A Meta-Analysis of Gene Expression Data Identifies a Molecular Signature Characteristic for Tumor-Stage Mycosis Fungoides

Marloes S. van Kester[1], Martin K. Borg[1], Willem H. Zoutman[1], Jacoba J. Out-Luiting[1], Patty M. Jansen[2], Enno J. Dreef[2], Maarten H. Vermeer[1], Remco van Doorn[1], Rein Willemze[1] and Cornelis P. Tensen[1]

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 publicly available gene expression data sets 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 of which (718 genes) are statistically significantly more expressed in MF compared with normal skin, inflamed skin, and normal T cells. As expected, the signature contains genes reflecting the highly proliferative characteristic 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 partly explain the enhanced activity of NF-κB, which is a hallmark of MF and other CTCLs.

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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% (van Doorn *et al.*, 2000; Kim *et al.*, 2003). Although for MF numerous genetic and genomic studies are described, ranging from investigating individual gene (mutation)s (Dereure *et al.*, 2002; Scarisbrick *et al.*, 2002) to genome-wide (array-based) analyses (Karenko *et al.*, 2007; van Doorn *et al.*, 2009; Laharanne *et al.*, 2010; Salgado *et al.*, 2010), 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 (MF-TS) 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 (Dijkman *et al.*, 2007; van Kester *et al.*, 2010), different stages of the disease (Shin *et al.*, 2007), or analyzing copy number effect on mRNA expression (van Doorn *et al.*, 2009). 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 (Tracey *et al.*, 2003).

In this study, we performed a meta-analysis on raw gene expression data available in public repositories, selecting high-quality data sets from normal T-cell (subset)s, skin, inflamed skin, and MF-Ts, generated with commercially available Gene chips. Subsequently, these data sets were corrected for inaccurate gene annotations (Dai *et al.*, 2005). 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 (subset)s and normal skin, as well as inflamed skin from experimentally induced allergic contact dermatitis simultaneously. Finally, we confirmed altered expression of selected

[1]*Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands and* [2]*Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands*

Correspondence: Cornelis P. Tensen, Department of Dermatology, Leiden University Medical Center, Room T2-03, Einthovenweg 20, 2333 ZC Leiden, The Netherlands. E-mail: c.p.tensen@lumc.nl

Abbreviations: CED, chronic eczematous dermatitis; CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; MF-T, MF tumor biopsies; miRNA, microRNA; qPCR, quantitative PCR

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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 into the pathogenesis of this lymphoma, a description of its (immuno) phenotype, and the discovery of previously unreported putative diagnostic markers and therapeutic targets.

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 differentially expressed (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 (e.g., 22 MF-Ts; van Doorn *et al.*, 2009) were subjected to the Affymetrix QC metrics and individual samples passing the control (Table 1 and Supplementary Table S1 online) were included for further analyses. Next, we compared gene expression profiles of MF-Ts 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, whereas comparison between MF-T biopsies 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 MF-T (''unique genes''), we decided to perform a comparison between the lists of DE genes, identified by the pairwise comparisons, using an ''AND'' operator (Watkins *et al.*, 2009) and to look for genes that were consistently upregulated or downregulated. To that end, the MF-T 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 downregulated and 718 were overexpressed in MF-Ts. The top lists are given in Table 2a and b, whereas a full searchable table is available in the Supplementary Information Table S2 online. This list was used for the detailed analysis described below. To further validate our approach, we also carried out a similar comparison for MF-T, T cells, and the data set from Yao *et al.* (2008), containing normal skin, uninvolved skin, and lesional skin from psoriasis patients. This analysis showed that the majority of genes (but not all) found to be characteristic for MF-T are also consistently and DE compared with these data sets (596 up and 195 down; results are provided in Supplementary Information Table S3 online).

