MicroRNA Expression Profiling and DNA Methylation Signature for Deregulated MicroRNA in Cutaneous T-Cell Lymphoma

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MicroRNAs usually regulate gene expression negatively, and aberrant expression has been involved in the development of several types of cancers. Microarray profiling of microRNA expression was performed to define a microRNA signature in a series of mycosis fungoides tumor stage (MFt, $n=21$) and CD30+ primary cutaneous anaplastic large cell lymphoma (CD30+ cALCL, $n=11$) samples in comparison with inflammatory dermatoses (ID, $n=5$). Supervised clustering confirmed a distinctive microRNA profile for cutaneous T-cell lymphoma (CTCL) with respect to ID. A 40 microRNA signature was found in MFt including upregulated onco-microRNAs (miR-146a, miR-142-3p/5p, miR-21, miR-181a/b, and miR-155) and downregulated tumor-suppressor microRNAs (miR-200ab/429 cluster, miR-10b, miR-193b, miR-141/200c, and miR-23b/27b). Regarding CD30+ cALCL, 39 differentially expressed microRNAs were identified. Particularly, overexpression of miR-155, miR-21, or miR-142-3p/5p and downregulation of the miR-141/200c clusters were observed. DNA methylation in microRNA gene promoters, as expression regulatory mechanism for deregulated microRNAs, was analyzed using Infinium 450K array and approximately one-third of the differentially expressed microRNAs showed significant DNA methylation differences. Two different microRNA methylation signatures for MFt and CD30+ cALCL were found. Correlation analysis showed an inverse relationship for microRNA promoter methylation and microRNA expression. These results reveal a subgroup-specific epigenetically regulated microRNA signatures for MFt and CD30+ cALCL patients.

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Abbreviations: cALCL, cutaneous anaplastic large cell lymphoma; CTCL, cutaneous T-cell lymphoma; dmmiRNA, differentially methylated microRNA; ID, inflammatory dermatose; MF, mycosis fungoide; MFt, mycosis fungoides tumor stage

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INTRODUCTION

Mycosis fungoides (MF) and primary cutaneous CD30+ T-cell lymphoproliferative disorders (lymphomatoid papulosis and the CD30+ primary cutaneous anaplastic large cell lymphoma (CD30+ cALCL)) are the most frequent subtypes of primary cutaneous T-cell lymphomas (CTCL) (Willemze *et al.*, 2005; Swerdlow *et al.*, 2008). MF and CD30+ cALCL are clinically and pathologically different, but both are derived from effector memory T cells. MF usually follows an indolent clinical evolution, but up to 20% of patients may develop a progressive disease with tumoral lesions (MFt), extracutaneous spread, and an aggressive behavior (5-year survival of <40% in advanced stages) (Agar *et al.*, 2010). Unlike MFt, CD30+ cALCL cases are associated with an excellent prognosis with survival rates of >90% in patients after a 5-year follow-up (Kempf *et al.*, 2011).

Different studies have focused on the genetic characterization of MF and CD30+ cALCL, applying conventional cytogenetics and array comparative genomic hybridization techniques. These approaches have identified possible prognostic markers and putative targets for directed therapeutic intervention (van Doorn *et al.*, 2009; Salgado *et al.*, 2010; Laharanne *et al.*, 2010; van Kester *et al.*, 2010; Sánchez-Schmidt *et al.*,

2011; Szuhai *et al.*, 2013). MF patients harboring frequent genomic imbalances or genomic instability with multiple DNA aberrations seem to show a shorter overall survival (van Doorn *et al.*, 2009; Salgado *et al.*, 2010). Interestingly, genetic aberrations in CD30+ cALCL differ from chromosomal alterations observed in other forms of CTCL. However, genetic aberrations are not sufficient to explain the full complexity of CTCL biology.

Epigenetic regulation, whose mechanisms involve many different factors, is also mediated by noncoding RNAs. MicroRNAs are a class of short noncoding RNAs that negatively regulate gene expression post transcriptionally, usually inducing degradation or inhibition of specific targeted genes. It is known that microRNA expression is deregulated in several types of cancers, disrupting biological processes related to carcinogenesis including cell proliferation and differentiation, invasiveness, or apoptosis. MicroRNAs have become of special interest as specific biomarkers in identifying cancer subtypes (Lu *et al.*, 2005).

The microRNA-targeted genes have an important role in the pathogenesis of CTCL. In addition to oncogenic or tumor-suppressor properties, microRNAs participate in many biological processes in T cells (Allan and Nutt, 2014). Resistance of T cells to chemotherapy-induced apoptosis, aberrant cytokine expression deregulating downstream signal transducer and activator of transcription-5 signaling, and impaired immune response have been reported as altered mechanisms in CTCL that may be mediated by microRNA regulation (Persson, 2013). The usefulness of some CTCL-related microRNAs as specific diagnostic or prognostic markers has been postulated, and their potential role as promising targets has been proposed (Schneider, 2012; Maj *et al.*, 2012; Manfè *et al.*, 2012; Kopp *et al.*, 2013a; Moyal *et al.*, 2013; Manfè *et al.*, 2013; McGirt *et al.*, 2014; Ito *et al.*, 2014).

Recently, some studies have identified different microRNA signatures for MF and CD30+ cALCL (van Kester *et al.*, 2011; Ralfkiaer *et al.*, 2011; Benner *et al.*, 2012), but the mechanisms involved in microRNA expression deregulation among these putative mechanisms have not been fully elucidated. DNA methylation in microRNA gene promoters has been identified as a regulatory process that induces downregulation of microRNA expression in cancer. DNA methylation describes the covalent addition of a methyl group to the 5′ carbon of cytosine within cytosine–guanine dinucleotides. Aberrations in the DNA methylation patterns in microRNAs, particularly the hypermethylation of the CpG island sequences located in the promoter regions of tumor-suppressor microRNAs, have been identified in different cancer cells (Lujambio *et al.*, 2008).

