Diagnostic and prognostic markers in tumor stage mycosis fungoides and Sézary syndrome

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Epigenomic analysis of Sézary syndrome defines patterns of aberrant DNA methylation and identifies diagnostic markers

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ABSTRACT

Sézary syndrome (SS) is a malignancy of skin-homing CD4+ memory T cells that is clinically characterized by erythroderma, lymphadenopathy, and blood involvement. Distinction of SS from erythroderma secondary to inflammatory skin diseases (erythrodermic inflammatory dermatoses [EID]) is often challenging. Recent studies identified recurrent mutations in epigenetic enzymes involved in DNA modification in SS. Here we defined the DNA methylomes of purified CD4+ T cells from patients with SS, EID, and healthy controls subjects. SS showed extensive global DNA methylation alterations, with 7.8% of 473,921 interrogated autosomal CpG sites showing hypomethylation and 3.2% hypermethylation. Promoter CpG islands were markedly enriched for hypermethylation. The 126 genes with recurrent promoter hypermethylation in SS included multiple candidate tumor suppressors that showed transcriptional repression, implicating aberrant methylation in the pathogenesis of SS. Validation in an independent sample set showed promoter hypermethylation of *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, and *PAM* in 94-100% of SS samples but not in EID samples. Notably, promoter hypermethylation of a single gene, the chemokine-like factor *CMTM2*, was sufficient to accurately distinguish SS from EID in all cases. This study shows that SS is characterized by widespread yet distinct DNA methylation alterations, which can be used clinically as epigenetic diagnostic markers.

INTRODUCTION

Sézary syndrome (SS) is an aggressive type of cutaneous T-cell lymphoma (CTCL) that is characterized clinically by erythroderma, generalized lymphadenopathy, and the presence of malignant T cells in the skin, lymph nodes, and peripheral blood.[1] SS is a malignant clonal proliferation of mature CD4+ skin-homing central memory T cells.[2] Malignant T cells of patients with SS commonly display a Foxp3+, CD25+ phenotype, and it has been postulated that SS represents a malignancy of regulatory T cells in a subset of patients.[3;4] Differentiation of SS from erythroderma secondary to inflammatory skin diseases (EID) can be challenging, but it is important for the therapeutic management of patients. Especially in early stages of the disease, clinical, cytological, and histopathological findings in SS are often nonspecific. Because circulating atypical lymphocytes with immunophenotypic alterations and T cell clonality can be found in EID as well as SS, there is a need for molecular markers to reliably diagnose SS. The use of combined flow cytometry markers and combined gene transcript analysis has been proposed to aid in diagnosing SS.[5-8]

The molecular pathogenesis of SS is not fully understood, and targets for directed therapeutic intervention remain to be defined. Recent exome sequencing studies in SS have described a broad spectrum of genetic alterations, including mutations in genes involved in DNA methylation, histone modification, and chromatin remodelling.[9-12] Epigenetic modifier genes with recurrent mutations in SS include *DNMT3a*, *TET1*, and *ARID1A*. These observations are in line with experimental evidence showing that epigenetic mechanisms, such as aberrant DNA methylation, have a causative role in various hematopoietic malignancies.[13;14] In SS, promoter CpG island hypermethylation of several tumor suppressor genes, including *CDKN2A* and *FAS*, has been found.[15;16] The therapeutic efficacy of histone deacetylase inhibitors provides additional evidence for a causative role of epigenetic alterations in the molecular pathogenesis of SS.[17;18]

In this study we report on distinct genome-wide DNA methylation characteristics of CD4+ T cells isolated from patients with SS as compared with those from patients with EID and healthy individuals. This analysis enabled us to define patterns of aberrant DNA methylation with potential relevance for the pathogenesis of SS and to identify epigenetic markers that can be used in the diagnosis of this T-cell malignancy.

METHODS

SELECTION OF PATIENTS

DNA isolated from peripheral blood CD4+ T cells of 15 patients with SS, three patients with EID, and four age-matched healthy volunteers was subjected to Illumina 450k (Illumina) DNA methylation analysis. Differential methylation was validated in a second patient series of 20 patients with SS, 10 patients with EID, and seven healthy volunteers. Diagnosis of SS was based on criteria defined in the World Health Organization - European Organization for Research and Treatment of Cancer (WHO-EORTC) classification.[1] Patients with SS presented with erythroderma, highly elevated CD4/CD8 ratios, and

clonality of malignant T cells in peripheral blood (**Table 1**). EID was secondary to atopic dermatitis, psoriasis, and paraneoplastic or idiopathic syndromes (**Supplementary Table S1**). CD4+ T cells were isolated by negative selection from Ficoll density centrifuged peripheral blood mononuclear cells (CD4+ T-cell Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany). DNA methylation profiling was additionally performed on the SeAx and HuT78 cell lines, derived from patients with SS. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Approval for these studies was obtained from the institutional review board of Leiden University Medical Center. Written informed consent was provided according to the Declaration of Helsinki.

GENOME-WIDE DNA METHYLATION DATA GENERATION AND ANALYSIS

The Infinium methylation assay was performed at a certified Illumina service provider (Service XS, Leiden, The Netherlands). DNA was bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Orange, CA) and hybridized onto Illumina 450k

Table 1. Clinical characteristics of the 15 patients with Sézary syndrome subjected to genome-wide DNA methylation analysis and 20 patients analyzed to validate epigenetic diagnostic biomarkers.

Characteristics at diagnosis of SS	Confirmation SS, n=15	Validation SS, n=20
Male:female ratio	6:9	16:4
Age in years, median (range)	64 (46-78)	68 (32-89)
Findings, n/total		
Erythroderma	14/15	17/20
Pruritus	13/15	17/20
Ectropion	2/14	3/20
Hyperkeratosis hands/feet	8/13	3/20
Palpable lymphadenopathy	6/15	5/20
Lymphadenopathy confirmed by CT scan	5/11	3/14
Leukocytes ≥ 10.0 x10^9/L	12/15	14/20
CD4/CD8 ratio ≥ 10.0	14/15	20/20
Absolute Sézary cell count ≥ 1000 per mm^3	5/5	13/16
T-cell clone in peripheral blood	15/15	20/20
Identical T-cell clone in blood and skin	6/6	9/9
Status at last date of follow-up, n		
Alive with disease	9	15
Died of disease	5	4
Died of other cause than linked to SS	1	0
Died of unknown cause	0	1
Duration of follow-up in months, median (range)	49 (8-120)	20 (1-33)