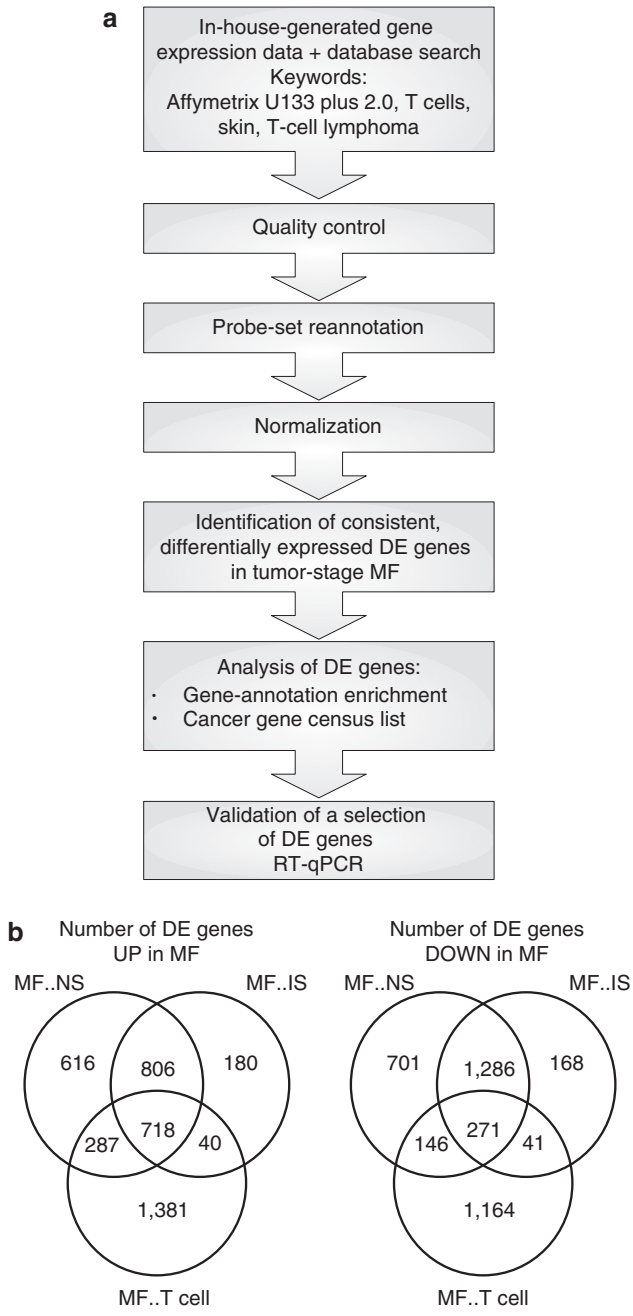


Figure 1. Strategy and results of the *in silico* identification of the molecular signature of mycosis fungoides (MF) tumor stage. (**a**) Workflow (1) data sets were obtained by searching for Affymetrix U133 versus 2 Gene chip (GPL570). (2) The quality of the data sets was checked using a series of QC metrics. (3) Gene expression data were reannotated according to the Entrez genome annotation using CDF files. (4) GC-RMA (robust multiarray averaging) normalization was applied. (5) Differentially expressed (DE) genes were identified with LIMMA using a log 2 fold change $\geqslant 1$ as a threshold, an adjusted *P*-value of <0.01, and the ''AND'' operator to identify consistently upregulated or downregulated genes. (6) Analysis of DE genes. (7) Quantitative PCR (qPCR) was used to validate differential expression in additional samples. (**b**) Venn diagram illustrating the number of genes that show altered expression in MF tumor stage compared with normal skin (NS), inflamed skin (IS), and T cells. Values indicate the number of genes significantly (left) upregulated or (right) downregulated. The intersecting regions represent number of genes that are common to the specific comparisons.

Table 1. Samples included in the comparative analyses

Group	Subtype	Reference	No. of samples (passing QC)
MF	MF tumor skin biopsy	van Doorn *et al.* (2009)	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.* (2007)	16
Inflamed skin	ACD skin biopsy (clinical signs)	Pedersen *et al.* (2007)	9
Skin	Normal skin biopsy	Yao *et al.* (2008)	17
Psoriasis	Uninvolved skin psoriasis biopsy	Yao *et al.* (2008)	27
Psoriasis	Lesional skin psoriasis biopsy	Yao *et al.* (2008)	31

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

Systematic and integrative analysis of DE genes

Gene-annotation enrichment analysis. Next, Internet-based gene-annotation category enrichment analysis programs (PPI spider, DAVID, Panther, Webgestalt; Thomas *et al.*, 2003; Zhang *et al.*, 2005; Antonov *et al.*, 2009; Huang *et al.*, 2009a, b) were applied to gain further insight into the (clinical) relevance of the MF DE genes and all gave similar results. For example, using PPI spider classification (Supplementary Table S4a–c online), we observed that genes associated with ''mitosis'', ''cell division'', ''cell cycle'', ''spindle'', and ''spindle organization'' are over-represented 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 online revealed over-expression of several IFN-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 downregulation of *IL11RA*. Functional annotation analysis of downregulated genes identified a single category: RNA processing (Supplementary Table S4c online).