In this work, we evaluated the epigenetic mechanisms involved in CTCL performing a large-scale global microRNA expression analysis using a microarray platform. In addition, the promoter methylation of these differentially expressed microRNAs was explored using the Infinium 450K DNA methylation BeadChip array. Our study has characterized the microRNA landscape and the interplay with DNA methylation for CTCL, revealing common and subgroup-specific features for MF and CD30+ cALCL patients.

RESULTS

MicroRNA expression signature for CTCL

We first evaluated a global genome-wide expression profile of 937 identified microRNAs comparing inflammatory dermatoses (ID $n=5$) and a series of CTCL patients ($n=32$; MFt = 21 and CD30+ cALCL = 11). The unsupervised hierarchical clustering analysis identified a distinctive microRNA expression pattern for CTCL samples. However, the clustering of the complete microRNA profile did not discriminate between MFt and CD30+ cALCL subtypes (Figure 1a). The most relevant differentially expressed microRNAs that discriminate CTCL from ID were characterized by performing a supervised analysis with the following criteria: Δ-value >1.2 and false discovery rate <5%. We obtained a signature of 61 differentially expressed microRNAs that clearly segregated CTCL from ID samples (Figure 1b).

In order to validate the microRNA gene expression microarray analysis, we analyzed the expression of five representative downregulated (miR-193b and miR-10b) and upregulated (miR-142-3p, miR-142-5p, and miR-146a) microRNAs by quantitative real-time reverse-transcriptase–PCR in an independent cohort of CTCL patients ($n=10$) and ID controls (subacute cutaneous lupus erythematosus, $n=6$ and psoriasis, $n=7$). The observed microRNA expression in this cohort of CTCL patients was similar to that found in the microarray (Supplementary Figure S1 online), supporting the reliability of the microarray analysis previously performed.

The differentially expressed microRNAs showed an enrichment in different functional categories with major biological roles in cancer development. The most highly populated KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways altered by aberrant microRNAs expression were cell cycle, DNA replication, NOD-like receptor signaling pathway, mismatch repair, phosphatidylinositol 3 kinase/Akt signaling pathway, the p53 signaling pathway, transcriptional dysregulation, viral carcinogenesis, or Notch signaling pathway. Importantly, many of these biological pathways have been related to neoplastic T-cell biology (Allan and Nutt, 2014).

Distinctive microRNA expression profile in MFt

To elucidate whether MFt displays a distinctive microRNA expression profile compared with ID, we performed a supervised analysis following the same previously mentioned criteria and using exclusively the MFt cohort. A total of 40 microRNAs were differentially expressed in MFt samples with respect to ID: 13 upregulated and 27 downregulated. Among these microRNAs more significantly upregulated, a subset of well-known cancer-related microRNAs was found, such as miR-142-3p/5p and miR-146a, miR-155, miR-21, miR-181a/miR-181b, and others. Among downregulated microRNAs with tumor-suppressor properties, miR-193b, miR-10b, miR-141/200c, and miR-200ab/429 clusters showed particularly low expression levels. Other interesting lymphoma-related microRNAs such as miR-203, miR-205, miR-23b/27b, and others were also downregulated (Table 1a and Figure 2a).

Distinctive microRNA expression profile in CD30+ cALCL

Using a similar approach comparing CD30+ cALCL with ID, we identified 39 deregulated microRNAs in this lymphoma

