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

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Abstract

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

Introduction

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

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

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

Material & Methods

In silico analysis

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

The quality of all datasets were checked using a series of QC metrics recommended by Affymetrix (using the R-script described by Heber and Sick[14]) in order to confirm that arrays were hybridized correctly, that sample quality was acceptable and batches of datasets could reliable be compared in a meta analysis. Data sets in which more than 30% of the individual samples required exclusion solely based on RNA quality, were discarded entirely. Next, raw gene expression data from Affymetrix CEL files passing all QC controls were reannotated according to the Entrez genome annotation using CDF-files[13] followed by GC-RMA (robust multiarray averaging) normalization. Samples passing QC which were included in the final normalization are summarized in Supplementary Table S1. For subsequent analysis, all T-cell expression data from healthy volunteers were grouped to create a reference of normal T cells. Gene expression profiles of skin biopsies were clustered and labeled according to the supplementary information given in GEO or ArrayExpress or accompanying papers[15,16] (Table 1), resulting in groups labeled as normal skin or inflamed skin (Pedersen data) or normal skin, uninvolved skin psoriasis and lesional skin psoriasis (Yao data). Differentially expressed (DE) genes between groups and MF tumor stage were identified with LIMMA[17] using a log 2 fold change ≥ 1 as threshold and were considered statistically significant at an adjusted *P*-value of < 0.01 (using Benjamini-Hochberg multiple testing correction). Subsequently, the "AND" operator[18] was used to perform a comparison between the lists of DE genes from the individual comparisons to identify consistently up- or down-regulated transcripts in MF. This comparison resulting in a list of genes enriched or depleted in MF. Gene enrichment analyses of DE genes were performed with PPI spider, DAVID, Panther and Webgestalt.[19-23]

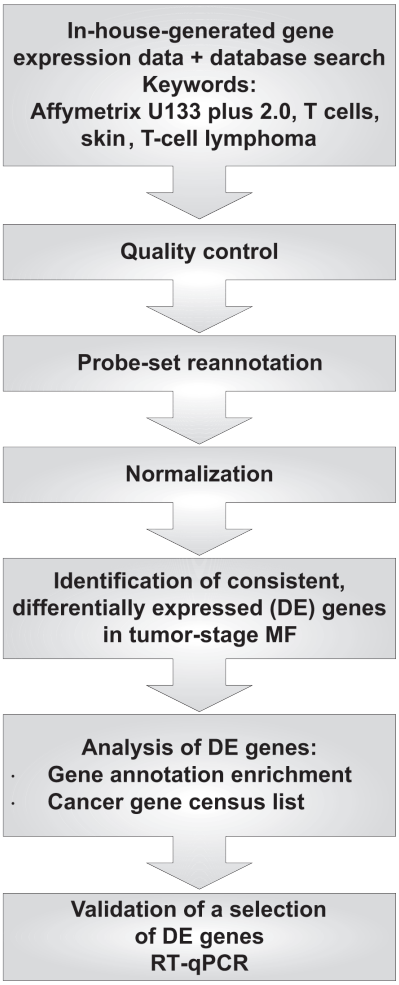


Figure 1a Workflow of the strategy used to identify the molecular signature of MF tumor stage
1) Data sets were obtained by searching for Affymetrix U133 vs 2 Gene chip (GPL570). 2) The quality of the data-sets was checked using a series of QC metrics. 3) Gene expression data were reannotated according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

Reverse transcription-coupled quantitative PCR (RT-qPCR)

Array results were validated by RT-qPCR on RNA isolated from fresh-frozen skin biopsies of 21 MF tumors, 6 chronic discoid lupus erythematosus (CDLE) lesions, 8 early-stage MF (IA/B) patients, 4 patients with chronic eczematous dermatitis (CED), normal skin (n=6), freshly isolated $CD4^+$ T cells of 6 healthy donors as described previously[24] and MyLa cells. cDNA was synthesized of 1 µg total RNA, after treatment with RQ1 DNase I (Promega, Madison, WI), using IScript reverse transcriptase (Bio-Rad, Veenendaal, the Netherlands), oligo$(dT)_{12\text{-}18}$, and random hexamer priming (Bio-Rad) in a final volume of 20 µl. RT-qPCR was performed with the MyIQ Detection System and the SYBR Green Supermix (Bio-Rad).