CT, computed tomographic; SS, Sézary syndrome

arrays (Illumina, San Diego, CA). Analyses were performed using R Statistics, version 2.15.1. Data were normalized for color bias using Lumi package. CpG sites on the sex chromosomes were excluded for analysis. After quality control, 473,921 CpG sites were used to test for differences between healthy T cells and SS T cells. Individual CpGs were tested for an association between SS and benign T-cell samples using a linear model, and *P*-values were adjusted for multiple testing using the false discovery rate. CpG sites with *P*-value adjusted for multiple testing using the false discovery rate less than 0.05 and absolute β-value difference greater than 0.2 were considered significant. Statistical testing using the Welch *t* test and multiple testing correction using Bonferroni correction (P_{BON}) for promoter CpG islands were performed using the Limma package. For promoter CpG islands, methylation was calculated as average β-values of all CpG sites located in CpG islands of proximal promoter regions. Promoters with P_{BON} less than 0.05 and absolute average β-value difference greater than 0.2 between SS and benign T cells were considered differentially methylated. CpG sites were annotated to a combined gene- and CpG island-centric annotation. For the gene-centric annotation, CpGs were classified as distal promoter (-10 kilobase pairs to -1.5 kilobase pairs), proximal promoter (-1.5 kilobase pairs to +500 base pairs), gene body (+500 base pairs to 3'), or downstream region (3' to +5 kilobase pairs).[19] The β-value denotes the ratio of fluorescence intensities between methylated and unmethylated alleles and was used to estimate the methylation level at each interrogated CpG site. For promoter CpG islands the methylation was calculated as average β-values of all CpG sites located in CpG islands of the proximal promoter region.

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COMPARATIVE ANALYSIS OF METHYLATION DATA

For comparison with Illumina 450k array data generated by The Cancer Genome Atlas Research Network (http://cancergenome.nih.gov/), cancer and control IDAT files were obtained for thyroid carcinoma (509 tumor biopsy samples, 56 normal tissue samples), stomach adenocarcinoma (427, 2), kidney renal clear cell cancer (312, 160), bladder cancer (266, 19), lung adenocarcinoma (434, 32), colon adenocarcinoma (297, 38), and melanoma (427, 2). Quality control was performed using the R package MethylAid, and data were normalized using functional normalization in the Minfi package.[20;21] Average DNA methylation and their difference per CpG site were calculated for tumor and normal tissues, with absolute β-value difference greater than 0.2 considered as differentially methylated. To allow comparison of DNA methylation data of SS with published data from T-cell subsets, obtained using similar 450k arrays (Gene Expression Omnibus accession: GSE49667), quality control and normalization were performed as for The Cancer Genome Atlas samples.[22] Raw methylation data from T-cell subsets were kindly provided by Leonard C. Harrison. Average methylation of 1,689 CpG sites, identified as discriminative between activated regulatory T cells and activated naïve T cells, was calculated for SS and external T-cell samples.

METHYLATION-SPECIFIC MELTING CURVE ANALYSIS

Bisulfite primers were designed to amplify CpG islands located in the promoter region of the genes of interest, encompassing the corresponding significant probe sequences on the Illumina 450k array (**Supplementary Materials** online). Amplification was

performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands) using a touchdown PCR protocol followed by melting curve analysis.[23] A promoter was considered methylated if melting temperatures exceeded half of the maximum temperature difference as determined for the reference control samples.

5-AZA-2'-DEOXYCYTIDINE TREATMENT

The SeAx cell line was treated with 0.5µmol/L 5-aza-2'-deoxycytidine (Sigma, St. Louis, MO) for 3 weeks, during which multiple cell divisions occurred. Culture medium containing 5-aza-2'-deoxycytidine was replaced every 3-4 days. Cells were harvested for RNA and DNA extraction.

QUANTITATIVE REAL-TIME PCR

Expression of differentially methylated genes was examined in 32 SS and nine EID patients and the SeAx cell line using quantitative real-time PCR. RNA was isolated using the RNeasy Mini Kit (Qiagen). Complementary DNA synthesis was performed by using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed with SYBR Green Supermix (Bio-Rad). Data were normalized according the ΔΔCq method and presented as relative messenger RNA expression.[24] Primer sequences are given in **Supplementary Materials** online.

RESULTS

DEFINING THE SÉZARY SYNDROME METHYLOME

DNA samples from CD4+ T cells of 15 patients with SS, three patients with EID, and four healthy control subjects were subjected to genome-wide DNA methylation analysis using the Illumina 450k array that interrogates cytosine positions in 99% of annotated genes (**Table 1** and **Supplementary Table S1**). Multidimensional scaling analysis showed substantial differences in global DNA methylation patterns between samples from SS patients and samples from EID patients and healthy volunteers (**Figure 1a**). Hypomethylation was more common in the SS samples (37,139 CpG sites; 7.8%; β-value difference > 0.2; *P*-value adjusted for multiple testing using the false discovery rate ≤ 0.05) than hypermethylation (15,056 CpG sites, 3.2%). The methylation patterns of the SS cell lines SeAx and HuT78 differed substantially from those of our SS patient samples. In patients with lower CD4+ T-cell counts, indicative of peripheral blood tumor burden, the frequency of aberrant methylation events was not significantly higher (data not shown). All individual differentially methylated CpG sites are listed in **Supplementary Materials** online. The location of differentially methylated CpG sites across all human autosomes showed an even distribution, with no preferential hyper- or hypomethylation at specific chromosomes (**Figure 1b** and **Supplementary Figure S1**). Comparison of DNA methylation data of SS with that of solid tumor types generated by The Cancer Genome Atlas Research Network using the same array platform showed that both hypermethylation and hypomethylation were strikingly more common in SS (**Figure 1c**).