Comparison of molecular signatures. Comparison of the ''MF-T signature'' with the most recent Cancer Gene census list (updated 15 November 2011; first described by Futreal *et al.* (2004)) identified 32 *bona fide* cancer genes (see Supplementary Table S5 online). We also determined whether differential expression of genes could be related to known genomic alterations. However, none of the ''top 30'' DE genes (Table 2a and b) resides within previously described minimal common regions of genetic imbalances (van Doorn *et al.*, 2009).

Promoter hypermethylation

We investigated the promoter sites of downregulated 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 downregulated 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 (Worm *et al.*, 2001; Senff *et al.*, 2009). 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-curve analyses of PCR products (see Supplementary Figure S1 online) could methylation be demonstrated, although all contained PCR products representing unmethylated DNA. We therefore concluded that promoter hypermethylation does not have a role in the downregulation of these genes in MF tumor stage.

Verification of MF DE genes using qPCR

Differential expression for a selection of genes (as 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 online). We included mRNA isolated from skin biopsies of patients with chronic discoid lupus erythematosus, early-stage MF (IA/B), chronic eczematous dermatitis (CED), 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

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)

Down in MF		Log 2 fold change			Up in MF		Log 2 fold change		
HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cell	HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cell
(a)									
TAOK1	57551	−5.30	**−5.20**	−4.26	*ADAMDEC1*	27299	6.93	**7.20**	6.72
PSAPL1	768239	−4.56	**−4.77**	−1.00	*IL32*	9235	7.97	**7.12**	3.20
NR1D2	9975	−5.31	**−4.55**	−5.46	*GTSF1*	121355	6.64	**6.65**	4.98
ZBTB20	26137	−4.72	**−4.25**	−3.66	*CXCL13*	10563	6.44	**6.50**	4.58
ATXN7	6314	−3.24	**−4.13**	−5.45	*RRM2*	6241	6.92	**6.21**	4.95
UGCG	7357	−3.70	**−3.82**	−3.28	*PTPRCAP*	5790	6.86	**6.04**	1.09
EPHA4	2043	−3.68	**−3.68**	−2.66	*CXCL9*	4283	5.83	**5.81**	8.22
KIAA0754	643314	−2.99	**−3.65**	−3.02	*SPP1*	6696	5.81	**5.69**	6.22
SLC16A7	9194	−3.26	**−3.61**	−3.07	*PTPN7*	5778	6.61	**5.43**	1.15
MAST4	375449	−2.98	**−3.59**	−1.83	*DLGAP5*	9787	4.72	**5.30**	4.95
ABCA5	23461	−4.05	**−3.50**	−3.06	*UBD*	10537	5.77	**5.22**	7.99
ZBTB16	7704	−3.64	**−3.47**	−1.95	*CKS2*	1164	4.08	**5.15**	3.33
NKTR	4820	−3.26	**−3.46**	−3.86	*CCL18*	6362	7.36	**5.04**	8.57
NFKBIZ	64332	−2.75	**−3.42**	−4.07	*MMP1*	4312	5.79	**4.81**	5.74
RPS27P19	100129905	−2.62	**−3.39**	−5.51	*CEP55*	55165	4.73	**4.77**	4.52
DICER1	23405	−3.42	**−3.36**	−3.25	*TMEM163*	81615	4.68	**4.68**	4.17
ZBTB43	23099	−2.77	**−3.26**	−2.70	*UBE2C*	11065	4.96	**4.66**	5.79
MALAT1	378938	−2.48	**−3.26**	−2.75	*IDO1*	3620	4.17	**4.57**	3.99
IL6ST	3572	−3.18	**−3.22**	−2.64	*HMMR*	3161	4.17	**4.55**	4.27
ATP7A	538	−3.81	**−3.17**	−2.63	*MAD2L1*	4085	4.21	**4.54**	2.53
EIF2C2	27161	−2.83	**−3.16**	−3.82	*PLA2G2D*	26279	4.54	**4.54**	4.50
NSUN6	221078	−2.83	**−3.12**	−3.53	*IQCG*	84223	4.74	**4.45**	4.44
PLEKHA1	59338	−3.37	**−3.11**	−2.10	*AURKB*	9212	4.49	**4.41**	4.36
EIF2C3	192669	−2.78	**−3.03**	−2.74	*TRIP13*	9319	4.84	**4.39**	4.75
TCF7L2	6934	−3.93	**−2.94**	−1.35	*ASF1B*	55723	4.58	**4.38**	4.16
GSK3B	2932	−2.36	**−2.92**	−1.61	*CDC20*	991	4.56	**4.38**	5.20
DKFZP586I1420	222161	−2.57	**−2.92**	−2.71	*CCNA2*	890	4.50	**4.37**	4.34
PIK3R1	5295	−3.27	**−2.91**	−3.46	*CXCL10*	3627	4.95	**4.31**	6.79
C2orf40	84417	−3.79	**−2.91**	−1.34	*CENPA*	1058	3.97	**4.30**	4.22
LOC654340	654340	−2.70	**−2.89**	−2.89	*APOBEC3B*	9582	3.87	**4.27**	3.59
(b)									
FGFBP2	83888	−3.62	−2.45	**−5.93**	*CCL18*	6362	7.36	5.04	**8.57**
RPS27P19	100129905	−2.62	−3.39	**−5.51**	*KRT6C*	286887	3.92	4.19	**8.46**
NR1D2	9975	−5.31	−4.55	**−5.46**	*IFI27*	3429	1.66	1.19	**8.28**
ATXN7	6314	−3.24	−4.13	**−5.45**	*CXCL9*	4283	5.83	5.81	**8.22**
TAOK1	57551	−5.30	−5.20	**−4.26**	*UBD*	10537	5.77	5.22	**7.99**
NFKBIZ	64332	−2.75	−3.42	**−4.07**	*C1QC*	714	2.70	2.61	**7.59**
GPRASP1	9737	−2.87	−1.76	**−4.06**	*C1QA*	712	2.54	2.91	**7.54**
LOC100132279	100132279	−1.61	−1.50	**−4.00**	*C1QB*	713	3.15	3.41	**7.17**