Table 1 Samples included in the comparative analyses

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

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

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

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

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

methylated and non-methylated DNA will be amplified. MS-MCA PCR reactions were performed as described earlier[26] with the MyIQ Detection System and the SYBR Green Supermix (Bio-Rad) in a 25 µl reaction volume. Cycle parameters for all analyzed CpG islands were as followed: denaturing at 96 ˚C for 30 s, annealing at temperatures varying from 65 ˚C to 58 ˚C depending on the primer set used for 40 s and extension at 72 ˚C for 40 s for eight cycles; followed by denaturing for 30 s, annealing at 60 ˚C for 40 s and extension at 72 ˚C for 40 s for 35 cycles. DNA melting curves were acquired directly after amplification by measuring the fluorescence of SYBR Green Supermix (Bio-Rad) during a linear temperature transition from 65 ˚C to 94.8 ˚C at 0.2 ˚C /s.
Sensitivity and specificity of the MS-MCA was validated for all primer sets using (mixtures of) methylated human DNA (Chemicon, Hampshire, UK) or unmethylated human semen DNA as input. Approval for these studies was obtained from the institutional review board of the Leiden University Medical Center. Informed consent was provided according to the Declaration of Helsinki Principles.

Results

In silico analysis: identification of differentially expressed genes

Screening and filtering strategy for the identification of MF tumor-specific genes
To identify MF tumor genes we designed a strategy (the workflow is shown in Figure 1a) eventually generating a list of DE genes characteristic for MF tumor stage. First, gene expression data sets were obtained by searching for Affymetrix U133 plus 2.0 Gene chip (designated as GPL570 in the Gene Expression Omnibus, GEO) in combination with the keywords skin and/or (activated) T cells and/or T-cell lymphoma, revealing approximately 15 suitable hits. Downloaded CEL files and previously generated expression profiles (amongst which 22 T-MF[8]) were subjected to the Affymetrix QC metrics and individual samples passing the control (Table 1 and Supplementary Table S1) were included for further analyses. Next, we compared gene expression profiles of T-MF one-to-one with skin or (reference) T cells. Not surprisingly, comparing MF tumor samples with skin (or inflamed skin only) revealed a large number of typical T-cell (related) genes, while comparison between MF tumor samples and T cells produced a long list of DE genes indicative for skin (results not shown). To identify genes that are specifically enriched or depleted in T-MF ("unique genes") we decided to perform a comparison between the lists of DE genes, identified by the pairwise comparisons, using an "AND" operator[18] and to look for genes that were consistently up- or down-regulated. To that end the T-MF

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

Systematic and integrative analysis of differentially expressed genes

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

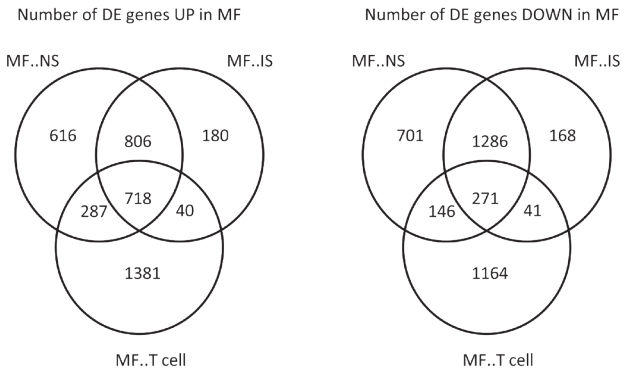


Figure 1b Venn diagram illustrating the number of genes that show altered expression in MF tumor stage compared to normal skin, (NS) inflamed skin (IS) and T cells. Values indicate the number of genes significantly up-regulated (left) or down-regulated (right). The intersecting regions represent number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

