



The MicroRNA Expression Profile Differs Between Erythrodermic Mycosis Fungoides and Sézary Syndrome

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It is difficult to distinguish erythrodermic mycosis fungoides from Sézary syndrome due to their similar clinical and histological features. The main purpose of this study was to investigate whether microRNA expression profiles in lesional skin could discriminate patients with erythrodermic mycosis fungoides from those with Sézary syndrome. A further aim was to assess whether the microRNA expression profiles in erythrodermic mycosis fungoides skin was more comparable to microRNA expression profiles of Sézary syndrome or early-stage mycosis fungoides. RNA was extracted from diagnostic skin biopsies, followed by quantitative reverse transcription polymerase chain reaction analysis of 383 microRNAs. Twenty-seven microRNAs were significantly differentially expressed between erythrodermic mycosis fungoides and Sézary syndrome. Moreover, erythrodermic mycosis fungoides showed microRNA features overlapping with Sézary syndrome and early-stage mycosis fungoides, although hierarchical cluster analysis co-clustered erythrodermic mycosis fungoides with early-stage mycosis fungoides rather than with Sézary syndrome. These findings underscore that erythrodermic mycosis fungoides and Sézary syndrome are different diseases.

Key words: mycosis fungoides, Sézary syndrome, cutaneous T-cell lymphoma, microRNA.

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Cutaneous T-cell lymphoma (CTCL) is a group of rare heterogeneous lymphoproliferative disorders primarily confined to the skin. The most prevalent clinical form of CTCL is mycosis fungoides (MF) (1, 2). Early-stage MF comprises patch and plaque skin lesions, whereas advanced stages involve skin tumours and erythrodermic MF (eMF) (3, 4). eMF and the more aggressive leukemic variant, Sézary syndrome (SS), are characterized by chronic erythroderma with $\geq 80\%$ skin involvement (4). eMF and SS are difficult to distinguish because of their similar clinical features, with erythroderma and lymphadenopathy combined with symptoms such as pruritus, skin burning, and chills (5). However, eMF sometimes progresses through patch- and/or plaque-stage disease

SIGNIFICANCE

Erythrodermic mycosis fungoides and Sézary syndrome share clinical and histological features, making it difficult to distinguish these diseases. It has been discussed previously whether erythrodermic mycosis fungoides and Sézary syndrome are different stages of the same disease. However, differences in treatment and prognoses indicate that the diseases should be considered separately. MicroRNAs are small sequences of RNA, which have the potential to discriminate clinically similar diseases. This study showed that 27 microRNAs discriminated erythrodermic mycosis fungoides from Sézary syndrome. These data support the perception of erythrodermic mycosis fungoides and Sézary syndrome as different diseases.

and has various levels of blood involvement, whereas SS usually presents with erythroderma and significant blood involvement (6, 7). Controversies still exist regarding whether MF and SS are distinct diseases or different manifestations of a single disease (8, 9), which has led to substantial discussions about the distinction between eMF and SS (6, 9, 10). Discrimination between SS and eMF is essential due to differences in treatment recommendations and prognosis (7, 11, 12).

MicroRNAs (miRNAs) may have the potential to discriminate between SS and eMF. miRNAs are short non-coding RNA molecules that regulate gene expression by modulating translation of messenger RNA (13). Thus, miRNAs are involved in important biological functions, and altered regulation of miRNAs plays a key role in cancer development, progression and metastasis (14). Many miRNAs are differentially expressed in CTCL compared with normal skin (15–17). They have been proposed to discriminate CTCL from benign skin diseases as a diagnostic marker (18–20), and a prognostic 3-miRNA classifier was developed recently for patients diagnosed with early-stage MF (21). Moreover, single miRNAs (e.g. miR-155) may have essential regulatory functions in CTCL (22, 23), and a drug targeting miR-155, cobomarsen, was developed recently and tested in a clinical trial with promising outcomes (24).

This study examined the miRNA expression profiles of diagnostic lesional skin biopsies from early-stage MF, eMF, and SS, with the aim of: (i) examining whether a distinct miRNA expression profile can discriminate eMF from SS; and (ii) addressing whether the miRNA

signature in eMF is more similar to the signature in SS than to the miRNA profile in early-stage MF skin.