To further capture the distribution of aberrant methylation in SS, we visualized

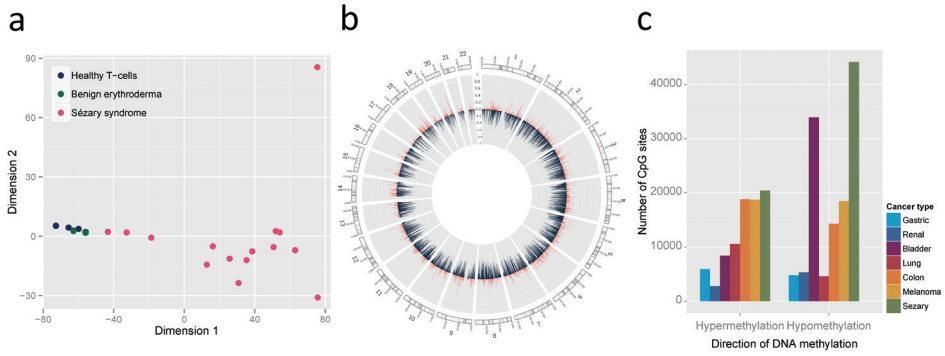

Figure 1. Genome-wide distribution of DNA methylation in Sézary syndrome (SS) patients, erythrodermic inflammatory dermatoses (EID) patients and, healthy control subjects.
a. Multidimensional scaling of all normalized methylation data for all autosomal CpG sites among all patient samples. The largest differences were found between SS samples and benign T-cell samples (dimension 1), and among SS patients variation was found as well (dimension 2). b. Circos representation of the location of differential DNA methylation between SS and benign T cells from EID patients and healthy volunteers across all autosomes. Red indicates hypermethylation and black hypomethylation. Median difference in DNA methylation values were calculated per 100 kilobase pair bins. c. Number of CpG sites with hypermethylation or hypomethylation (absolute β-value difference > 0.2, no *P*-value threshold) between healthy and tumor tissue of thyroid, stomach, kidney, bladder, lung, colon and skin melanoma and SS. CpG, 5'-cytosine-phosphate-guanine-3'; EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome.

the correlation between average methylation in the SS samples and the benign T-cell samples, considering CpG sites located in a CpG island context and non-CpG islands separately (**Figure 2a**). This showed a contrast between CpG sites within CpG islands that were predominantly hypermethylated and CpG sites not located in CpG islands that were more frequently hypomethylated. CpG sites at various specific loci showed selective hyper- and hypomethylation in SS, as illustrated for the *GNMT* and *SPON2* loci (**Figure 2b**). The differentially methylated CpG sites were then assigned to CpG islands, shores and non-CpG islands (NCs) in different regions of a gene, namely the intergenic (IG), distal promoter (DP), proximal promoter (PP), gene body (GB), or downstream (DS) region. This gene-centric analysis showed that CpG sites located in CpG islands in the proximal promoter region of genes showed striking enrichment of hypermethylation in SS (odds ratio = 38, $P \leq 0.0001$; **Figure 2c**). Unsupervised clustering of promoter CpG island methylation separated SS and benign T-cell samples, as evidenced by multidimensional scaling analysis of CpG sites located in promoter CpG islands (**Supplementary Figure S2**).

Given the strong enrichment of hypermethylation in promoter CpG islands in SS and because this epigenetic alteration is often associated with transcriptional repression, we focused on aberrant methylation of promoter CpG islands in SS when compared with EID patients and healthy volunteers. In total, 126 protein-coding genes showed significant and frequent promoter CpG island hypermethylation in SS, whereas only the *LCN6* gene had a hypomethylated promoter CpG island (Welch *t* test, adjusted $P \leq 0.05$ and β-value difference > 0.2) (**Supplementary Materials**). A heat map showing the methylation patterns of 46 promoter CpG islands illustrates the specificity of these methylation alterations for SS (**Figure 2d**). The promoter CpG islands with the highest average β-value

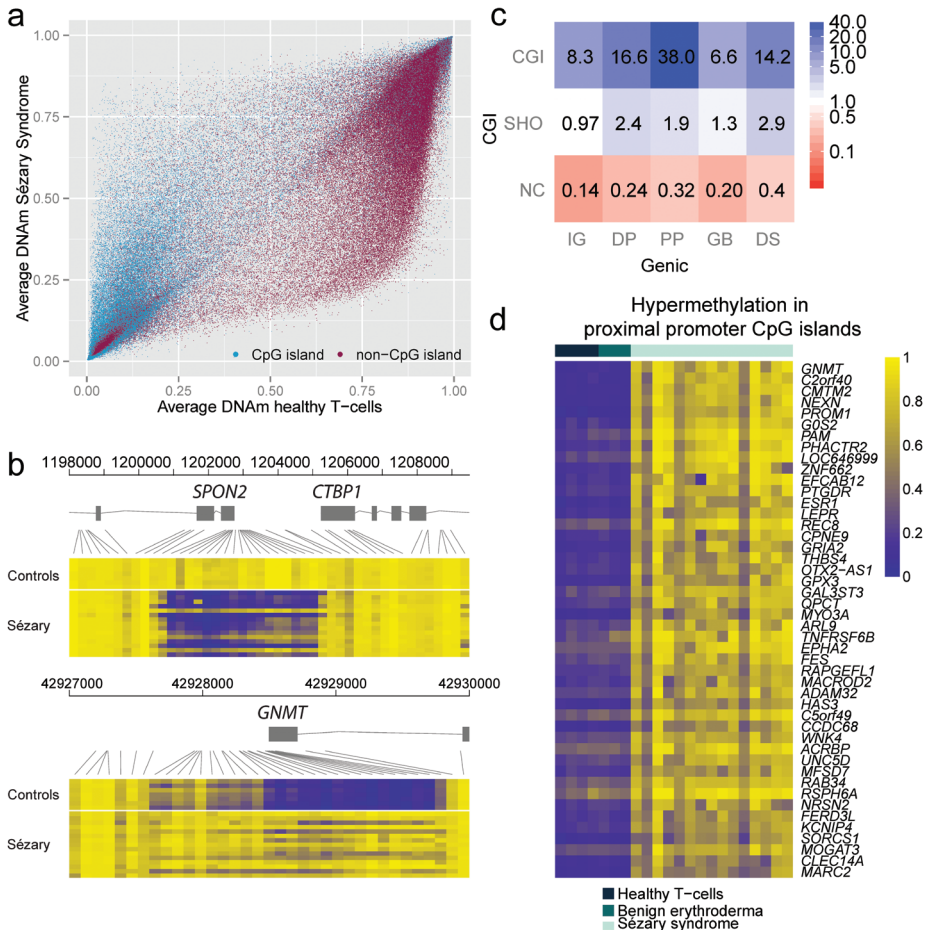

Figure 2. Gene-centric analysis of aberrant DNA methylation of CpG sites within and outside CpG islands. a. Scatterplot illustrating the relation between average DNA methylation in Sézary syndrome (SS) T cells and benign T cells for CpG sites located in a CpG island and CpG sites not located in a CpG island context, that is, not in a CpG island or 4-kilobase pair flanking region. b. Top: promoter of *SPON2*, which is hypomethylated in SS T cells and methylated in healthy T cells. Bottom: promoter of *GNMT*, which is hypermethylated in SS T cells and hypomethylated in healthy T cells. c. Enrichment (as odds ratio) of hypermethylation over hypomethylation in CpG island- and gene-centric annotations. CpG island shores were located within a 2-kilobase pair distance of CpG islands. d. Heat map highlighting the top promoter CpG islands identified as hypermethylated in SS T cells ($P \leq 0.05$, β-value difference ≥ 0.4) when compared with benign T cells, ranked according to average β-value difference. Genes are clustered based on euclidean distance. CGI, CpG island; CpG, 5′-cytosine-phosphate-guanine-3′; DP, distal promoter; DS, downstream; GB, gene body; IG, intergenic; NC, non-CpG island; PP, proximal promoter; SHO, CpG island shore.