Table 2 continued on the following page

Table 2. Continued

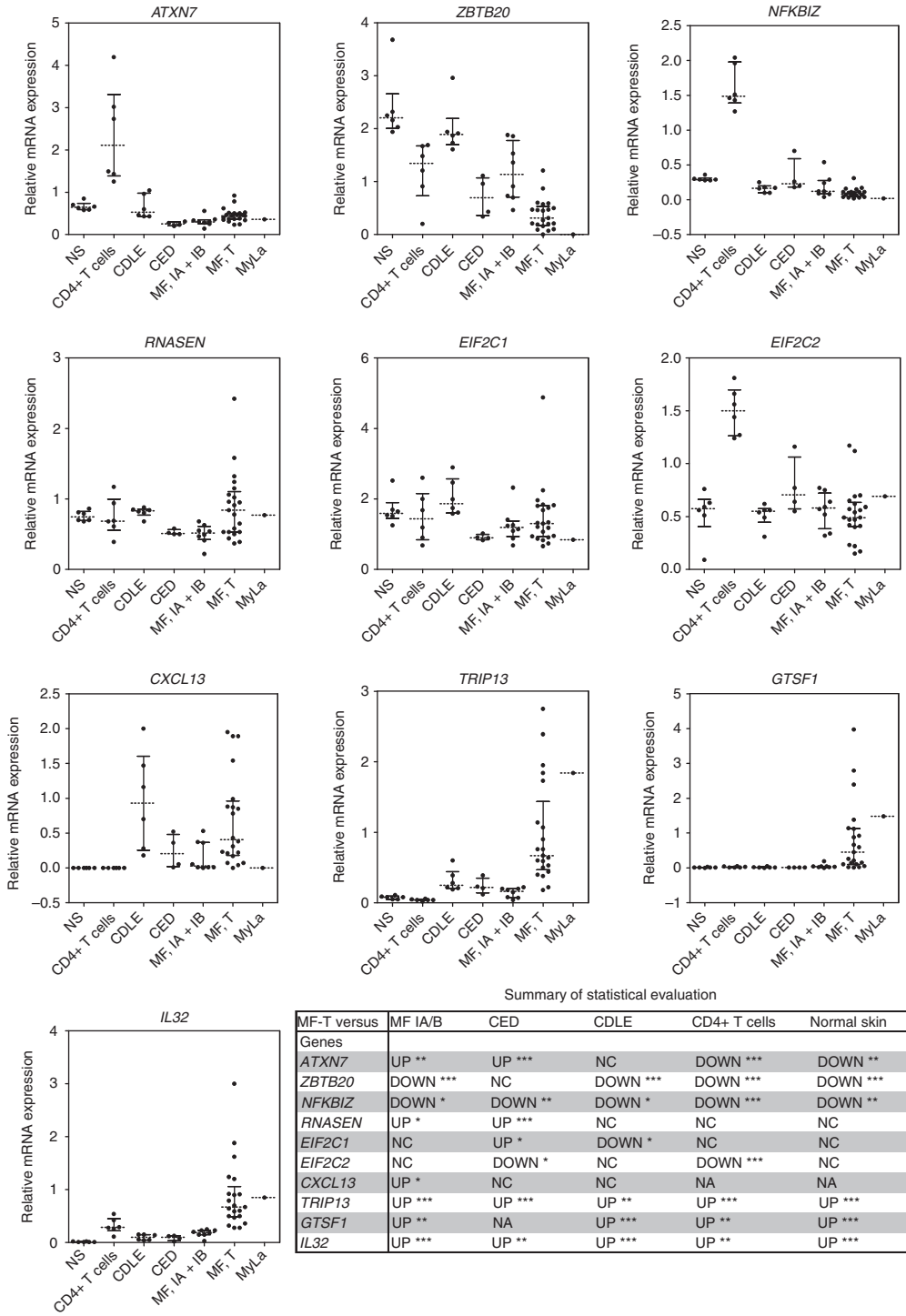
Down in MF		Log 2 fold change			Up in MF		Log 2 fold change		
HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cell	HGNC symbol	Entrez ID	MF versus NS	MF versus IS	MF versus T cell
NKTR	4820	−3.26	−3.46	**−3.86**	*CXCL10*	3627	4.95	4.31	**6.79**
EIF2C2	27161	−2.83	−3.16	**−3.82**	*ADAMDEC1*	27299	6.93	7.20	**6.72**
ZNF331	55422	−1.38	−1.36	**−3.82**	*APOE*	348	1.01	1.00	**6.64**
UHMK1	127933	−2.32	−2.42	**−3.76**	*VCAM1*	7412	2.88	2.40	**6.23**
ZBTB20	26137	−4.72	−4.25	**−3.66**	*SPP1*	6696	5.81	5.69	**6.22**
RASA2	5922	−1.28	−1.50	**−3.61**	*UBE2C*	11065	4.96	4.66	**5.79**
BEX2	84707	−1.70	−1.07	**−3.57**	*TMEM176B*	28959	2.62	2.94	**5.77**
NSUN6	221078	−2.83	−3.12	**−3.53**	*MMP1*	4312	5.79	4.81	**5.74**
ZNF44	51710	−1.78	−1.99	**−3.52**	*CTSZ*	1522	4.59	4.18	**5.22**