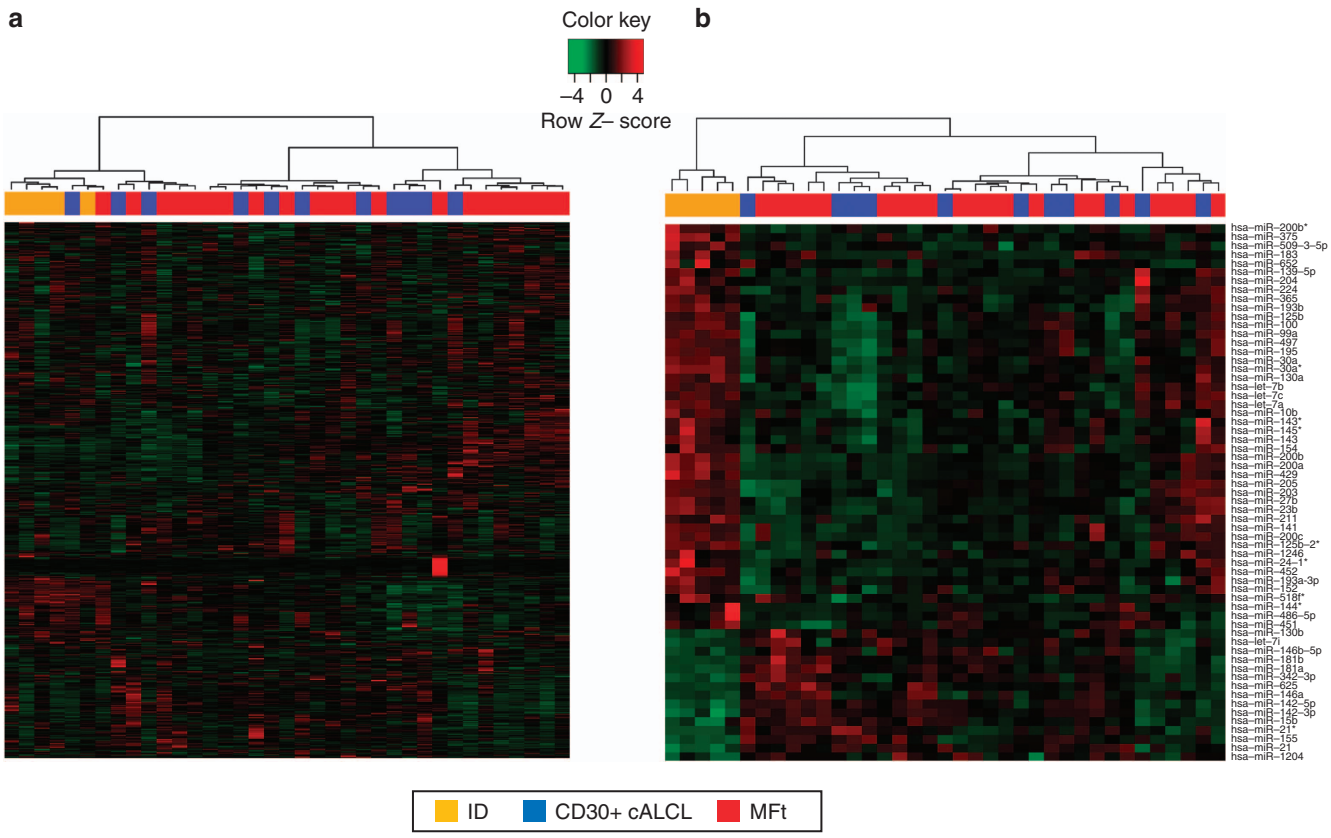

Figure 1. MicroRNA expression profile in cutaneous T-cell lymphomas (CTCL). (**a**) Unsupervised analysis of microRNA expression microarray of mycosis fungoides tumor stage (MFt), CD30+ cutaneous anaplastic T-cell lymphoma (CD30+ cALCL), and inflammatory dermatoses (ID). (**b**) Supervised analysis differentiating MFt and CD30+ cALCL from ID. Each column represents patients, and each row represents the different microRNAs.

subtype: 13 upregulated and 26 downregulated. Several onco-microRNAs including miR-155, miR-21, and miR-142-3p/5p were highly overexpressed, together with other relevant pro-oncogenic microRNAs (let-7i, miR-424, miR-431, miR-542-5p, miR-29b-1, miR-342-p, and miR-484). On other hand, the miR-141/200c cluster was clearly downregulated. There were other interesting tumor-suppressor microRNAs with low expression levels such as miR-23b/miR-27b, miR-203, miR-205, or miR-125b (complete microRNA profile is illustrated in Table 1b and Figure 2b).

Distinctive microRNA signature for MFt and CD30+ cALCL

Although global differences among MFt and CD30+ cALCL were not detected, using an individual comparative analysis we found distinctively expressed microRNAs for each disease (see Table 1a and b). The obtained results disclosed common microRNAs in both groups: 6 upregulated and 20 down-regulated. We detected seven differentially upregulated micro-RNAs in MFt but not in CD30+ cALCL: miR-146a, miR-181a/b, miR-15b, miR-130b, miR-62,5 and miR-103. Six micro-RNAs were downregulated exclusively in MFt: miR-204, miR-224, miR-152, miR-143, miR-652, and miR-486-5p. Five microRNAs were upregulated in CD30+ cALCL but not in MFt samples: miR-424, miR-542-5p, miR-29b, miR-431, and miR-484. Five microRNAs were downregulated exclusively in CD30+ cALCL: miR-125b, miR-96, let-7b, miR-193a-3p, and miR-211.

We further compared the microarray results for the overall analysis (MFt plus CD30+ cALCL vs. ID) with those observed for each tumor subset and grouped in a Venn diagram in order to identify those eligible disease-related microRNAs. Specific microRNA subsets and intersections are detailed in Supplementary Table S1 and Supplementary Figure S2 online. Twenty-seven microRNAs were significantly altered in all three analyses. There were 17 deregulated microRNAs in the joint analysis (CTCL group) when compared with ID. Twelve microRNAs shared MFt and CTCL joint analyses and only one was found in MFt analysis. Five microRNAs in the CTCL group were also found deregulated in the CD30+ cALCL group but not in the MFt analysis; seven unique microRNAs were only present in the CD30+ cALCL group.

Analysis of the promoter microRNA methylation in CTCL

Considering DNA methylation mechanism as a major check-point in regulating microRNA expression (Lujambio *et al.*, 2008; Quintavalle *et al.*, 2013), we evaluated the epigenetic profile of these previously identified differentially expressed microRNAs in CTCL. DNA methylation mapping was performed in 11 available samples, including 4 MFt, 4 CD30+ cALCL, and 3 ID samples as controls. Unsupervised

Table 1a. MicroRNA signature in MFt

MicroRNA	Log FC	Adjusted *P*-value	Chromosome location
Upregulated			
hsa-miR-142-3p	***5.9902***	***0.0005***	***17q22***
hsa-miR-21	***2.6217***	***0.0016***	***17q23.1***
hsa-miR-146a	5.5520	0.0020	5q34
hsa-miR-342-3p	2.6223	0.0020	14q32.2
hsa-miR-142-5p	***5.2473***	***0.0023***	***17q22***
hsa-miR-181a	***2.9545***	***0.0033***	***9q33.3***
hsa-miR-155	4.5118	0.0103	21q21.3
hsa-miR-625	1.4718	0.0218	14q23.3
hsa-miR-181b	***2.0274***	***0.0226***	***9q33.3***
hsa-let-7i	1.5800	0.0375	12q14.1
hsa-miR-130b	1.6729	0.0410	22q11.21
hsa-miR-103	1.4544	0.0410	5q34
hsa-miR-15b	1.8579	0.0421	3q25.33
Downregulated			
hsa-miR-429	−1.6484	0.0000	1p36.33
hsa-miR-200a	−2.1491	0.0000	1p36.33
hsa-miR-30a*	−1.2511	0.0000	6p13
hsa-miR-452	−1.2279	0.0000	Xq28
hsa-miR-200b	−2.7533	0.0001	1p36.33
hsa-miR-30a	−1.7810	0.0020	6q13
hsa-miR-193b	−1.6595	0.0022	16p13.12
hsa-miR-23b	***−2.8029***	***0.0037***	***9q22.32***
hsa-miR-27b	***−2.4158***	***0.0075***	***9q22.32***
hsa-miR-204	***−1.2910***	***0.0075***	***9q21.12***
hsa-miR-224	−1.4480	0.0083	Xq28
hsa-miR-99a	−2.5959	0.0103	21q21.1
hsa-miR-183	−1.3477	0.0149	7q32.2
hsa-miR-130a	−1.7556	0.0158	11q12.1
hsa-miR-200c	−2.5097	0.0216	12p13.31
hsa-miR-100	−2.0314	0.0226	11q24.1
hsa-miR-152	−1.2207	0.0226	17q21.32
hsa-miR-203	−4.4807	0.0272	14q32.33
hsa-miR-143	−1.4855	0.0272	5q32
hsa-miR-195	***−1.8171***	***0.0293***	***17p13.1***
hsa-miR-205	−4.4161	0.0316	1q32.2
hsa-miR-141	−2.9076	0.0316	12p13.31
hsa-miR-652	−1.2260	0.0322	Xq23
hsa-miR-10b	−1.5321	0.0360	2q31.1
hsa-miR-486-5p	−1.6457	0.0375	8p11.21
hsa-let-7a	***−1.5740***	***0.0403***	***9q22.32***
hsa-miR-497	***−1.5925***	***0.0437***	***17p13.1***

