overexpressed in our patients. The discrepant observations of

KIR3DL2 may be because only about half of the expanded T-cell clones express CD158k and that CD158k expression is heterogeneous even within the malignant clones (31). Taking together, our data provide evidence for the down-regulation of several cytotoxicity-associated genes in Sezary syndrome.

We found *IL-2Rβ* to be down-regulated 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 up-regulated by IL-2. The high/intermediate affinity IL-2R (α/p55/CD25$^+$ β/p75/CD122$^+$ γ/p64/CD132$^+$ chains/β + γ chains) is expressed on ~50% of CTCL cells (32). Consequently, IL-2-targeted therapy has been used for CTCL, most recently with a fusion protein denileukin diftitox (ONTAK; ref. 33). Interestingly, the retinoid X receptor retinoid, bexarotene, a new therapeutic agent for mycosis fungoides (34), up-regulates both the p55 and p75 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 (35).

It is general experience that true CTCL cell lines are extremely difficult to propagate, but some success has been gained with the growth factors IL-7 and IL-15 (36). IL-7 production was shown recently to be elevated in CTCL skin (37), although the source of IL-7 remained obscure. We found the expression levels of IL-7R, but not IL-7, up-regulated in the lesional skin biopsies of mycosis fungoides patients. Immunohistology revealed that the origin of the increased IL-7R was in the basal keratinocytes and lymphocytes in CTCL lesions. Because IL-7R signalling promotes proliferation and survival of T cells and IL-7 has a role in peripheral T-cell homeostasis (38), we assume that the IL-7/IL-7R balance influences the homing of malignant lymphocytes to the epidermis in CTCL. Thus, our findings revealed a plausible molecular explanation for the proliferative response of CTCL cells to skin-derived IL-7 (36).

Another membrane antigen, CD52, expressed on all lymphocytes, was found and confirmed up-regulated in the lesional skin samples of early-stage mycosis fungoides (stage IA-IB). The actual function of CD52 in lymphocytes is unclear, but humanized anti-CD52 monoclonal antibody (Campath-1H, alemtuzumab) has been used to treat advanced forms of CTCL (Sezary syndrome and late-stage mycosis fungoides; ref. 39). Our finding might warrant the use of alemtuzumab also for earlier stages of mycosis fungoides, in case its adverse effects allow. As the expression of IL-7R and CD52 was not significantly changed in Sezary syndrome blood samples, their differential expression may represent a signature for the skin-infiltrating malignant lymphocytes.

In addition to the genes involved in immunoregulation, two genes, commonly up-regulated in our samples, were of interest. The *SNCA* gene, mapping to chromosome 4q21, was up-regulated in all studied tissues or cell types of mycosis fungoides and Sezary syndrome patients. Amplifications of 4q are frequent in CTCL (40), and in our integrated analysis, 4q turned out to be one of the areas with overexpressed genes and DNA copy number amplifications. Overexpression of *SNCA* has been shown to increase cell proliferation (41). The *MMP-9* was overexpressed in Sezary syndrome and mycosis fungoides PBMC samples. This provides new aspects for the pathogenesis of CTCL, because MMPs, in addition to their role in facilitating tumor cell invasion and metastasis, may be involved in cancer initiation by causing genomic instability (42). Our observation thus suggests that the role of MMPs should be studied further at the early stages of CTCL carcinogenesis.

Previously, several studies on CTCL have pointed out chromosomal instability as a hallmark of the disease (10, 39, 43); thus, chromosomal aberrations may affect gene expression (11). The *DLG5* tumor suppressor gene, down-regulated in our Sezary syndrome samples, is located in 10q23, a chromosomal area often deleted in CTCL (10, 40). In this study, we have for

Table 2. Genes found to be differentially expressed and relevant to CTCL pathogenesis

Gene designation	Finding	Molecular function	Presumed function or expected functional consequence
TBX21 (T-bet)	Down-regulated	Transcription factor	Th1 down
SCYA5 (RANTES)	Down-regulated	Chemokine	Th1 down
NKG7	Down-regulated	Th differentiation	Th1 down
XCL1 (lymphotactin)	Down-regulated	Chemokine, T-cell cytotoxicity (CTL)	Th1 down, lack of CTL activity
TXK	Down-regulated	Transcription factor	Th1 down
GZMB (granzyme B)	Down-regulated	T-cell cytotoxicity	Th1 down, lack of CTL activity
S100P	Up-regulated	Th2 polarization, cell cycle and differentiation	Th2 up
LIR9	Up-regulated	Membrane receptor, induces IL-1β, tumor necrosis factor-α and IL-6	Th2 up
KIR3DL2	Down-regulated	Membrane receptor	Lack of CTL activity
IL-2Rβ	Down-regulated	Cytokine receptor	Impaired immune response
VAV3	Down-regulated	Signal transduction, activates Rho family	Disturbed T-cell activation
DLG5	Down-regulated	Tumor suppressor	Increases cell proliferation
MMP-9	Up-regulated	MMP	Carcinogenesis and tumor spread
IL-7R	Up-regulated (basal keratinocytes)	Cytokine receptor	Lymphocyte activation and homing to epidermis
CD52	Up-regulated	Membrane antigen	(Target molecule of alemtuzumab)
MS4A4A	Up-regulated	Membrane antigen	Signal transduction in hematopoietic cells

the first time correlated the chromosomal changes with aberrations observed in gene expression level in the same patient subset. We identified seven chromosome arms, where both gene expression and DNA copy number was changed to the same direction (i.e., 1q, 3p, 3q, 4q, 12q, 16p, and 16q). All these arms contain overexpressed genes and amplified chromosomal areas in our data set. Additionally, 4q and 12q also contain down-regulated genes and deleted areas. We have observed previously gains of chromosome arms 1q, 3p, 3q, 4q, and 16q by CGH in CTCL,[6] and diverse aberrations of these chromosomes are common by other cytogenetic or molecular cytogenetic methods (11, 40, 43). Thus, the above chromosome arms are potential targets for searching for further recurrent gene aberrations in CTCL.

In summary, our results revealed several new potential target genes for CTCL pathogenesis and eventual therapy, widening the knowledge of gene expression profiles on different CTCL subtypes. The diversity of the published results might reflect the heterogeneity of the cell populations studied thus far. In the future, studying morphologically malignant cells picked by microdissection might reveal gene expression profiles specific for the malignant clones. Our study reinforced the role of T helper cell balance in the pathogenesis of CTCL. Down-regulation of the major Th1-polarizing factor, TBX21, was for the first time associated with CTCL. Some of the membrane proteins up-regulated on the malignant cells are potential targets for monoclonal antibody-mediated therapy.

[6] Karenko and Kähkönen, unpublished observation.

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