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

Comparison of molecular signatures

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

Promoter hypermethylation

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

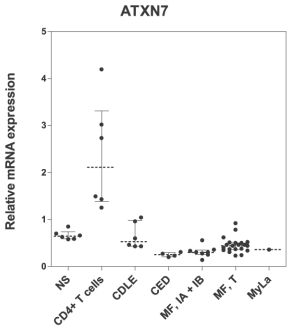
Verification of MF DE genes using qPCR

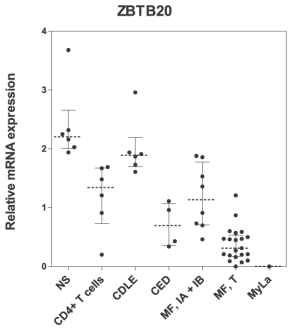
Differential expression for a selection of genes since material is limited) was verified using RT-qPCR (results summarized in Figure 2; *P*-values from statistical evaluation (Mann-Whitney) can be found in Supplementary Table S6). We included mRNA isolated from skin biopsies of patients with chronic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

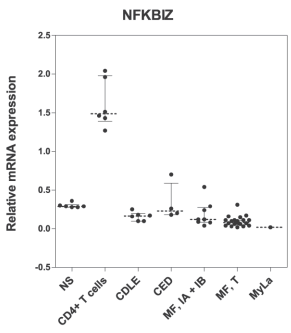
MF (IA/B), chronic eczematous dermatitis, normal skin and freshly isolated CD4+ T cells of healthy donors as additional samples. Instead of solely confirming the highest differences, we focused on genes that represent different classes, which might give further insights into the disease and/or previously unreported putative targets for diagnosis/therapy. We noticed that the array comparison indicated dysregulation of multiple genes involved in the miRNA biogenesis/machinery and aberrant expression of proteins involved in miRNA processing has been observed in T-cell lymphoma including Sézary syndrome.[29] We therefore included also *RNASEN, DICER, EIF2C1, EIF2C2, EIF2C3* and *EIF2C4* in the RT-qPCR validation. GeNorm analysis [25] revealed that *EIF2C4* was stably expressed in all samples and therefore in addition to *ARF5, TMEM87a* and *ERCC3* (See Materials and Methods) used as a reference gene. Initial experiments could not detect any significant differences in *DICER* and *EIF2C3* expression between samples and controls; therefore, these genes were excluded from subsequent analysis. With RT-qPCR down-regulation of *ATXN7, ZBTB20, NFKBIZ*, in T-MF in comparison to CD4+ T cells and normal skin could be affirmed. *ZBTB20* was also significantly less expressed in T-MF compared to CLE and early-stage MF, whereas *NFKBIZ* is lower in T-MF compared to all controls. Expression of *ATXN7*, however, was higher in T-MF versus CED and early-stage MF.

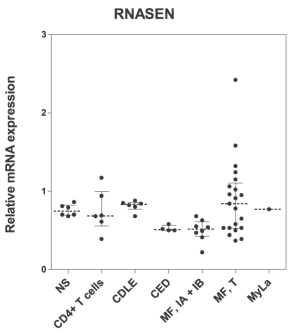
We observed that the expression of *RNASEN* and *EIF2C1* in T-MF is not different from control CD4+ T cells, but *RNASEN* is higher in T-MF compared to early-stage MF and CED, whereas that of *EIF2C1* is lower in T-MF compared to CLE. *EIF2C2* expression in T-MF was lower in CD4+ T cells and CED, but not different from early-stage MF, CED and normal skin. With RT-qPCR the over expression of *CXCL13, TRIP13, GSTF1* and *IL32*, in T-MF compared to freshly isolated CD4+ T cells and normal skin was confirmed (Figure 2). *CXCL13*, a marker for follicular helper T cells, showed variable expression among all biopsies but is over expressed in T-MF versus early-stage MF. *TRIP13*, a gene encoding a key protein for chromosome development, is highly up-regulated in T-MF versus control biopsies, whereas *IL32*, a gene belonging to the immune cluster, and the gene encoding the gametocyte specific protein, *GTSF1* (gametocyte specific factor 1), are nearly exclusively expressed in T-MF patients.