METHODS

Patients

Formalin-fixed paraffin-embedded skin biopsies, used for first-time diagnosis of early-stage MF, eMF and SS were collected. The study included 15 patients with early-stage MF, 14 with eMF and 11 with SS diagnosed in the period between 1981 and 2013. Patients with early-stage MF (stages IA–IB) were diagnosed and grouped in accordance with the International Society for Cutaneous Lymphomas/European Organization of Research and Treatment of Cancer (ISCL/EORTC) recommendations from 2007 (4). Early-stage patients have also been included in a previous study by Lindahl et al. (21). eMF and SS were categorized in accordance with the clinical guidelines at the time of diagnosis. A diagnosis of SS required erythroderma, compatible histology, and blood involvement of at least 5% of Sézary cells (25, 26). Relevant clinical variables, such as sex, age, and treatment at the time of diagnosis, were obtained from the patient files.

The study was approved by the local ethics committee (1-10-72-91-13) and the Danish Data Protection Agency (Datatilsynet 1-16-02-478-15).

RNA extraction and quantitative reverse transcription PCR (qRT-PCR) profiling

Biopsies were cut into 10- μ m tissue sections, and RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit (ThermoFisher Scientific/Applied Biosystems, Waltham, MA, USA) according to the manufacturer's guidelines. Total RNA quantity and quality controls were performed using a NanoDrop-1000 spectrophotometer. The extracted RNA was used for qRT-PCR-based miRNA profiling covering 383 human miRNAs, as described previously (21).

Statistical analysis

The miRNAs with signals close to or below the detection limit ($C_t > 37$) in most or all samples were excluded from further analyses, leaving 264 specific miRNAs for further analyses. Data were normalized by a global mean approach.

Data were visualized by heatmaps and unsupervised hierarchical clustering in QluCore Omics Explorer v.3.4 (QluCore AB, Lund, Sweden). Analysis of variance (ANOVA) was used to detect differentially expressed miRNAs between the 3 groups. Differences in miRNA expression levels were considered significant at > 1.5 fold-change (FC) and p -value < 0.05 . Adjustment for multiple testing was carried out by estimation of the false discovery rate (Q value). Target prediction and pathway analysis were performed by applying DIANA-miRPath v.3.0 (27).

RESULTS

Patient characteristics

Patient characteristics are listed in **Table I**. A total of 40 patients were identified; 15 with early-stage MF, 14 with eMF and 11 with SS. Median age at diagnosis was 66 years (range 47–88 years) in patients with early-stage MF, 76 years (range 51–94 years) in patients with eMF, and 74 years (range 55–86 years) in patients with SS

and did not differ between the patient groups (1-way analysis of variance (ANOVA), $p = 0.12$). Eleven patients with early-stage MF, 6 with eMF, and 3 with SS did not receive CTCL-directed therapy at the time of diagnosis. Topical treatment was assigned to 4 patients with early-stage MF, 8 with eMF, and 5 with SS. Systemic therapy was given to 3 patients with eMF, 3 with SS, and none of the early-stage MF patients at the time of diagnosis.

miRNA expression profile can discriminate erythrodermic mycosis fungoides from Sézary syndrome

The miRNA expression profile was distinctly different in skin biopsies from patients with eMF compared with patients with SS (**Fig. 1**). Twenty-seven miRNAs with strong ($FC > 1.5$) and significantly different expression in eMF vs. SS (**Fig. 1**) were identified. Fourteen miRNAs