Abbreviations: FC, fold change; MFt, mycosis fungoides tumor stage.
Expression (log fold change), *P*-value, and chromosome location are shown. In bold and italics are loci with reported recurrent genomic alterations. The miR-30a*/miR-30a were categorized as separate microRNAs.

hierarchical clustering of the differentially expressed microRNA-associated CpG methylation data indicate that CTCL show a distinct pattern in comparison to ID (Figure 3). However, this analysis was unable to segregate both lymphoma subtypes (MFt and CD30+ cALCL) into two different clearcut groups.

Next, in order to identify particular lymphoma-related differences, we determined differentially methylated microRNAs (dmmiRNAs) between controls and each particular tumor type. The Δβ-values of >0.2 and multiple testing corrected *P*-value of <0.1 were used as the criteria for this analysis. Approximately 36% of the differentially expressed microRNAs showed DNA methylation differences between MFt and the CD30+ cALCL group (Figure 4a and d and Supplementary Tables S2 and S3 online). Two specific signatures for CD30+ cALCL and MFt represented by 17 and 33 differentially methylated CpGs were, respectively, identified. Moreover, 16 differentially methylated CpGs were common to both disease groups. In particular, we found 16 dmmiRNAs (including 15 CpGs hypermethylated in 8 microRNAs and 18 CpGs hypomethylated in 8 microRNAs) and 17 dmmiRNAs (with 22 CpGs hypermethylated in 5 microRNAs and 27 CpGs hypomethylated in 12 microRNAs) in CD30+ cALCL and MFt, respectively (Supplementary Tables S3 and S4 online). Among the dmmiRNAs identified, we observed a specific and significant hypermethylation pattern for miR-141/200c in CD30+ cALCL (Figure 4b) and for miR-193b and miR-10b in MFt (Figure 4e). MiR-21 and miR-429 were detected as specific and significant hypomethylated microRNAs in CD30+ cALCL and MFt, respectively, whereas miR-142 was found hypomethylated in both lymphoma subtypes. Other significant dmmiRNAs for MFt and CD30+ cALCL are included in Supplementary Tables S3 and S4 online.

Furthermore, to obtain more information about the influence of DNA methylation on the regulation of the microRNA expression pattern, a correlation analysis between the microRNA promoter methylation and the differentially expressed microRNA levels was performed (Figure 4c and f). An inverse correlation between the levels of DNA methylation at CpG sites in the promoter region and microRNA expression for both the MFt and the CD30+ cALCL groups was found. Consequently, the promoter hypermethylation of microRNAs highly correlated with the decrease in their expression in MFt and CD30+ cALCL, suggesting a possible role as epigenetically regulated tumor-suppressor microRNAs.

The microRNAs miR-10b, miR-193b, and miR-141 have shown an epigenetic regulation by DNA methylation in diverse cutaneous tumors (Rauhala *et al.*, 2010; Vrba *et al.*, 2010; Biagioni *et al.*, 2012). Similarly, we observed that these microRNAs are methylated throughout their promoters in the human CTCL cell lines Myla, HuT-78, and HH (Figure 5a and Supplementary Figure S3 online). Importantly, after the treatment of these cell lines with the demethylating agent 5-aza-2′-deoxycytidine, the methylation levels of the microRNAs decreased (data not shown) and the expression of these microRNAs was significantly restored (Figure 5b). These results indicate that DNA methylation has a functional role in the transcriptional control of miR-10b, miR-193b,