PIK3R1	5295	−3.27	−2.91	**−3.46**	*CDC20*	991	4.56	4.38	**5.20**
NLRP1	22861	−1.19	−1.53	**−3.45**	*MMP9*	4318	5.29	3.74	**5.11**
C2orf82	389084	−1.82	−1.32	**−3.44**	*GTSF1*	121355	6.64	6.65	**4.98**
ADRB2	154	−2.84	−2.50	**−3.36**	*CDC2*	983	3.88	4.08	**4.95**
NR3C2	4306	−3.65	−2.61	**−3.35**	*DLGAP5*	9787	4.72	5.30	**4.95**
UGCG	7357	−3.70	−3.82	**−3.28**	*RRM2*	6241	6.92	6.21	**4.95**
FLJ10213	55096	−1.80	−2.37	**−3.27**	*TOP2A*	7153	3.61	3.94	**4.84**
DICER1	23405	−3.42	−3.36	**−3.25**	*TYMP*	1890	4.84	3.01	**4.79**
C5orf41	153222	−2.24	−1.37	**−3.22**	*TRIP13*	9319	4.84	4.39	**4.75**
LOC731484	731484	−1.44	−1.21	**−3.20**	*MS4A4A*	51338	1.60	2.37	**4.67**
ZCCHC2	54877	−1.96	−2.23	**−3.13**	*BUB1B*	701	3.68	3.96	**4.61**
LCOR	84458	−1.97	−1.91	**−3.11**	*TYMS*	7298	4.48	3.98	**4.61**
SLC16A7	9194	−3.26	−3.61	**−3.07**	*SLAMF8*	56833	3.78	3.12	**4.60**