Table I. Clinical characteristics

Pat. No.	Sex	Age at biopsy (years)	Treatment at time of diagnosis	Stage	T-stage
Mycosis fungoides T1 + T2					
1	F	70	No treatment	IB	2
2	M	66	No treatment	IA	1
3	F	88	No treatment	IB	2
4	M	66	Ultraviolet B	IA	1
5	M	71	No treatment	IB	2
6	M	60	Topical steroid	IA	1
7	M	67	No treatment	IA	1
8	F	58	Topical steroid	IA	1
9	M	71	No treatment	IB	2
10	F	72	No treatment	IB	2
11	M	85	Ultraviolet B	IB	2
12	M	50	No treatment	IB	2
13	F	51	No treatment	IB	2
14	F	65	No treatment	IB	2
15	M	47	No treatment	IA	1
Mycosis fungoides T4					
16	F	82	Oral steroid Topical steroid	IIIA	4
17	M	79	Topical steroid	IIIA	4
18	M	51	Etretinate Topical steroid	IIIA	4
19	F	83	Topical steroid	IIIA	4
20	M	68	No treatment	IIIA	4
21	M	74	Topical steroid	IIIA	4
22	M	84	Topical steroid	IIIA	4
23	M	76	Methotrexate Topical steroid	IIIA	4
24	M	60	No treatment	IIIA	4
25	M	75	No treatment	IIIA	4
26	F	94	No treatment	IIIA	4
27	M	69	No treatment	IIIA	4
28	M	80	No treatment	IIIA	4
29	M	65	Topical steroid	IIIA	4
Sézary syndrome					
30	M	55	Topical steroid		4
31	F	85	No treatment		4
32	M	75	No treatment		4
33	M	64	Topical steroid		4
34	M	65	Topical steroid		4
35	F	59	Topical steroid		4
36	F	83	No treatment		4
37	F	80	Topical steroid		4
38	M	80	Alemtuzumab		4
39	M	74	Oral steroid		4
40	M	64	Oral steroid Ciclosporin		4

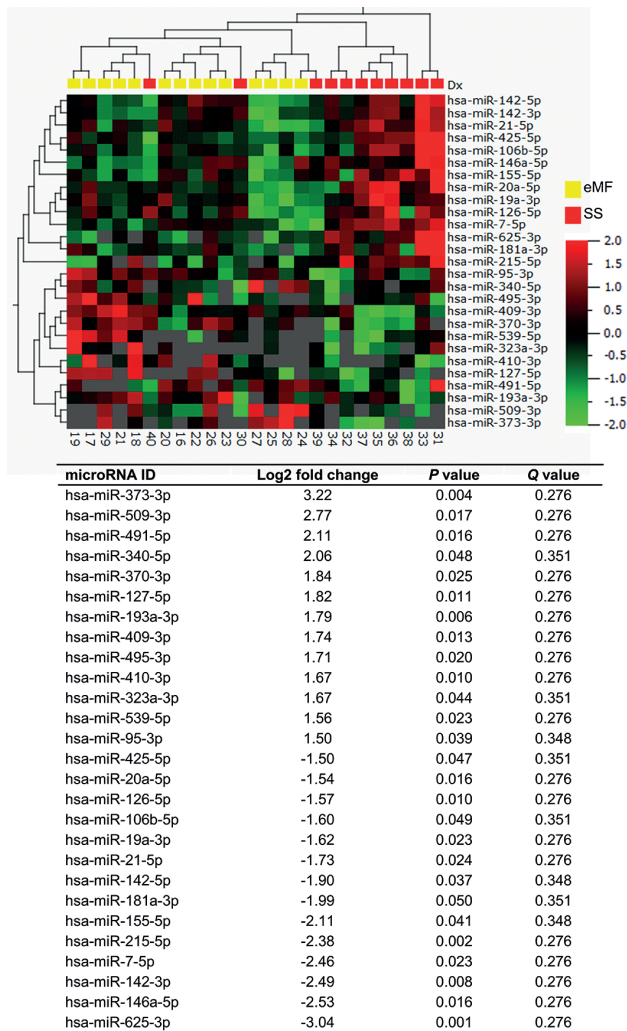


Fig. 1. Heatmap of significantly differentially expressed microRNAs between erythrodermic mycosis fungoides (eMF) and Sézary syndrome (SS). Heatmap and unsupervised 2-way hierarchical clustering based on the 27 microRNAs with strong (fold-change > 1.5) and significantly ($p < 0.05$) differential expression between eMF and SS.