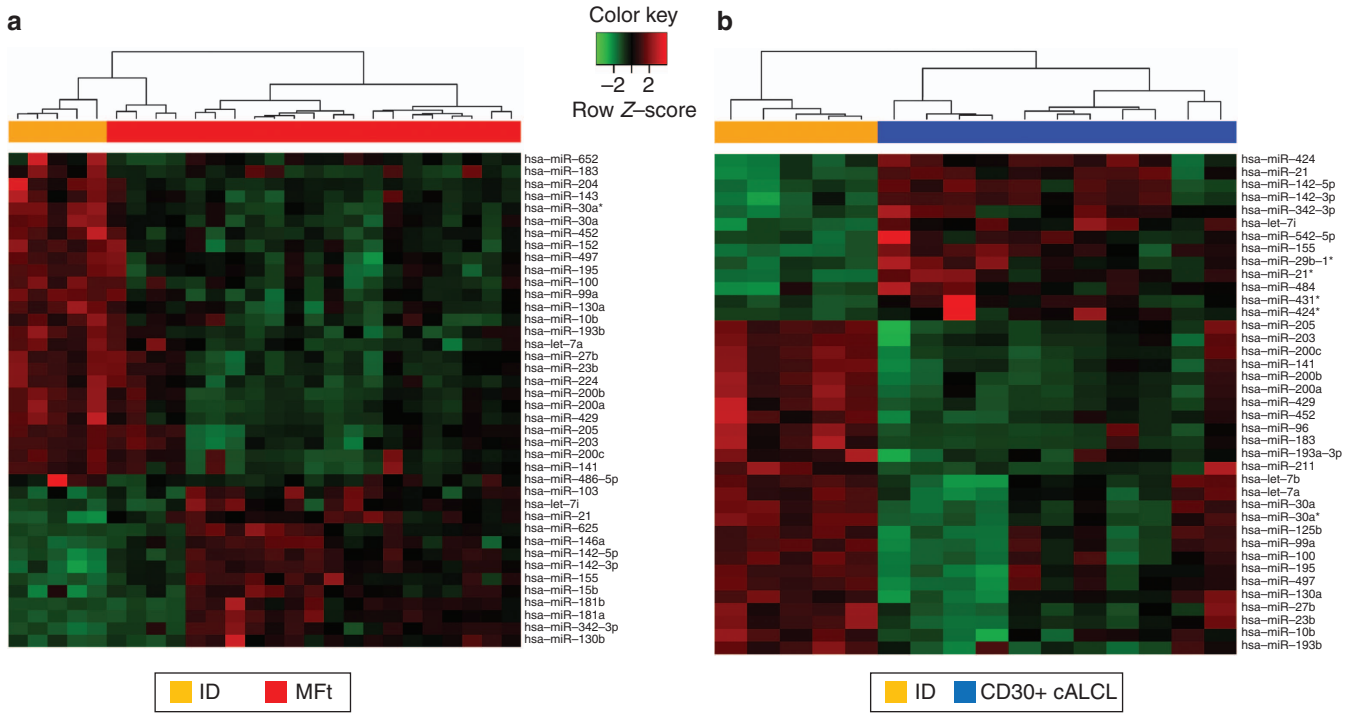

Figure 2. Distinctive expression profiles in mycosis fungoides tumor stage (MFt) and CD30+ cutaneous anaplastic T-cell lymphoma (CD30+ cALCL). (**a**) Heatmap representing the supervised clustering corresponding to the comparison between MFt and control samples (inflammatory dermatoses (ID)). (**b**) Heatmap representing the distinctive microRNAs in supervised clustering corresponding to the comparison between CD30+ cALCL and control samples (ID).

and miR-141 in CTCL. This also suggests that restoring the expression of DNA-methylated microRNAs might have a potential therapeutic role in CTCL.

DISCUSSION

There is increasing evidence that altered microRNA pattern expression is associated with distinct clinical aspects in many benign and malignant diseases (Calin and Croce, 2006; Kent and Mendell, 2006; Cowland *et al.*, 2007; Croce, 2009; O Connell *et al.*, 2010). Our results identified a distinctive microRNA expression signature in MFt and CD30+ cALCL. Importantly, we have found unreported microRNAs with altered expression and confirmed others that have been previously described in both MFt and cALCL (Ballabio *et al.*, 2010; Ralfkiaer *et al.*, 2011; van Kester *et al.*, 2011; Benner *et al.*, 2012). Moreover, a set of three microRNAs detected in our series (miR-155, miR-203, and miR-205) has been proposed as a diagnostic tool to discriminate CTCL from ID samples (Ralfkiaer *et al.*, 2011; Marstrand *et al.*, 2014).

Although both CTCL groups shared many altered microRNAs in our work, we have also found specific microRNAs for each CTCL subtype. In MFt, upregulated microRNAs found in our study (miR-142-5p, miR-155, and mir146a) present pro-oncogenic activities and regulatory properties within the immune system and lymphocyte homeostasis (Lawrie, 2013; Jardin and Figeac, 2013). MiR-155 is one of the most investigated microRNAs in cancer that drives some functions of interest in CTCL development. This microRNA promotes an aberrant activation of the signal transducer and activator of transcription-5 pathway (Persson, 2013; Kopp *et al.*, 2013b) and has a critical role for T-cell response, throughout regulating CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), promoting T-cell proliferation in atopic dermatitis (Sonkoly *et al.*, 2010). Other interesting onco-microRNAs are miR-142-5p, a suppressor of pro-apoptotic gene *TP53INP1* (Saito *et al.*, 2012), and the regulators of the Notch signaling pathway: miR-146a (negative regulator of Numb-Notch) and miR-181a/miR-181b (controlling the strength and threshold of Notch oncogenic activity and targeting multiple anti-apoptotic genes) (Wang *et al.*, 2010). Therefore, these microRNAs seem to be of great interest to further investigations in CTCL (Kamstrup *et al.*, 2010a, b). Some relevant downregulated tumor-suppressor microRNAs in MFt included members of the Notch-related miR-141/200c cluster, miR-10b with apoptotic regulatory properties through TWIST-1 (Li *et al.*, 2013a), and miR-193b that represses cell proliferation controlling critical genes as cyclin D1 (Chen *et al.*, 2010). Moreover, other deregulated microRNAs may also be potential biomarkers suitable for further studies in MF: miR-23b (*c-myc*-driven microRNA targeting pro-apoptotic Fas in thymus lymphoma) (Gao *et al.*, 2009; Li *et al.*, 2013b) and miR-204 (B-cell lymphoma 2 (BCL2) protein regulator and AKT/mammalian target of rapamycin (mTOR) signaling activator) (Sacconi *et al.*, 2012; Imam *et al.*, 2012).

Regarding CD30+ cALCL, we detected overexpression of the onco-microRNAs miR-155, miR-21, or miR-142 and low expression of miR-141/200c cluster as the most relevant features. Previously, Benner *et al.* (2012) also found