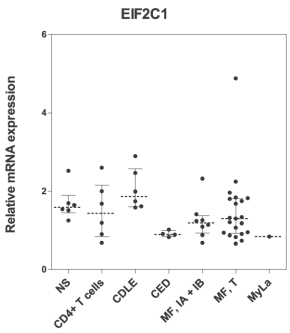
Figure 2 Relative mRNA expression levels for a selection of genes in normal skin (NS), CD4+ T cells, cutaneous discoid lupus erythematosus (CDLE), chronic eczematous dermatitis (CED), early-stage MF (MF, IA/B) MF tumor (MF, T) samples and the cell line MyLa. The mRNA expression levels were measured by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

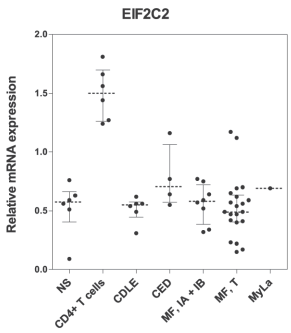


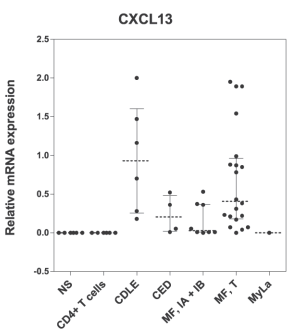


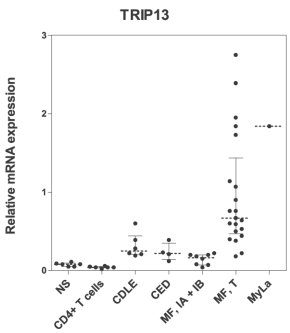


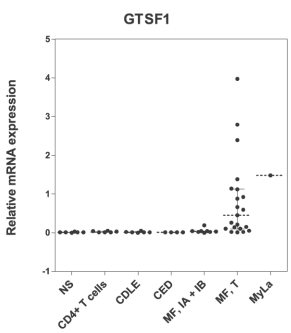


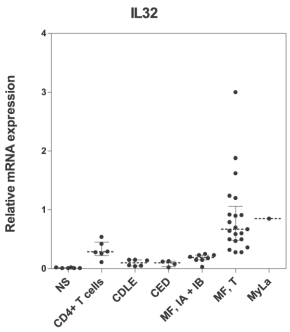












Summary of statistical evaluation

MF-T vs	MF IA/B	CED	CDLE	CD4+ T cells	Normal Skin
Genes					
ATXN7	UP **	UP ***	NC	DOWN ***	DOWN **
ZBTB20	DOWN ***	NC	DOWN ***	DOWN ***	DOWN ***
NFKBIZ	DOWN *	DOWN **	DOWN *	DOWN ***	DOWN **
RNASEN	UP *	UP ***	NC	NC	NC
EIF2C1	NC	UP *	DOWN *	NC	NC
EIF2C2	NC	DOWN *	NC	DOWN ***	NC
CXCL13	UP *	NC	NC	NA	NA
TRIP13	UP ***	UP ***	UP **	UP ***	UP ***
GTSF1	UP **	NA	UP ***	UP **	UP ***
IL32	UP ***	UP **	UP ***	UP **	UP ***

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

A

Down in MF		Log^2 Fold change		
HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cells
TAOK1	57551	-5,30	**-5,20**	-4,26
PSAPL1	768239	-4,56	**-4,77**	-1,00
NR1D2	9975	-5,31	**-4,55**	-5,46
ZBTB20	26137	-4,72	**-4,25**	-3,66
ATXN7	6314	-3,24	**-4,13**	-5,45
UGCG	7357	-3,70	**-3,82**	-3,28
EPHA4	2043	-3,68	**-3,68**	-2,66
KIAA0754	643314	-2,99	**-3,65**	-3,02
SLC16A7	9194	-3,26	**-3,61**	-3,07
MAST4	375449	-2,98	**-3,59**	-1,83
ABCA5	23461	-4,05	**-3,50**	-3,06
ZBTB16	7704	-3,64	**-3,47**	-1,95
NKTR	4820	-3,26	**-3,46**	-3,86
NFKBIZ	64332	-2,75	**-3,42**	-4,07
RPS27P19	100129905	-2,62	**-3,39**	-5,51
DICER1	23405	-3,42	**-3,36**	-3,25
ZBTB43	23099	-2,77	**-3,26**	-2,70
MALAT1	378938	-2,48	**-3,26**	-2,75
IL6ST	3572	-3,18	**-3,22**	-2,64
ATP7A	538	-3,81	**-3,17**	-2,63
EIF2C2	27161	-2,83	**-3,16**	-3,82
NSUN6	221078	-2,83	**-3,12**	-3,53
PLEKHA1	59338	-3,37	**-3,11**	-2,10
EIF2C3	192669	-2,78	**-3,03**	-2,74
TCF7L2	6934	-3,93	**-2,94**	-1,35
GSK3B	2932	-2,36	**-2,92**	-1,61
DKFZP586I1420	222161	-2,57	**-2,92**	-2,71
PIK3R1	5295	-3,27	**-2,91**	-3,46
C2orf40	84417	-3,79	**-2,91**	-1,34
LOC654340	654340	-2,70	**-2,89**	-2,89