were expressed at lower levels, and 13 were expressed at higher levels in eMF compared with in SS. Notably, miRNAs, such as miR-106b, miR-142, miR-155 and miR-21, which may play an important role in progression of CTCL (21, 22, 28, 29) had significantly lower expression levels in eMF compared with in SS. Unsupervised hierarchical clustering showed that the 27 differentially expressed miRNAs clearly separated eMF from SS (Fig. 1). Three patients with SS clustered with the eMF patients. Of note, 2 of these patients (patients 39 and 40) were treated with oral corticosteroids (daily dose 25 and 15 mg, respectively), whereas none of the other patients with SS received this treatment.

miRNA expression in erythrodermic mycosis fungoides (MF) and Sézary syndrome compared with early-stage MF

Next, miRNA expression in eMF and SS were compared with that in patients with early-stage MF. Twenty-eight

significantly differentially expressed miRNAs were identified in skin lesions from eMF compared with early-stage MF ($p < 0.05$, $FC > 1.5$) (Fig. 2a). Two-way unsupervised hierarchical clustering based on the 28 differentially expressed miRNAs discriminated eMF from early-stage MF, albeit with some overlap. Patients 16, 20, 22, 25 and 26 diagnosed with eMF sub-clustered with 4 patients with early-stage MF before clustering with the rest of the patients with eMF. This subdivision of the eMF disease group did not depend on disease stage (IA/IB) of the early-stage MF group, treatment at the time of diagnosis, or sex.

The miRNA expression in SS also differed from the expression pattern observed in early-stage MF. Thirty-four significantly differentially expressed miRNAs were identified (Fig. 2b, $p < 0.05$, > 1.5 FC, $Q < 0.30$), which separated the 2 disease entities, as illustrated by the unsupervised hierarchical clustering (Fig. 2b). However, the 2 patients with SS who were treated with oral corticosteroids (patients 39 and 40) clustered with the patients with early-stage MF. Thus, oral corticosteroid-induced skin improvement seems to induce changes in the miRNA expression pattern toward what is observed in early-stage MF.

Eleven miRNAs separated both eMF and SS from early-stage MF. Of these 11 miRNAs, 4 were expressed at significantly higher levels in SS and eMF vs. early-stage MF (miR-22-3p, miR-199a-3p, miR-199a-5p, miR-199b-5p), and 7 were expressed at significantly lower levels (miR-27b-5p, miR-328-3p, miR-342-3p, miR-433-3p, miR-483-3p, miR-484, and miR-663a).

Differentially expressed miRNAs between all patient groups

Fifty-four miRNAs were significantly differentially expressed between all patient groups ($p < 0.05$, $Q = 0.24$). In the heatmap and unsupervised hierarchical clustering based on these 54 miRNAs, the 3 disease entities, eMF, SS and early-stage MF, separated into distinct clusters, although with some overlap (Fig. 3). Interestingly, eMF displays miRNA features overlapping with both those of SS and of early-stage MF, although the hierarchical cluster analysis suggests eMF co-clustering with early-stage MF rather than with SS.

Differences in predicted miRNA-induced downstream pathway activation

Differences in the miRNA expression profile between eMF and SS may reflect differences in downstream pathway activation between eMF and SS. Therefore, we performed a pathway analysis using DIANA-tools and miRPath v3.0 based on the 27 miRNAs that were significantly differentially expressed between eMF and SS. Pathways of particular interest in CTCL are shown in Table II, indicating possible involvement of signalling