Table 1b. MicroRNA signature in CD30+ cALCL

MicroRNA	Log FC	Adjusted *P*-value	Chromosome location
Upregulated			
hsa-miR-155	9.2955	0.0003	21q21.3
hsa-miR-21*	***2.6247***	***0.0003***	***17q23.1***
hsa-miR-21	***3.0527***	***0.0006***	***17q23.1***
hsa-miR-142-3p	***4.8551***	***0.0047***	***17q22***
hsa-miR-424	2.5167	0.0067	Xq26.3
hsa-miR-542-5p	1.1619	0.0096	Xq26.3
hsa-miR-29b-1	***1.1953***	***0.0203***	***7q32.3***
hsa-miR-142-5p	***4.1513***	***0.0205***	***17q22***
hsa-miR-424*	1.1305	0.0226	Xq26.3
hsa-miR-431	1.1505	0.0255	14q32.2
hsa-miR-342-3p	2.1726	0.0275	14q32.2
hsa-miR-484	1.1556	0.0297	16p13.11
hsa-let-7i	1.6132	0.0470	12q14.1
Downregulated			
hsa-miR-429	***−1.6811***	***0.0000***	***1p36.33***
hsa-miR-200a	***−2.2797***	***0.0000***	***1p36.33***
hsa-miR-30a*	−1.2898	0.0000	6p13
hsa-miR-200b	***−3.0737***	***0.0001***	***1p36.33***
hsa-miR-452	−1.2281	0.0002	Xp28
hsa-miR-30a	−2.0813	0.0003	6p13
hsa-miR-183	−1.4675	0.0037	7q32.2
hsa-miR-141	−4.4498	0.0037	12p13.31
hsa-miR-200c	−3.3745	0.0037	12p13.31
hsa-miR-100	−2.5358	0.0043	11q24.1
hsa-miR-193b	***−1.6812***	***0.0043***	***16p13.12***
hsa-miR-23b	−2.8684	0.0073	9q22.32
hsa-miR-497	***−1.8667***	***0.0094***	***17p13.1***
hsa-miR-195	***−2.0687***	***0.0118***	***17p13.1***
hsa-miR-99a	−2.7552	0.0119	21q21.1
hsa-miR-203	−5.8234	0.0147	14q32.33
hsa-miR-27b	−2.4004	0.0156	9q22.32
hsa-miR-125b	−2.9092	0.0170	11q24.1
hsa-miR-205	−5.4667	0.0205	1q32.2
hsa-let-7a	−1.7179	0.0205	9q22.32
hsa-miR-96	−2.0958	0.0216	7q32.2
hsa-miR-10b	−1.5941	0.0328	2q31.1
hsa-miR-130a	−1.7167	0.0364	11q12.1
hsa-miR-193a-3p	−1.3866	0.0414	17q11.2
hsa-let-7b	−2.0900	0.0425	22q13.31
hsa-miR-211	−1.5881	0.0425	15q13.3

Abbreviations: cALCL, cutaneous anaplastic T-cell lymphoma; FC, fold change.
Expression (log fold change), *P*-value, and chromosome location are shown. In bold and italics are loci with reported recurrent genomic alterations. The miR-30a*/miR-30a, miR-21*/miR-21 and miR-424*/miR-424, were categorized as separate microRNAs.

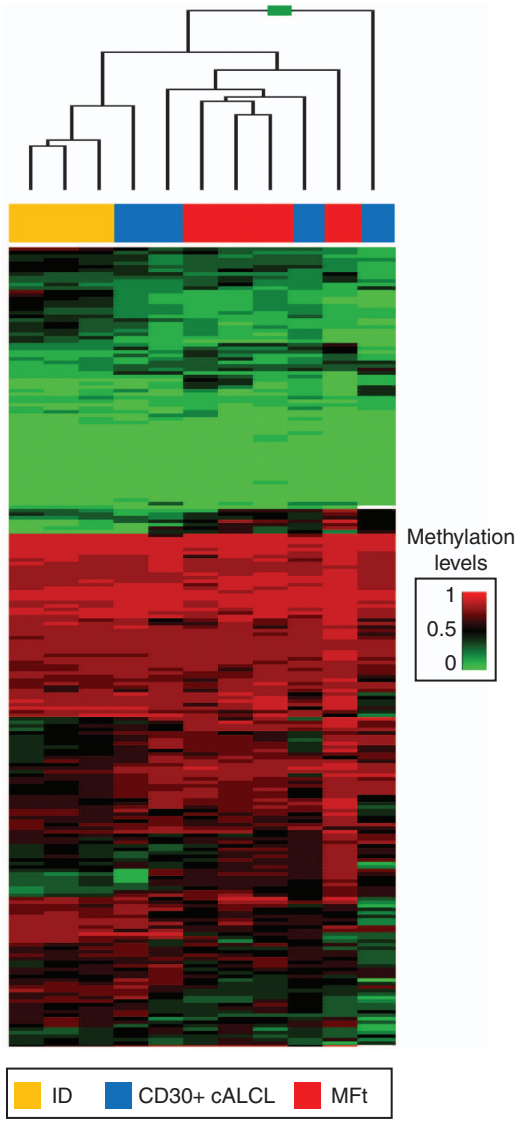

Figure 3. Unsupervised DNA methylation analysis using CpGs from differentially expressed microRNA cutaneous T-cell lymphoma (CTCL) samples with respect to inflammatory dermatoses (ID). Unsupervised hierarchical clustering and heatmap associated with the methylation profile (according to the color scale shown) of the sample specimens based on the β-values of the 229 differentially expressed microRNA CpGs. Three different types of samples are represented: ID, mycosis fungoides tumor stage (MFt), and CD30+ cutaneous anaplastic T-cell lymphoma (cALCL). Each column represents an individual patient and each row an individual CpG.

indistinguishable microRNA expression profile for CD30+ ALCL and MFt, although subtle differences were found by quantitative real-time reverse-transcriptase–PCR. The microRNA profile reported in systemic ALCL differs considerably from those reported in CD30+ cALCL, suggesting different underlying pathogenic mechanisms (Lawrie, 2008; Liu *et al.*, 2013).

The mechanisms regulating microRNA expression are diverse and include somatic mutations or genomic alterations, indicating a general link between the microRNA deregulation