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

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 microRNA (miRNA) biogenesis/machinery, and aberrant expression of proteins involved in miRNA processing has been observed in T-cell lymphoma including Sézary syndrome (Lawrie *et al.*, 2009). We therefore also included *RNASEN*, *DICER*, *EIF2C1*, *EIF2C2*, *EIF2C3*, and *EIF2C4* in the RT–qPCR validation. GeNorm analysis (Vandesompele *et al.*, 2002) revealed that *EIF2C4* was stably expressed in all samples, and therefore in addition to *ARF5*, *TMEM87a* and *ERCC3* (see Materials and Methods) was 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 MF-T in comparison to $CD4^+$ T-cells and normal skin could be affirmed. *ZBTB20* was also significantly less expressed in MF-T compared with CLE and early-stage MF, whereas *NFKBIZ* is lower in MF-T compared with all controls. Expression of *ATXN7*, however, was higher in MF-T versus CED and early-stage MF.

We observed that the expression of *RNASEN* and *EIF2C1* in MF-T is not different from control CD4+ T cells; however, the expression of *RNASEN* is higher in MF-T compared with early-stage MF and CED, whereas that of *EIF2C1* is lower in MF-T compared with CLE. *EIF2C2* expression in MF-T was lower in CD4+ T cells and CED, but not different from early-stage MF, CED, and normal skin. With RT–qPCR, the overexpression of *CXCL13*, *TRIP13*, *GSTF1*, and *IL32* in MF-T compared with 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 overexpressed in MF-T versus early-stage MF. *TRIP13*, a gene encoding a key protein for chromosome development, is highly up-regulated in MF-T 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 MF-T patients.



Summary of statistical evaluation

MF-T versus	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 ***

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 mycosis fungoides (MF, IA/B), MF tumor (MF, T) samples, and the cell line MyLa. The mRNA expression levels were measured by reverse transcriptase–coupled quantitative PCR (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, and *** < 0.001; complete data are provided in Supplementary Table S6 online. NC = no change; NA = Mann–Whitney not applicable as the gene is not expressed in this group, resulting in *ex aequo* values.

DISCUSSION

The purpose of this study was to distill the gene expression profile of MF tumor stage, aiming to gain more insight into the pathogenesis and the molecular basis of this disease. We performed a meta-analysis using high-quality data sets generated with commercially available Gene chips, a robust

statistical analysis, and compared/studied expression data of MF tumor stage 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 DE in MF-T, the majority of which (718) are more expressed and 271 genes are less expressed in MF tumor stage. 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 that 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 overexpression of MCM7 protein, a member of the minichromosome maintenance complex, in tumor-stage MF (Gambichler *et al.*, 2008). Our results, however, suggest that not only MCM7 but also MCM2–6, ORCL1, CDC6, and CDC7 belonging to the MCM7 complex are upregulated. These results are in line with the notion that (collective) upregulation of kinetochore and proliferation genes can lead to aberrant chromosome separation, and hence contribute to genomic instability in tumors including lymphoma (Sanchez-Aguilera *et al.*, 2006). On the basis of our results, especially the high expression of *UBE2C*, one of the key regulators of cell cycle completion and marker of grade of malignancy in lymphoma (Troncone *et al.*, 2009), might have a central role in chromosomal instability as observed in MF (van Doorn *et al.*, 2009; Salgado *et al.*, 2010). In analogy, the overexpression of *TRIP13* in MF-T (confirmed by RT–qPCR) is of interest. *TRIP13* has a prominent role in chromosome recombination and chromosome structure development, and mRNA overexpression was recently correlated with prostate cancer progression (Larkin *et al.*, 2011).