difference, indicative of the level of hypermethylation, were those of *GNMT*, *C2orf40*, *CMTM2*, *NEXN*, *PROM1*, and *G0S2*. The hypermethylated promoter CpG islands in SS included those of the tumor suppressor genes *GNMT*, *C2orf40*, *GPX3*, *FES*, *NRSN2*, *THRB*, *TGFBI*, *IRX1*, *ASCL1*, and *CDK2AP1*, listed in the TSGene database.[25] Assessment of genes previously reported as hypermethylated in SS and other CTCL types showed promoter

hypermethylation of *THBS4* (93% of patients), *TP73* (27%), *NEUROG1* (13%), *RARB* (7%), and *PPARG* (7%) in the SS patients (**Supplementary Table S2**). However, we could not confirm promoter hypermethylation of other genes, including *MLH1*, *BCL7A*, and *SOCS1*, previously reported in CTCL. The discrepancies might be related to the fact that particular promoter CpG sequences were not interrogated by the array probes and to differences in promoter methylation patterns between SS and other CTCL types analyzed in the reported studies.

It has been reported that SS T cells show similarity to regulatory T cells.[3] We compared the SS methylomes to those of regulatory T-cell subsets generated using the same array platform.[22] In their study, 1,689 CpG sites were identified that were significantly differentially methylated between activated naïve and activated regulatory T-cell subsets. Joint analysis of DNA methylation of these 1,689 CpG sites in our SS samples and in the published T-cell subsets showed that methylation in most SS samples differed from that of activated naïve T cells and bore more resemblance to that of activated regulatory T cells (**Supplementary Figure S3**). In two SS samples, the methylation pattern appeared more similar to that of activated naïve T cells, suggesting variability of T-cell phenotype among patients with SS.

HYPERMETHYLATED PROMOTER CPG LOCI AS DIAGNOSTIC MARKERS FOR SÉZARY SYNDROME

Among the 126 hypermethylated genes in SS, we selected 12 genes for validation with methylation-specific melting curve analysis. These included the seven gene promoter CpG islands with the highest average β-value difference (>0.6) and the *TFAP2A*, *ID4*, *TGFB2*, *HSPB6*, and *CPEB3* genes based on gene function. Promoter hypermethylation has been shown previously for these genes in other tumor types, except for *NEXN* and *CPEB3*.[26-29] First, we confirmed the Illumina 450k array results by examining promoter methylation using methylation-specific melting curve analysis of the 12 genes in the 15 SS and seven benign control samples previously subjected to genome-wide methylation analysis (**Table 2**). The frequency of methylation of these genes ranged from 40 –100% in SS samples, whereas methylation of these genes was absent in all benign T-cell samples. Next, we investigated the methylation status of these genes in an independent sample set consisting of DNA isolated from CD4+ T cells of 20 patients with SS, 10 patients with EID, and seven healthy volunteers. Detailed results of methylation analysis studies are shown for the *CMTM2* gene as example in **Figure 3**. Methylation-specific melting curve analysis profiles show a clear additional peak corresponding with the melting temperature of the methylated sequence after bisulphite conversion in SS samples, whereas only a peak corresponding to unmethylated DNA is seen in the EID samples (**Figure 3b**). The methylation frequencies in SS samples in the independent sample group were largely similar to those obtained in the primary sample set (**Table 2**). None of the 12 selected genes was affected by promoter CpG island methylation in the benign T-cell samples from patients with EID or from healthy volunteers. The *CMTM2* gene was methylated in all SS samples of the validation group and of the initial group subjected to epigenomic analysis. Detection of promoter CpG island hypermethylation of a single gene, *CMTM2*, therefore allowed accurate diagnosis of SS and distinction from EID with 100% sensitivity

Table 2. Promoter CpG island hypermethylation of selected genes.

Gene		Frequency of aberrant promoter hypermethylation		
		Confirmation		
Symbol	**Function**	**SS, n=15**	**EID, n=3**	**HC, n=4**
CMTM2	Chemokine-like factor gene	**100%**	0%	0%
C2orf40	ECRG4; tumor suppressor gene	**93%**	0%	0%
G0S2	Regulates proliferation and apoptosis; tumor suppressor gene	**93%**	0%	0%
HSPB6	Heat shock protein 20; promotes apoptosis	**93%**	0%	0%
PROM1	CD133; transmembrane protein expressed by tumor stem cells	**100%**	0%	0%
PAM	Multifunctional enzyme; activates neuropeptides	**100%**	0%	0%
CPEB3	Regulates mRNA translation by binding to 3'-UTR	**93%**	0%	0%
GNMT	Metabolic modulator of DNA methylation; tumor suppressor gene	**93%**	0%	0%
TFAP2A	Transcription factor AP-2 alpha; tumor suppressor gene	**93%**	0%	0%
NEXN	Actin-binding protein with role in migration	**87%**	0%	0%
ID4	Transcription factor; tumor suppressor gene	**73%**	0%	0%
TGFB2	Multifunctional cytokine; regulates proliferation and differentiation	**40%**	0%	0%

Boldface is meant to place emphasis on the results of SS.
AP-2, activator protein; CpG, 5'-cytosine-phosphate-guanine-3'; EID, erythrodermic inflammatory dermatoses; HC, healthy control subjects; mRNA, messenger RNA; SS, Sézary syndrome; UTR, untranslated region.

and specificity in these patient groups. Promoter hypermethylation of *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, *PAM*, and the *CPEB3* gene was observed in at least 90% of cases in the validation group as well as in the initial group.