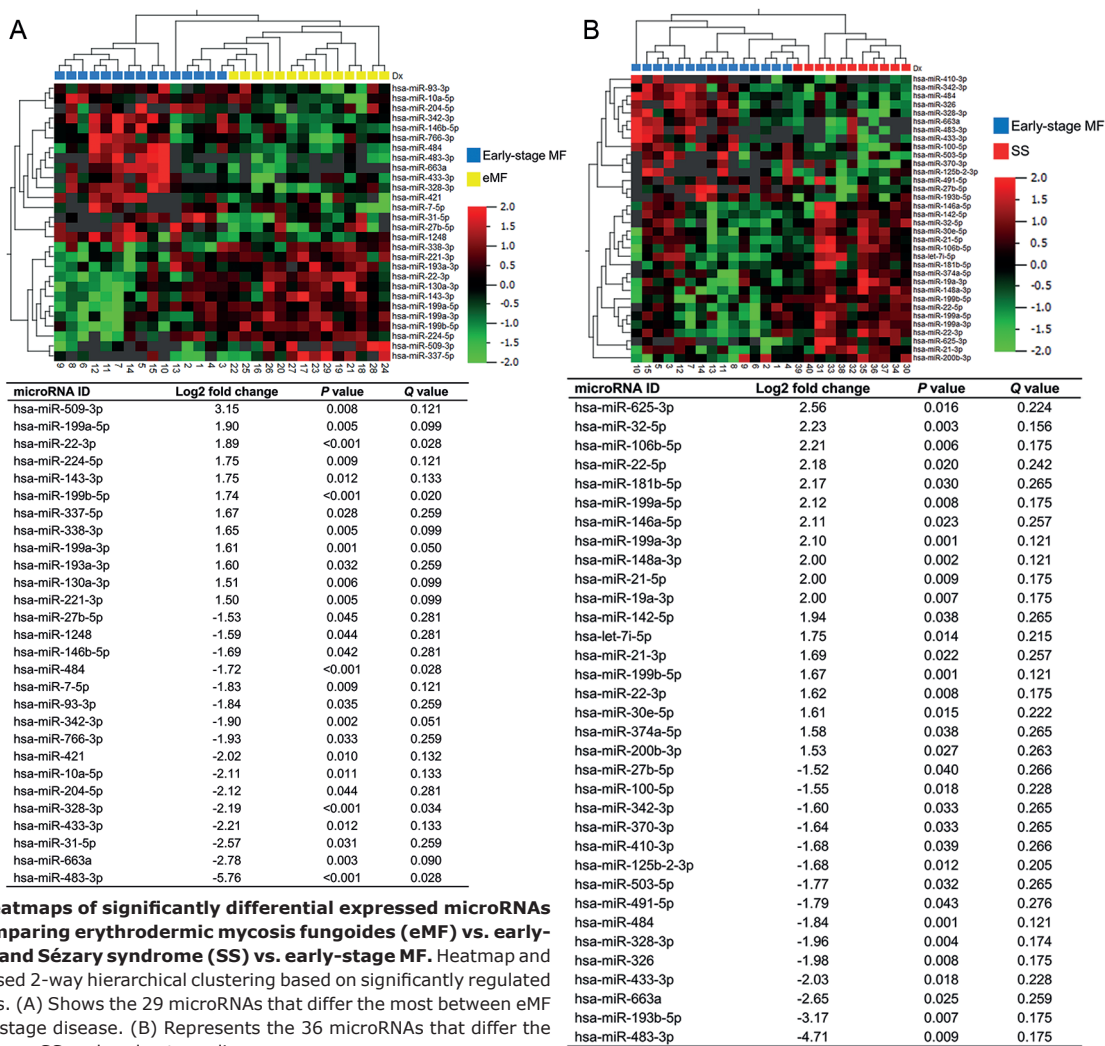


Fig. 2. Heatmaps of significantly differential expressed microRNAs when comparing erythrodermic mycosis fungoides (eMF) vs. early-stage MF and Sézary syndrome (SS) vs. early-stage MF. Heatmap and unsupervised 2-way hierarchical clustering based on significantly regulated microRNAs. (A) Shows the 29 microRNAs that differ the most between eMF and early-stage disease. (B) Represents the 36 microRNAs that differ the most between SS and early-stage disease.

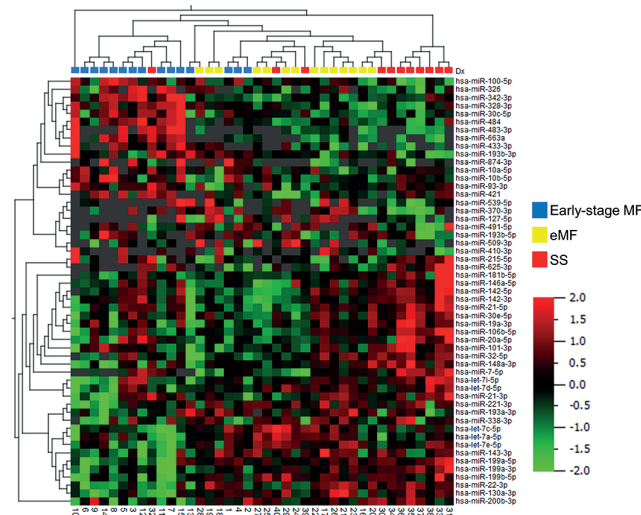
pathways associated with cancer (i.e. mitogen-activated protein kinase (MAPK), tumour necrosis factor (TNF), T-cell receptors, mechanistic target of rapamycin kinase (mTOR), and phosphatidylinositol 3-kinase-serine/threonine kinase (PI3K-Akt).