We noticed that a large proportion of downregulated genes in tumor MF contained CpG islands in their promoter region. As DNA methylation of tumor suppressor genes has been found in MF (van Doorn *et al.*, 2005), we screened several of these genes for DNA promoter hypermethylation. For none of the genes tested, however, could promoter DNA hypermethylation be confirmed, indicating that other mechanisms are responsible for downregulation of these genes (e.g. aberrant expressed transcription factors or miRNA-induced mRNA degradation; Croce, 2009). Among the downregulated genes, in comparison with healthy CD4+ T cells and benign dermatoses, we also detected (and confirmed by PCR) decreased expression of Argonaute 2 (*EIF2C2*), a protein belonging to the RNA-induced silencing complex and an essential component of the miRNA machinery (Esquela-Kerscher and Slack, 2006). As aberrant expression of other proteins involved in miRNA processing has been observed in T-cell lymphoma, including Sézary syndrome (Lawrie *et al.*, 2009), we also determined the expression of Argonaute 1, 3, and 4 (encoded by *EIF2C1*, *EIF2C3*, and *EIF2C4*), *DICER*, and Drosha (*RNASEN*) using RT–qPCR. We could only demonstrate upregulation of *RNASEN* in MF tumor stage compared with early-stage MF and downregulation of *EIF2C1* in MF-T in comparison with chronic discoid lupus erythematosus. When comparing the MF tumor ''signature'' with signatures for CTCL (Shin *et al.*, 2007; Litvinov *et al.*, 2010), we identified nine genes in the so-called ''cluster 1'' that are shared with our study: *IL26*, *PTPN7*, *TNFSF14*, *TNFSF4*, *CCR8*, *FUT7*, *CXCL13*, *LILRB4*, and *ST8SIA4* (all upregulated in cluster 1). Comparison of the MF tumor DE genes with the Cancer Gene census database (Futreal *et al.*, 2004) revealed differential expression of 32 *bona fide* cancer genes in tumor-stage MF.

Immunophenotype

Our results show upregulation of both IFN-responsive genes (e.g. STAT1; Fantin *et al.*, 2008) and several interleukin/chemokines genes previously demonstrated to be upregulated in MF tumor cells (*IL10* (Asadullah *et al.*, 1996), *IL15* (Asadullah *et al.*, 2000)), or surrounding cells (*CXCL9, 10* in keratinocytes (Tensen *et al.*, 1998); *CCL18* in macrophages (Gunther *et al.*, 2011)). Our analysis could not confirm elevated expression of *CCL17* (Kakinuma *et al.*, 2003). With regard to the cytokine receptors, high expression of *IL15RA* is in line with the reported sensitivity of MF cells for IL15 (Dobbeling *et al.*, 1998), 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 (Colantonio *et al.*, 2002), although no data on CCR8 (protein) expression in MF are available as yet. In contrast to FACS-based data of Campbell *et al.* (2010) and Clark *et al.* (2011), our gene expression data do not show upregulation of CCR4 in MF-T. 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 (Ciree *et al.*, 2004): increased IL-26 mRNA levels even though increased expression of IL-17 is not detected, whereas genesis from Th22 (no increase in IL-22), Treg (no FoxP3 overexpression), or T follicular helper cells (no upregulation of ICOS or programmed cell death 1) is unlikely. Instead, we observed a large degree of heterogeneity in the expression of another putative T-follicular helper marker, *CXCL13*, as well as increased expression of programmed cell death 1 ligand 2.

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 MF-T 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, especially as recent studies described IL-32 production by fibroblasts (in rheumatoid synovium; Alsaleh *et al.*, 2010), keratinocytes (Meyer *et al.*, 2010), and mast cells (Kempuraj *et al.*, 2010).

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 (Krejsgaard *et al.*, 2009) and the described overexpression of Polo-like kinase 1 (*PLK1*; Nihal *et al.*, 2011). Here we also demonstrate that *NFKBIZ*, a gene encoding an NF-κB signaling inhibitor, is downregulated in MF (supported by RT–qPCR data), which might be an explanation for enhanced NF-κB activity, a hallmark of MF (Izban *et al.*, 2000). In this respect, targeting of RMM2 (among the DE genes upregulated in MF-T), which induces NF-κB-dependent MMP9 activation (upregulated in MF-T and in agreement with published protein data; Rasheed *et al.*, 2010) and thereby enhances cellular invasiveness (Duxbury and Whang, 2007), warrants further studies; several potent inhibitors for RRM2 protein were recently described that cause growth suppression of tumors (Davis *et al.*, 2010). *CD74* overexpression could be targeted by milatuzumab, a humanized antibody currently tested on lymphoma and multiple myeloma patients in phase I trials (Mark *et al.*, 2009). Neither *CD52* nor *NOTCH-1* or *-3* overexpression could be reproduced in our analysis. *CD52* is a target of alemtuzumab (also known as Campath), and although previously described as being upregulated at the mRNA level in CTCL (Hahtola *et al.*, 2006) its use in the treatment of MF and Sézary syndrome is with varying results (Gribben and Hallek, 2009). *NOTCH-1* is overexpressed at the protein level in advanced MF (Kamstrup *et al.*, 2010), but data on mRNA expression are solely obtained from CTCL cell lines. Overexpression of *TOP2A*, also identified in nodal peripheral T-cell lymphoma (Cuadros *et al.*, 2007), 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 overexpressed in MF tumor stage. A recent study demonstrated that the small-molecule inhibitor INCB024360 is able to inhibit *IDO2* protein and can act as an effective immunotherapeutic agent (Liu *et al.*, 2010).