DIMINISHED EXPRESSION OF HYPERMETHYLATED GENES IN SÉZARY SYNDROME

Comparison of promoter methylation and messenger RNA levels in 32 CD4+ T-cell samples from SS patients and nine CD4+ T-cell samples from EID patients showed that the *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, *GNMT*, and *NEXN* genes, hypermethylated in most of the analyzed SS samples, were expressed at significantly lower levels in SS than in benign T-cell samples (**Table 3**). For *CMTM2*, the results of messenger RNA expression analysis using quantitative real-time PCR are depicted in **Figure 3c** and **d**. Treatment of the SeAx cell line with the demethylating agent 5-aza-2'-deoxycytidine and analysis of gene expression further showed an association between promoter methylation and transcriptional repression for the *CMTM2* and *NEXN* genes. Genes previously

Frequency of aberrant promoter hypermethylation				
	Validation		Results (combined)	
SS, n=20	EID, n=10	HC, n=7	SS, n=35	EID/HC, n=24
100%	0%	0%	**100%**	0%
100%	0%	0%	**97%**	0%
95%	0%	0%	**94%**	0%
95%	0%	0%	**94%**	0%
90%	0%	0%	**94%**	0%
90%	0%	0%	**94%**	0%
90%	0%	0%	**91%**	0%
85%	0%	0%	**89%**	0%
85%	0%	0%	**89%**	0%
75%	0%	0%	**80%**	0%
70%	0%	0%	**71%**	0%
40%	0%	0%	**40%**	0%

shown to be epigenetically regulated were not analyzed in 5-aza gene reactivation experiments. These results show that for most of these genes, including the candidate tumor suppressors *GNMT* and *C2orf40*, promoter hypermethylation is associated with transcriptional repression.

DISCUSSION

In this study we characterized the DNA methylomes of CD4+ T cells from patients with SS and from patients with EID and healthy control subjects. The extent of aberrant methylation in SS was high and exceeded that in solid malignancies analyzed by The Cancer Genome Atlas Research Network. This observation fits well with recently described mutations in epigenetic modifier genes such as *DNMT3a* and *TET1* in SS.[11] Although recent DNA methylation studies in cancer have highlighted the occurrence

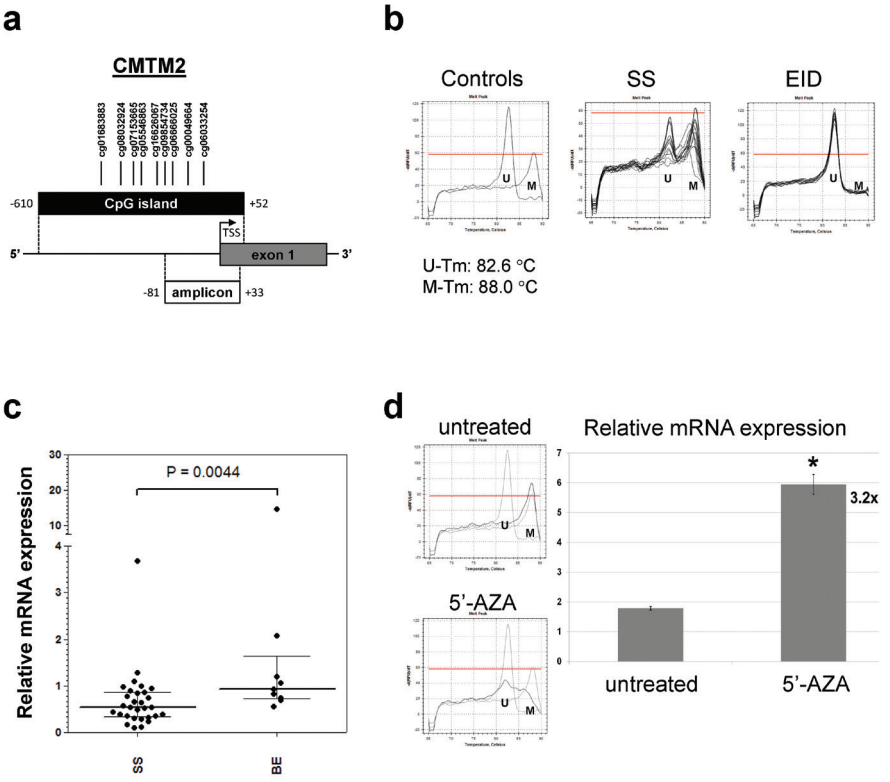

Figure 3. Validation of promoter hypermethylation and transcriptional down-regulation of the *CMTM2* gene in Sézary syndrome (SS).
a. Schematic depiction of the CpG island in relation to the promoter and gene body with location of the significant CpG sites interrogated by the array and amplicon for MS-MCA relative to the transcription start site. b. MS-MCA curve profiles of DNA samples plotted jointly in single graphs. Left panel: fully methylated and unmethylated control samples. The SS samples in the middle panel show a peak in their melting curve patterns at the melting temperature of 88.0°C for methylated DNA, whereas EID samples in the left panel show a peak exclusively at the temperature for unmethylated DNA of 82.6°C. c. Relative mRNA expression data of 32 SS and nine EID patients depicted as dot plots. Median and error bars are generated according to the standard interquartile range where an one-tailed Mann-Whitney test was applied to prove significant differential expression. d. MS-MCA curve plots before and after treatment of the SeAx cell line with 5-aza-2'-deoxycytidine showing relative demethylation (left panel). The effect of demethylation on *CMTM2* expression in the SeAx cell line cultured in the presence or absence of 0.5 mmol/L 5-aza-2'-deoxycytidine (right panel). Data are representative of duplicate treatment experiments; error bars indicate standard deviation from triplicate quantitative real-time PCR experiments. The fold induction in expression upon treatment with 5-aza-2'-deoxycytidine is indicated next to the bars. An one-sided t test was used for statistical analysis. *$P <$ 0.05. 5'-AZA, 5-aza-2'-deoxycytidine; BE, benign erythroderma; CpG, 5'-cytosine-phosphate-guanine-3'; EID, erythrodermic inflammatory dermatoses; M, methylated; mRNA, messenger RNA; MS-MCA, methylation-specific melting curve analysis; SS, Sézary syndrome; Tm, melting temperature; U, unmethylated.

of non-CpG island methylation, this unbiased genome-wide analysis shows that hypermethylation is particularly enriched for in CpG islands located in promoters and to a lesser extent in downstream regions in SS. Hypomethylation occurred predominantly at CpG sites not located in CpG islands in intergenic regions. This high-resolution view of

Table 3. Expression of selected genes.[26-28;34;43;44]

Gene	Differential expression 32 SS vs 9 EID	Up-regulation of demethylated SeAx cell line 5-aza-2'-deoxycytidine	
	P-value	Fold change	*P*-value
CMTM2	0.0044	x3.2	0.0011
G0S2	0.0045	x83.2	0.0021
HSPB6	0.0143	x2.2	ns
PAM	ns	n/a	n/a
CPEB3	ns	x1.9	0.0624
NEXN	0.0450	x2.9	0.0189
C2orf40	0.0003	Yue et al.	
PROM1	0.0005	Pleshkan et al.	
GNMT	0.0002	Huidobro et al.	
TFAP2A	Up-regulated	Douglas et al.	
ID4	Not expressed	Chen et al.	
TGFB2	ns	Hinshelwood et al.	