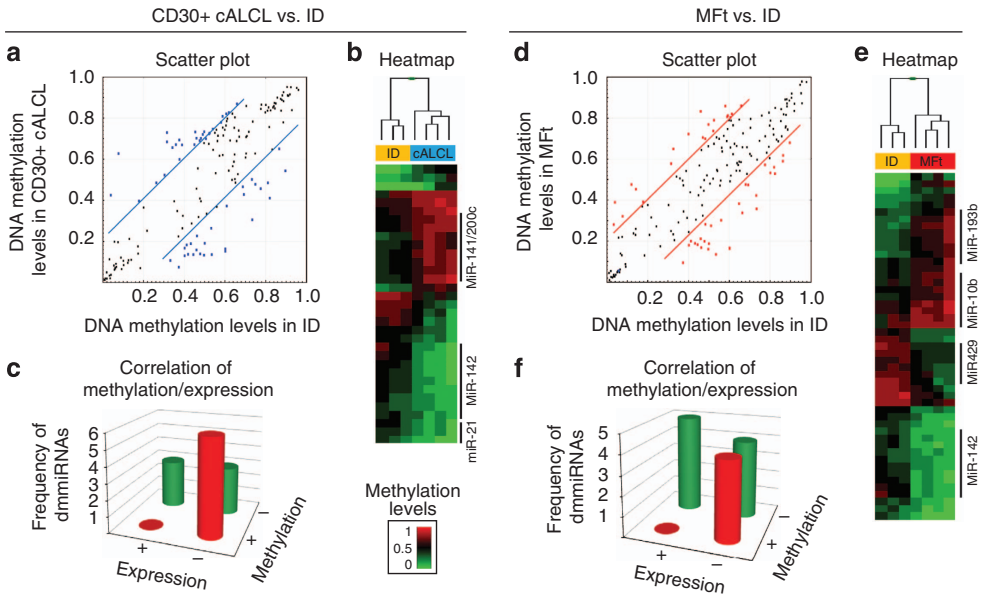

Figure 4. DNA methylation signatures for CD30+ cutaneous anaplastic T-cell lymphoma (CD30+ cALCL) and mycosis fungoides tumor stage (MFt) patients. (**a**, **d**) Scatter plots representing DNA methylation normalized level of differentially expressed CpG sites using the Infinium 450K DNA methylation assay. In red color (**a**) and blue color (**d**) are displayed the differentially methylated microRNA CpGs associated with MFt and CD30+ cALCL, respectively. (**b**, **e**) The hierarchical cluster and heatmap of differentially methylated microRNA CpGs in four CD30+ cALCL (blue) and four MFt (red) versus ID controls (orange), respectively. Selected differentially methylated microRNA (dmmiRNA) CpGs are highlighted. (**c**, **f**) Promoter methylation correlation with gene expression. The symbols (+) and (−) denote an increase and a decrease in expression or methylation, respectively.

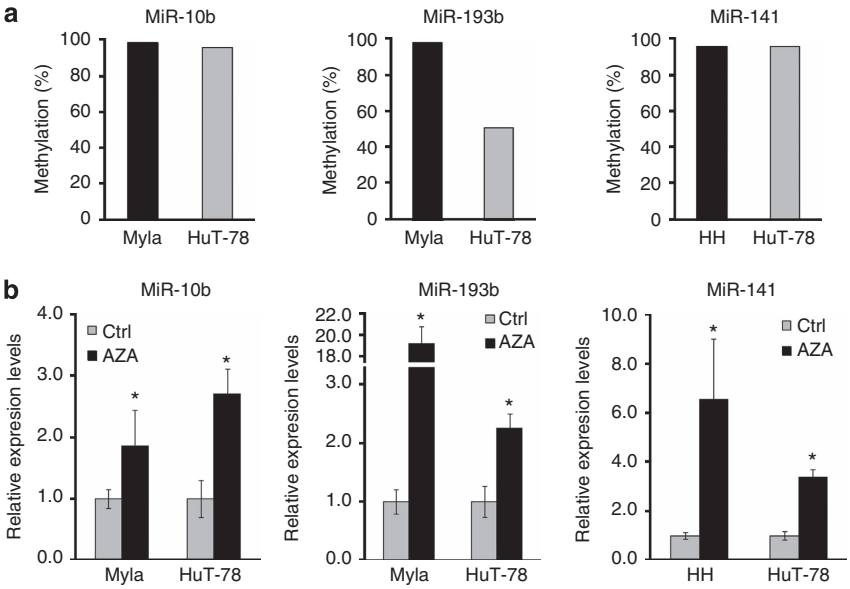

Figure 5. DNA methylation–associated silencing of miR-10b, miR-193b, and miR-141 in cutaneous T-cell lymphoma (CTCL) cell lines. (**a**) DNA methylation levels of miR-10b, miR-193b, and miR-141 determined by pyrosequencing. Data represent the mean of the methylation level of 2–3 consecutive CpGs. (**b**) Restored expression of DNA methylated microRNAs in CTCL cell lines after treatment with the DNA demethylating agent 5-aza-2′-deoxycytidine (AZA). Values were determined in triplicates by quantitative real-time reverse-transcriptase–PCR (qRT–PCR) and are expressed as mean ± SEM ($n = 3$). *$P < 0.05$ with respect to untreated cells (control).

and genome fragility (Calin and Croce, 2006b, 2007). Some of the upregulated microRNAs detected in our series are encoded in frequently gained genomic regions in both MFt (van Doorn *et al.*, 2009; Salgado *et al.*, 2010; Laharanne *et al.*, 2010) and CD30+ cALCL (Laharanne *et al.*, 2010; van Kester *et al.*, 2010; Sánchez-Schmidt *et al.*, 2011; Szuhai *et al.*,

2013). However, aberrant expression of microRNAs, non-related to genomic gains or losses, could be due to other regulatory mechanisms including epigenetic modifications. DNA methylation represents the most studied epigenetic regulatory mechanism of modifying gene expression in both coding and noncoding genes such as microRNAs (Lopez-Serra and Esteller, 2012, Sandoval and Esteller, 2012; Altorok *et al.*, 2014). Analyzing the DNA methylation profile in promoter regions of the differentially expressed microRNAs permitted us to identify a characteristic methylation pattern in CTCL. Prior studies have described DNA methylation signatures associated with different types of tumors related to both coding genes (Sandoval *et al.*, 2013) and microRNAs (Lujambio *et al.*, 2008). However, to our knowledge, the DNA methylation profile of differentially expressed microRNAs has not been previously reported in association with CTCL. Importantly, epigenetically deregulated microRNAs in our series have been also linked as a mechanism to disease in different cancer subtypes (Neves *et al.*, 2010; Vrba *et al.*, 2011; Li *et al.*, 2013c, 2014a, b).

Promoter DNA methylation is known to silence gene expression (Sanchez-Mut *et al.*, 2013; Chambwe *et al.*, 2014). In our series, an inverse correlation between microRNA expression and DNA methylation data in both MFt and the CD30+ cALCL groups was detected. Furthermore, demethylating agents restored the expression of methylated microRNAs, supporting the implication of DNA methylation on the regulation of the differentially expressed microRNAs in CTCL. In this sense, epigenetic therapy (methylation inhibitors or histone deacetylase inhibitors) has shown some benefit in cancer, including CTCL (Hassler *et al.*, 2012; Wong, 2013). Our results provide further evidence for the potential use of evolving epigenetic clinical strategies in the management of CTCL patients.