Up in MF		Log^2 Fold change		
HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cells
ADAMDEC1	27299	6,93	**7,20**	6,72
IL32	9235	7,97	**7,12**	3,20
GTSF1	121355	6,64	**6,65**	4,98
CXCL13	10563	6,44	**6,50**	4,58
RRM2	6241	6,92	**6,21**	4,95
PTPRCAP	5790	6,86	**6,04**	1,09
CXCL9	4283	5,83	**5,81**	8,22
SPP1	6696	5,81	**5,69**	6,22
PTPN7	5778	6,61	**5,43**	1,15
DLGAP5	9787	4,72	**5,30**	4,95
UBD	10537	5,77	**5,22**	7,99
CKS2	1164	4,08	**5,15**	3,33
CCL18	6362	7,36	**5,04**	8,57
MMP1	4312	5,79	**4,81**	5,74
CEP55	55165	4,73	**4,77**	4,52
TMEM163	81615	4,68	**4,68**	4,17
UBE2C	11065	4,96	**4,66**	5,79
IDO1	3620	4,17	**4,57**	3,99
HMMR	3161	4,17	**4,55**	4,27
MAD2L1	4085	4,21	**4,54**	2,53
PLA2G2D	26279	4,54	**4,54**	4,50
IQCG	84223	4,74	**4,45**	4,44
AURKB	9212	4,49	**4,41**	4,36
TRIP13	9319	4,84	**4,39**	4,75
ASF1B	55723	4,58	**4,38**	4,16
CDC20	991	4,56	**4,38**	5,20
CCNA2	890	4,50	**4,37**	4,34
CXCL10	3627	4,95	**4,31**	6,79
CENPA	1058	3,97	**4,30**	4,22
APOBEC3B	9582	3,87	**4,27**	3,59

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

B

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

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

Discussion

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

involved in miRNA processing has been observed in T-cell lymphoma including Sézary syndrome,[29] we also determined expression of Argonaute 1, 3 and 4 (encoded by *EIF2C1, EIF2C3* and *EIF2C4*), *DICER* and *Drosha* (*RNASEN*) using RT-qPCR. We could only demonstrate up-regulation of *RNASEN* in MF tumor stage compared to early-stage MF and down-regulation of *EIF2C1* in T-MF in comparison with CDLE. When comparing the T-MF "signature" with signatures for CTCL[11,37] we identified 9 genes in the so-called "cluster 1" that are shared with our study: *IL26, PTPN7, TNFSF14, TNFSF4, CCR8, FUT7, CXCL13, LILRB4* and *ST8SIA4* (all up-regulated in cluster 1). Comparison of the T-MF DE genes with the Cancer Gene census database[27] revealed differential expression of 32 *bona fide* cancer genes in T-MF.