EID, erythrodermic inflammatory dermatoses; n/a, not applicable; ns, not significant; SS, Sézary syndrome.

DNA methylation showed an array of epigenetic alterations common to SS patients with potential clinical applications. DNA methylation detection is increasingly applied in the clinical diagnosis of malignant tumors, because analysis of this stable epigenetic mark is straightforward and yields binary results.[30] Here we concentrated on the most robustly hypermethylated gene promoters to identify diagnostic markers. In two independent sample sets the promoters of *CMTM2*, *C2orf40*, *G0S2*, *HSPB6*, *PROM1*, and *PAM* were methylated in almost all SS samples but not in benign T-cell samples. Promoter hypermethylation of these genes was associated with transcriptional down-regulation in most cases. Promoter hypermethylation of a single gene, *CMTM2*, accurately distinguished all SS patients from EID patients and healthy control subjects with 100% sensitivity and specificity. SS-specific methylation of these selectively hypermethylated gene promoters, and *CMTM2* in particular, renders them useful epigenetic markers for the diagnosis of this lymphoid malignancy. The *CMTM2* (chemokine-like factor MARVEL transmembrane domain-containing 2) gene belongs to the chemokine-like factor superfamily and is primarily expressed in testis, bone marrow and peripheral blood cells.[31] *CMTM2* affects the function of the cyclic AMP response element-binding protein (AP-1) and CRE-binding protein (CREB) transcription factors in T cells.[32] Tumor suppressive properties have been reported for other members of this protein family.[33] The specificity of *CMTM2* promoter hypermethylation with concomitant transcriptional down-regulation might signify tumor suppressive functions in SS.

Among the 126 gene promoters with consistent hypermethylation in SS, there were multiple established and potential tumor suppressor genes. We found promoter hypermethylation of the *GNMT* tumor suppressor gene in most SS patients included

in this study, although it was expressed at significantly lower levels in SS than in EID. *GNMT* encodes a methyltransferase that catalyzes the conversion of the DNA methyl donor group *S*-adenosylmethionine to *S*-adenosylhomocysteine. Epigenetic silencing of *GNMT* leads to increased *S*-adenosylmethionine levels and has been found in hepatocellular cancer to promote establishment of DNA hypermethylation.[34] The consistent promoter hypermethylation and repression of *GNMT* in SS cells could indicate that a similar mechanism is operational in SS. Another potential tumor suppressor gene with recurrent promoter hypermethylation in SS is *ID4* (inhibitor of DNA binding protein 4), which is implicated in the development of chronic lymphocytic leukemia.[27] *G0S2* (G0/G1 switch gene 2), methylated and repressed in almost all SS samples, has been reported to regulate the proliferation of hematopoietic stem cells and leukemia cells.[35] In addition, in SS extensive DNA hypomethylation of CpG sites not located in CpG islands was found. DNA hypomethylation can result in chromosomal instability, and accordingly, the SS genome is characterized by gross chromosomal instability with many numerical and structural chromosomal alterations.[36;37] The silencing of multiple tumor suppressor genes and the extensive hypomethylation that is associated with chromosomal instability jointly point to a critical role for epigenetic mechanisms in the molecular pathogenesis of SS. This study therefore provides further rationale for epigenetic therapy of SS with histone deacetylase inhibitors (HDAC) and DNA demethylating agents. Accordingly, recent experiments that showed that exposure of the SS cell line HUT78 to a DNA demethylating agent restored sensitivity to apoptosis and reduced proliferation.[38] HDAC can cause reversal of gene promoter methylation and the clinical efficacy of vorinostat and romidepsin used in the treatment of patients with CTCL might be conferred in part by this property.[39]

SS cells have been proposed to represent a malignant clonal proliferation of the regulatory T cells in a subset of patients.[3] We found that in most patients with SS the methylation pattern differed from that of activated naïve T cells and bore more resemblance to that of activated regulatory T cells. The findings of this comparative analysis, although limited by technical variation, point to an epigenetic imprint in most SS samples with similarity to that of regulatory T cells. It would be worthwhile to perform a direct comparison of DNA methylation patterns of SS T cells with all T-cell subsets.

In summary, genome-wide methylation patterns of CD4+ T cells from patients with SS show extensive aberrant DNA methylation and show highly SS-specific hypermethylation events that can be used as epigenetic markers in the clinic for the diagnosis of this lymphoid malignancy. Several previous studies have tried to define additional immunophenotypic markers, copy number variations, gene, microRNA and long noncoding RNA expression profiles that can aid in the diagnosis of SS.[40-42] Our results indicate that measuring promoter methylation of a single gene, *CMTM2*, is sufficient to distinguish SS from benign conditions, rendering it an epigenetic marker that can readily be implemented in clinical practice. A prospective clinical study is justified to evaluate the diagnostic utility of *CMTM2* and other epigenetic markers in clinical practice. In addition, the widespread aberrant DNA methylation observed in this study strengthens the rationale for epigenetic therapies of SS using HDAC, possibly combined with demethylating agents.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Clinical characteristics of patients with Sézary syndrome and erythrodermic inflammatory dermatoses.
a. Blood cell counts and treatment at time of sample collection for each patient with Sézary syndrome.

Sézary patient, n=15	Leukocytes $x10^9/L$*	Lymphocytes % (including Sézary cells)	CD4 count $x10^6/L$*	CD8 count $x10^6/L$*	CD4/CD8 ratio*	Sézary cells /mm^3	Treatment at sample collection
1	9,70	33,00	3918	182	21,48	2910	prednisone
2	8,60	6,00	272	28	9,58	172	prednisone
3	12,00	28,60	3589	39	93,20	N/A	prednisone
4	14,10	48,00	6809	153	44,51	2820	prednisone
5	6,80	47,00	3277	173	18,90	1292	methotrexate
6	12,10	44,00	5372	313	17,18	1815	chlorambucil
7	8,40	44,10	3807	70	54,11	N/A	no therapy
8	20,40	72,20	15170	113	134,25	N/A	no therapy
9	17,10		11732	113	103,75	N/A	prednisone
10	23,20		17113	276	62,14	N/A	prednisone
11	11,74	55,70	3282	849	3,86	2113	acitretin
12	15,20	28,00	5051	26	194,27	N/A	prednisone
13	13,10	28,00	5110	66	77,42	N/A	prednisone and interferon-alpha
14	13,70	59,00	8612	378	22,78	N/A	no therapy
15	25,20	45,50	10606	122	86,93	N/A	prednisone and chlorambucil