DISCUSSION

This study provides evidence that the miRNA expression profile in lesional skin can discriminate eMF from SS. Twenty-seven miRNAs separated the 2 disease entities and were significantly differentially expressed between eMF and SS. Moreover, the miRNA expression profile of eMF differs from both SS and early-stage MF; however, hierarchical clustering showed that eMF co-clustered with early-stage rather than SS.

It is essential to discriminate eMF from SS due to differences in treatment recommendation and prognosis, with patients with SS in stage B2 having significantly higher mortality rates (4, 30). Several studies have indicated that miRNAs are involved in the pathogenesis and disease

progression and serve as markers of diagnosis and prognosis in CTCL (18–21, 23, 29, 31). In this study, 19 out of the 27 significantly differentially expressed miRNAs between eMF and SS have previously been linked to CTCL (15, 16, 18, 21, 28, 32). In particular, miR-106b, miR-155 and miR-21 showed significantly higher expression levels in SS and have previously been associated with progression of CTCL: miR-106b was included in the prognostic miRNA classifier developed for early-stage CTCL (21) and miR-21 is induced by IL15 and STAT5, which are important regulators of proliferation and cell survival in CTCL (29, 33). Most widely investigated is the STAT5 regulated miR-155 (22). There is a higher expression of miR-155 in SS compared with MF (34). Expression levels of miR-155 increases with MF stage (35) and a recent identified diagnostic- and prognostic classifier includes miR-155 expression levels (36). Due to its role in constitutive activation of important intracellular signalling pathways, and proliferation of malignant T cells, miR-155 is a promising therapeutic target in CTCL (22, 37). Another potential miRNA target in CTCL is miR-214



microRNA ID	P value	Q value	microRNA ID	P value	Q value
hsa-miR-199b-5p	<0.001	0.005	hsa-miR-200b-3p	0.016	0.145
hsa-miR-199a-3p	<0.001	0.009	hsa-miR-21-5p	0.016	0.145
hsa-miR-484	<0.001	0.009	hsa-miR-7-5p	0.017	0.145
hsa-miR-22-3p	<0.001	0.021	hsa-miR-146a-5p	0.017	0.145
hsa-miR-483-3p	0.001	0.037	hsa-miR-142-3p	0.018	0.152
hsa-miR-328-3p	0.001	0.037	hsa-miR-21-3p	0.020	0.163
hsa-miR-373-3p	0.002	0.046	hsa-miR-338-3p	0.021	0.165
hsa-miR-199a-5p	0.002	0.046	hsa-let-7c-5p	0.023	0.172
hsa-miR-148a-3p	0.002	0.046	hsa-miR-181b-5p	0.023	0.172
hsa-miR-342-3p	0.002	0.046	hsa-miR-338-5p	0.027	0.188
hsa-let-7e-5p	0.002	0.058	hsa-miR-370-3p	0.027	0.188
hsa-miR-625-3p	0.004	0.088	hsa-miR-142-3p	0.028	0.189
hsa-miR-30c-5p	0.006	0.094	hsa-miR-100-5p	0.029	0.189
hsa-miR-663a	0.006	0.094	hsa-miR-127-5p	0.032	0.205
hsa-miR-215-5p	0.006	0.094	hsa-miR-10a-5p	0.037	0.228
hsa-miR-106b-5p	0.006	0.094	hsa-miR-30e-5p	0.037	0.228
hsa-miR-509-3p	0.006	0.094	hsa-miR-410-3p	0.038	0.230
hsa-let-7d-5p	0.006	0.094	hsa-miR-32-5p	0.039	0.230
hsa-miR-874-3p	0.007	0.101	hsa-miR-539-5p	0.040	0.230
hsa-miR-221-3p	0.008	0.107	hsa-miR-101-3p	0.041	0.230
hsa-let-7i-5p	0.009	0.116	hsa-miR-20a-5p	0.043	0.232
hsa-miR-193b-5p	0.010	0.116	hsa-miR-93-3p	0.043	0.232
hsa-miR-326	0.011	0.128	hsa-miR-193b-3p	0.044	0.232
hsa-miR-19a-3p	0.012	0.128	hsa-miR-10b-5p	0.045	0.232
hsa-miR-130a-3p	0.012	0.128	hsa-miR-142-5p	0.046	0.232
hsa-miR-491-5p	0.013	0.128	hsa-miR-421	0.047	0.235
hsa-miR-433-3p	0.014	0.138	hsa-let-7a-5p	0.050	0.235