Immunophenotype

Our results show up-regulation of both interferon responsive genes (e.g. STAT1[38]) and several interleukin/chemokines genes previously demonstrated to be up-regulated in MF tumor cells (*IL10*[39], *IL15*[40]) or surrounding cells (*CXCL9, 10* in keratinocytes[41]; *CCL18* in macrophages[42]). Our analysis could not confirm elevated expression of *CCL17*.[43] With regard to the cytokine receptors, high expression of *IL15RA* is in line with the reported sensitivity of MF cells for *IL15*,[44] whereas expression of *IL13RA2* is not yet described for (cutaneous) lymphoma. We noticed increased expression of the chemokine receptors *CCR1*, *CCR8* and the skin-homing receptor *CCR10*. A role for CCR8 in localization of cutaneous memory T cells to the skin was proposed earlier[45] though no data on CCR8 (protein) expression in MF are not available yet. In contrast to FACS-based data of Campbell *et al.*,[46] and Clark *et al.*,[47] our gene expression data do not show up-regulation of CCR4 in T-MF. This might be explained by a high variable expression in either group (T-MF or controls) and consequently do not reach statistical significance. The gene expression data provide some evidence for the suggestion that MF is derived from Th17 cells[48]: increased IL-26 mRNA levels though increased expression of IL-17 is not detected, whereas genesis from Th22 (no increase in IL-22), Treg (no FoxP3 over expression) or T follicular helper cells (no up-regulation of ICOS, or PD1) is unlikely. Instead, we observed a large degree of heterogeneity in expression of another putative T-follicular helper marker, *CXCL13*, as well as increased expression of programmed cell death 1 ligand 2 (*PD1L2*).

We did find high and consistent expression of IL-32 mRNA in all patients which was confirmed by RT-qPCR data showing expression of IL-32 in T-MF and MyLa, but not in early-stage MF, benign controls and normal skin. Although these findings suggest that MF might originate from "Th32" cells, it remains to be proven that the tumor T cell is the source of IL-32 in particular since recent studies described IL-32 production by fibroblasts (in rheumatoid synovium,[49] keratinocytes[50] and mast cells.[51]

Therapeutic targets and diagnostic markers

Our data are in full agreement with the previously described aberrant expression of B-Lymphoid kinase (*BLK*) in MF, which enhances proliferation induced by constitutive activation of NF-κB[52] and the described over expression of Pololike kinase 1 (*PLK1*).[53] Here we also demonstrate that *NFKBIZ*, a gene encoding a NF-κB signaling inhibitor, is down-regulated in MF (supported by RT-qPCR data) which might be an explanation for enhanced NF-κB activity, a hallmark of MF.[54] In this respect, targeting of RMM2 (among the DE genes up-regulated in T-MF), which induces NF-κB -dependent MMP9 activation (up-regulated in T-MF and in agreement with published protein data[55]) and thereby enhances cellular invasiveness[56] warrants further studies; several potent inhibitors for RRM2 protein were recently described which leads to growth suppression of tumors.[57] *CD74* over expression, could be targeted by milatuzumab, a humanized antibody currently tested on lymphoma and multiple myeloma patients in phase I trials.[58] Neither *CD52* nor *NOTCH-1* or-*3* over expression could be reproduced in our analysis. *CD52* is a target of alemtuzumab (also known as Campath), and although previously described as being up-regulated on the mRNA level in CTCL[59] its use in the treatment of mycosis fungoides and Sézary syndrome is with varying results.[60] NOTCH-1 is over expressed on the protein level in advanced MF,[61] but data on mRNA expression are solely obtained from CTCL cell lines. Over expression of *TOP2A*, also identified in nodal peripheral T-cell lymphoma,[62] might serve as a target of anthracyclines, such as doxorubicin and etoposide. We observed that indoleamine 2,3-dioxygenase 2, *IDO2*, playing a role in immunomodulation and tumor escape is over expressed in T- MF. A recent study demonstrated that the small molecule inhibitor INCB024360 is able to inhibit IDO2 protein and can act as an effective immunotherapeutic agent.[63]

Finally, we identified a limited number of genes for which expression appears to be restricted to MF tumor stage and which might also serve as diagnostic (bio)markers. Amongst these is *GTSF1* (gametocyte specific factor 1). Expression thus far is only described for gametocytes[64] and according to our mRNA expression analysis (RT-qPCR) is limited to MF tumor samples. As male *GTSF1* knockout mice are sterile owing to massive apoptotic death of their germ cells, aberrant (over)expression of *GTSF1* might play a role in apoptosis resistance in MF.

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

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

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