N/A, not available.
*Median blood cell counts for the group of patients with Sézary syndrome: leukocytes 13.1 (range = 6.8-25.2); absolute CD4 count 5110 (range = 272-17113); absolute CD8 count 122 (range = 26-849); CD4/CD8 ratio median 54.11 (range = 3.86-194.27).

b. Clinical characteristics of patients with erythrodermic inflammatory dermatoses (EID).
Of the initial group of three EID patients, who were subjected to Infinium 450k Beadchip methylation analysis, two patients had erythroderma secondary to atopic dermatitis and one patient had an idiopathic erythroderma. The validation group, consisting of 10 patients with EID who were analyzed for methylation of 12 selected genes, included five patients with erythroderma secondary to atopic dermatitis, three patients with erythrodermic psoriasis, one patient with erythrodermic drug eruption and one patient with paraneoplastic erythroderma (colorectal cancer).

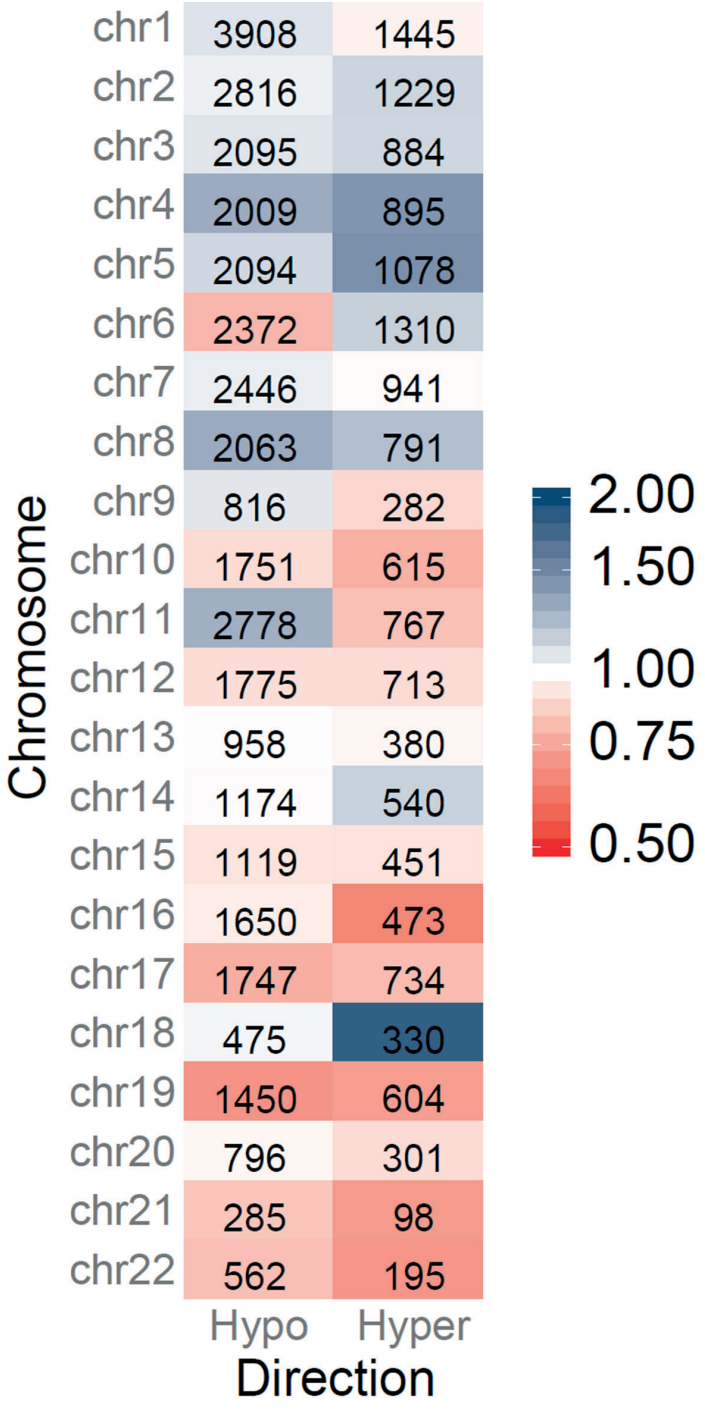
Characteristics of EID	**Confirmation EID, n=3**	**Validation EID, n=10**
Male:female ratio	2:1	9:1
Age in years, median (range)	41 (31-74)	71 (37-86)
Erythroderma	3/3	10/10
Pruritus	3/3	5/10
Ectropion	0/3	1/10
Hyperkeratosis hands/feet	1/3	0/10
Palpable lymphadenopathy	0/3	0/10
Lymphadenopathy confirmed by CT scan	0/0	0/1
Leukocytes ≥ 10.0 $x10^9$/L	0/2	4/6
CD4/CD8 ratio ≥ 10.0	0/0	0/4
Absolute Sézary cell count ≥ 1000 per mm^3	0/0	0/2
T-cell clone in peripheral blood	0/3	0/10
Identical T-cell clone in blood and skin	0/0	0/0
Status last date of follow-up		
Alive with disease	3	10
Died of disease		
Died of other cause than linked to SS		
Died of unknown cause		

EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome

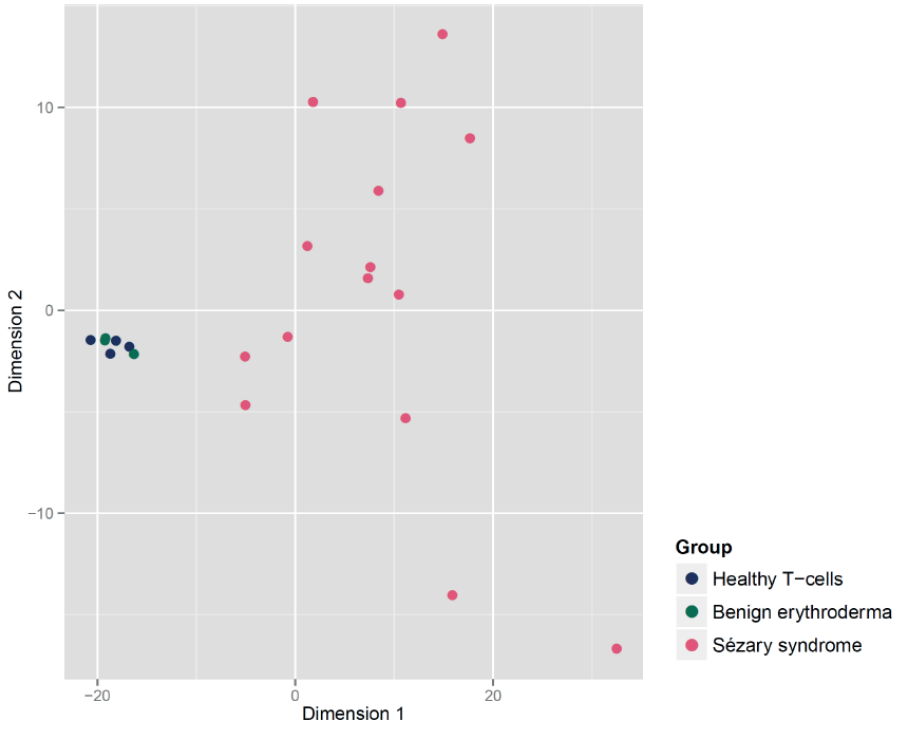
Supplementary Table S2. Promoter hypermethylation of genes reported in previous studies.[1-11]