Fig. 3. Heatmap of significantly differential expressed microRNAs between Sézary syndrome (SS), erythrodermic mycosis fungoides (eMF), and early-stage MF. Heatmap and unsupervised 2-way hierarchical clustering showing the 54 distinctive ($p < 0.05$) microRNAs between SS, eMF, and early-stage disease.

(38, 39). In addition, we found higher expression levels of miR-181a and miR-146a in the advanced stages of CTCL. Manso et al. showed a similar expression pattern and hypothesized miR-181a and miR-146a to play a role in disease progression (40).

Table II. Extrapolation of the significantly differently expressed microRNAs in the comparison of Sézary syndrome and erythrodermic mycosis fungoides skin using the mirPath v.3 database to enlighten the association to different genes in selected Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

KEGG pathway	p-value	Regulated genes n	miRNAs involved in KEGG pathways n
Pathways in cancer	1×10^{-6}	220	27
MAPK signalling pathway	8×10^{-5}	144	27
TNF signalling pathway	< 0.001	65	27
T-cell receptor signalling pathways	0.002	63	25
mTOR signalling pathway	0.002	39	25
PI3K-Akt signalling pathway	0.006	171	27

MAPK; mitogen-activated protein kinase; TNF; tumour necrosis factor; mTOR; mechanistic target of rapamycin kinase; PI3K-Akt; phosphatidylinositol 3-kinase-serine/threonine kinase.

Accordingly, the identified miRNA signature may play an important role in the pathogenesis and progression of CTCL. Moreover, the downstream signalling of the discriminative miRNAs between eMF and SS revealed signalling pathways fundamental in CTCL, which is indicative of the importance of these miRNAs (Table II).

Taken together, the present findings strongly indicate that miRNAs are important players in the CTCL pathogenesis and that the miRNA expression profile reflects the stage, aggressiveness, and disease entity of CTCL.

The study is limited by its retrospective design, and a prospective validation of the findings would have been preferred. Moreover, despite the rarity of CTCL, we would have preferred to include a larger number of patients.

The Danish registry system enabled identification of the specific biopsy used for the first diagnosis of eMF, SS, and early-stage MF and precise linkage to clinical characteristics of each individual patient, which strengthens the study. The technical and biological robustness of miRNAs in FFPE-preserved skin biopsies is a further strength of the study.

In conclusion, the miRNA expression profile in diagnostic skin biopsies can discriminate eMF from SS despite clinical and histological similarities. These results emphasize that eMF and SS represent distinct disease entities based on the miRNA signature of skin.

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Conflicts of interest. TL is employed at both University of Copenhagen and LEO Pharma A/S. NO is unpaid member of the Scientific Advisory Board, MiNDERA Corp., CA 94080, USA, with no financial interests or associations. LI served as a consultant and/or paid speaker for and/or participated in clinical trials sponsored by: AbbVie, Amgen, Astra Zeneca, BMS, Boehringer Ingelheim, Celgene, Centocor, Eli Lilly, Janssen Cilag, Kyowa, Leo Pharma, MSD, Novartis, Pfizer, Samsung, UCB. The other authors have nothing to declare.

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