In conclusion, the identification of expression profiles of microRNAs in CTCL and integrating these findings with data from epigenetic and genomic alterations may contribute to a better understanding of the pathogenic mechanisms involved in the development of both MF and CD30+ cALCL. This data set will allow to further evaluate the usefulness of microRNAs as diagnostic or progression markers in these diseases, and the possible design of more specific and selective treatments.

MATERIALS AND METHODS

Patients

This is a collaborative and retrospective study including a total of 32 CTCL samples obtained from different institutions in Spain (Hospital del Mar, Hospital de Bellvitge, Hospital Clínic, and Hospital de Sant Pau in Barcelona; Hospital General in Valencia; and Hospital 12 de Octubre in Madrid). Skin biopsy specimens were included in the study: from 21 patients with tumoral stage MF (MFt) and from 11 patients with CD30+ cALCL. All patients were diagnosed according to the World Health Organization/European Organization for Research and Treatment of Cancer classification (Willemze *et al.*, 2005). The MFt group included 9 females and 12 males with a median age of 63 years (range, 17–83). Six deaths were recorded in the MFt group at the end of the study. CD30+ cALCL cases were 6 males and 5 females with a median age of 52 years (range, 21–79). No deaths were recorded in this group. As a control group, we included a subset of 5 ID samples showing a dense lymphocytic infiltrate (subacute cutaneous lupus erythematosus, $n=3$ and psoriasis, $n=2$). An independent cohort of CTCL patients ($n=10$) and ID controls (subacute cutaneous lupus erythematosus, $n=6$ and psoriasis, $n=7$) was used for validation analyses.

MicroRNA expression microarray

For all samples, a hematoxylin–eosin staining of a frozen section was performed in order to confirm the presence of at least 70% of tumor cells. Total RNA was extracted for microRNA profiling from $20\times10\,\mu m$ snap-frozen samples using a commercial kit, MirVana miRNA Isolation Kit, following the manufacturer's protocol (Ambion, Austin, TX). RNAs were analyzed using the RNA nano 6000, (Bioanalyzer 2100, Agilent Technologies, Palo Alto, CA) in order to check the RNA integrity. Samples included in the microarray analysis had a quality index RIN (RNA integrity number) of ≥6.

Briefly, 100 ng of total RNA from each sample was hybridized using the Agilent Human microRNA microarrays v3 (ID021827; Agilent Technologies) as the manufacturer's protocol (see Supplementary Methods online). Unsupervised and supervised clustering to determine microRNAs differentially expressed compared with controls and between the two groups of CTCL (MFt and CD30+ cALCL) was analyzed. Linear models and empirical Bayes methods were employed for assessing differential expression in microarray experiments, and multiple testing adjust was performed (false discovery rate) (Smyth, 2004). MicroRNAs with false discovery rate adjusted *P*-value of $<5\%$ and additionally a fold change exceeding 1.2 in absolute value were selected as the relevant ones. All statistical analyses were performed with the Bioconductor project in the R statistical environment (version 3.0.2) (Gentleman *et al.*, 2004). Validation of microRNA expression was performed by quantitative real-time reverse-transcriptase–PCR. For further details see Supplementary Methods online.

Pathway enrichment analysis

MicroRNA pathway enrichment analysis was obtained from DIANA miRPath version 2.0 that uses DIANA-microT-CDS algorithm and/or experimentally validated microRNA interactions derived from DIANA-TarBase version 6.0 (Vlachos *et al.*, 2012).

Microarray-based DNA methylation analysis

Genome-wide DNA methylation analysis was performed in a subset of 11 samples (including 4 MFt, 4 CD30+ cALCL, and 3 ID) using the Infinium Human Methylation 450 BeadChip from Illumina (San Diego, CA) as previously described (Sandoval *et al.*, 2011; Bibikova *et al.*, 2011). The 450K DNA methylation array by Illumina is an established, highly reproducible method for DNA methylation detection and has been validated in three independent laboratories. The 450K DNA methylation array includes 485,764 cytosine positions of the human genome. From these cytosine sites, 4,168 are associated with noncoding RNA transcripts (1%), including 3,440 microRNAs that correspond to 834 unique microRNAs (Sandoval *et al.*, 2011). Only the CpGs associated with the subset of CTCL differentially expressed microRNAs were selected. We ended up with 230 CpGs. The intensities of the images were extracted and normalized using Genome Studio (2011.1) Methylation module

(1.9.0) software from Illumina. For determining differentially methylated microRNAs, nonparametric Wilcoxon tests were applied. Probes with multiple test corrected *P*-value of <0.1 and Δ-value threshold of 0.2 in absolute value were used for selecting the relevant CpGs. All statistical analyses were performed with the Bioconductor project (version 2.3) in the R statistical environment (version 2.8.1). Validation was performed by pyrosequencing analysis following the manufacturer's instructions. For further details see Supplementary Material online.

Cell lines and treatments for functional analysis

Three representative human cancer CTCL cell lines (American Type Culture Collection, Manassas, VA) were used to evaluate the DNA methylation of miR-10b, miR-193b, and miR-141: Myla (Mycosis fungoide), HuT-78 (the Sézary syndrome), and HH (non-MF aggressive CTCL). To restore the expression of DNA-methylated microRNAs, the cell lines were treated with the DNA demethylating agent 5-aza-2′-deoxycytidine (A3656, Sigma, St Louis, MO) at 3 μM for 72 hours.

Ethical considerations

The approval for the study was provided by the Comitè Ètic d'Investigació Clínica from l'Institut Mar d'Investigació Mèdica (CEIC-PSMAR), and written informed consent was obtained from all patients according to the National and International guidelines (code of ethics, Declaration of Helsinki Principles), and the legal regulations on data privacy (Law 15/1999 of 13 December on the Protection of Personal Data (Data Protection Act)) were considered.

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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