Gene	Frequency of aberrant promoter hypermethylation					
	SS, n=15	EID, n=3	HC, n=4	reported as	in CTCL type	reference
SAMHD1	**0%**	0%	0%	hypermethylated	Sezary syndrome	de Silva et al.
FAS	**0%**	0%	0%	hypermethylated	Sezary syndrome, CTCL	Jones et al., 2010; Wu et al.
CDKN2B	**0%**	0%	0%	hypermethylated	Sezary syndrome, CTCL	Scarisbrick et al., 2002; Gallardo et al.; van Doorn et al.
CDKN2A	**0%**	0%	0%	hypermethylated	Sezary syndrome, CTCL	Navas et al.; Scarisbrick at al., 2002; Gallardo et al,; van Doorn et al.; Ferrara et al.
MGMT	**0%**	0%	0%	hypermethylated	CTCL	Gallardo et al.; van Doorn et al; Ferrara et al.
MLH1	**0%**	0%	0%	hypermethylated	Mycosis fungoides	Scarisbrick et al., 2003; Ferrara et al.
BCL7A	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
PTPRG	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
THBS4	**93%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
TP73	**27%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
CHFR	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
PYCARD	**0%**	0%	0%	hypermethylated	CTCL	van Doorn et al.
CDKN1B	**0%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
IGF2	**80% (Hypomethylation)**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
NEUROG1	**13%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
PPARG	**7%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
RARB	**7%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
SOCS1	**0%**	0%	0%	hypermethylated	Mycosis fungoides	van Doorn et al.; Ferrara et al.
CDKN1C	**0%**	0%	0%	hypermethylated	Mycosis fungoides	Ferrara et al.
PLS3	**7% (Hypermethylation)**	0%	0%	hypomethylated	Sezary syndrome	Jones et al., 2012
FOXP3	**0%**	0%	0%	hypomethylated	Sezary syndrome, CTCL	Heid et al.

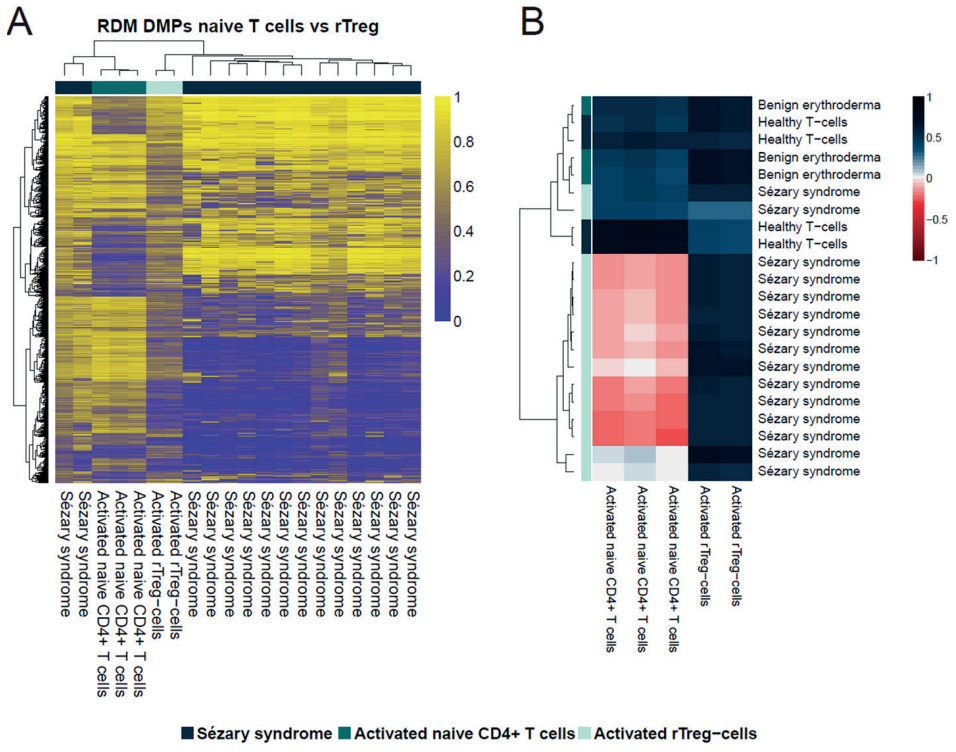
CTCL, cutaneous T-cell lymphoma; EID, erythrodermic inflammatory dermatoses; HC, healthy control; SS, Sézary syndrome



Supplementary Figure S1. Enrichment of hyper- and hypomethylation over chromosomes (absolute number of CG sites and odds ratios).



Supplementary Figure S2. Multidimensional scaling analysis of CpG sites located in CpG islands of proximal promoter regions of autosomal genes in Sézary syndrome, erythrodermic inflammatory dermatoses and healthy control subjects.



Supplementary Figure S3. Comparative analysis of DNA methylation with T-cell subsets.
a. DNA methylation for 1,689 CpG sites previously identified to be discriminative between activated naïve T cells and activated regulatory T cells in Sézary syndrome (SS) samples and external T-cell samples. The DNA methylation profile of SS resembles the methylation profile of activated regulatory T cells to a larger extent than that of activated naïve T cells. b. Correlation between DNA methylation of SS patients and activated naïve T cells and activated regulatory T cells. A high positive correlation was found between SS methylation profiles and regulatory T cells, suggesting it is more similar to regulatory T cells than activated naïve T cells.

Supplementary materials containing the remaining tables are available online at www.jidonline.org

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