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. Among these is *GTSF1*. Expression thus far is only described for gametocytes (Yoshimura *et al.*, 2007), 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 have a role in apoptosis resistance in MF.

In summary, we determined a molecular signature characteristic for MF tumor stage, offering more insight into the pathogenesis of this disease. Moreover, we uncovered more details of its immunophenotype: overexpression of IFN-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 downregulation of *IL11RA*. Finally, our data suggest previously unreported therapeutic targets and/or diagnostic markers: *IDO2*, *RRM2*, *CD74*, *TOP2A*, *GTSF1*, *TRIP13*, and *NFKBIZ*, which warrant further research.

MATERIALS AND 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 (http://www.ncbi.nlm.nih.gov/geo/) and ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) databases were screened for data sets, generated with Affymetrix U133 versus 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 MF-Ts generated previously (van Doorn *et al.*, 2009). In addition, gene expression data of two additional MF-Ts 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 data sets was checked using a series of QC metrics recommended by Affymetrix (using the R-script described by Heber and Sick (2006)) in order to confirm that arrays were hybridized correctly, that sample quality was acceptable, and that batches of data sets could reliably 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 (Dai *et al.*, 2005), followed by GC-RMA (robust multiarray averaging) normalization. Samples passing QC, which were included in the final normalization, are summarized in Supplementary Table S1 online. 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 (Pedersen *et al.*, 2007; Yao *et al.*, 2008) (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). DE genes between groups and MF tumor stage were identified with LIMMA (Smyth *et al.*, 2005) using a $\log 2$ fold change $\geqslant 1$ 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 (Watkins *et al.*, 2009) was used to perform a comparison between the lists of DE genes from the individual comparisons to identify consistently upregulated or downregulated transcripts in MF. This comparison resulted 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 (Thomas *et al.*, 2003; Zhang *et al.*, 2005; Antonov *et al.*, 2009; Huang *et al.*, 2009a, b).

Reverse transcription–coupled 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 lesions, 8 early-stage MF (IA/B) patients, 4 patients with CED, normal skin ($n=6$), freshly isolated $CD4^+$ T cells of 6 healthy donors as described previously (van Doorn *et al.*, 2004), and MyLa cells. cDNA was synthesized from 1 μg total RNA, after treatment with RQ1 DNase I (Promega, Madison, WI), using IScript reverse transcriptase (Bio-Rad, Veenendaal, The Netherlands),

oligo(dT)$_{12–18}$, and random hexamer priming (Bio-Rad) in a final volume of 20 µl. Quantitative real-time PCR (qPCR) was performed with the MyIQ Detection System and the SYBR Green Supermix (Bio-Rad).

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 online. The reference gene set was identified by testing several optimized primers on all samples included in the validation experiment and using GeNorm as described earlier (Vandesompele *et al.*, 2002). 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 follows: denaturing for 15 seconds at 97 °C, and annealing and extension for 20 seconds at 60 °C, for 40 cycles. The nonparametric Mann–Whitney *U*-test (one-tailed; Graphpad Prism 5; GraphPad Software, La Jolla, CA) was used for statistical evaluation of the RT–qPCR results.

Methylation-specific Melting-Curve Analysis PCR

For the bisulfite conversion by the EZ DNA methylation kit (Zymo Research, Orange, CA), 1 µg genomic DNA (isolated from MF-T skin biopsies; van Doorn *et al*, 2009) was used. Primers (Supplementary Table S5 online) 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. Methylation-specific melting-curve analysis PCR reactions were performed as described earlier (Worm *et al.*, 2001) 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 follows: denaturing at 96 °C for 30 seconds, annealing at temperatures varying from 65 to 58 °C depending on the primer set used for 40 seconds, and extension at 72 °C for 40 seconds for eight cycles, followed by denaturing for 30 seconds, annealing at 60 °C for 40 seconds, and extension at 72 °C for 40 seconds 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 to 94.8 °C at 0.2 °C s^{-1}.

Sensitivity and specificity of the methylation-specific melting-curve analysis 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.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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