MicroRNA Expression in Early Mycosis Fungoides Is Distinctly Different from Atopic Dermatitis and Advanced Cutaneous T-Cell Lymphoma

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Abstract. *Mycosis fungoides (MF) is the most common variant of cutaneous T-cell lymphoma (CTCL). MF is characterized by chronic inflammation dominated by cluster of differentiation 4-positive ($CD4^+$) T-cells and T helper 2 cytokines, and as the malignant T-cell clone is initially elusive, early diagnosis is often impossible. MF usually takes an indolent course, but for unknown reasons may turn into an aggressive disease with a poor prognosis. Herein, we used a global quantitative real-time polymerase chain reaction platform to study microRNA (miR) expression in patients with early MF (n=13), more advanced CTCL (n=42), and atopic dermatitis (AD, n=20). Thirty-eight miRs were differentially expressed (≥2-fold) in early MF vs. AD and 36 in early MF vs. more advanced disease. miRs that distinguish early MF from AD included both up-regulated (miR-155, miR-146a, 146b-5p, miR-342-3p, let-7i*) and down-regulated (miR-203, miR-205) miRs previously implicated in advanced CTCL. When comparing early MF to more advanced CTCL, additional miRs were significantly up-regulated including miRs which are part of the oncogenic miR-17/92, 106b/25 and 106a/363 clusters. In 16 patients for whom detailed follow-up data were available, 72 miRs were found differentially expressed between patients with progressive vs. those with non-progressive disease, again including miRs with a known relevance for lymphomagenesis, e.g. miR-155, miR-21, let-7i, miR-16, miR-142-3p, miR-146b-5p, miR-92a, miR-93 and miR-106a. In conclusion, we showed that early MF and AD display very different miR profiles despite their clinical, histological, and immunological similarities. During progression, an additional set of miRs becomes deregulated, suggesting their role in disease progression. These data suggest that miR profiling in CTCL may be a key to improving both diagnosis and risk prediction.*

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Key Words: Cutaneous T-cell lymphoma (CTCL), mycosis fungoides, microRNA, diagnosis.

Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphoma (CTCL). The neoplastic cells presumably originate from mature, skin-homing memory T-cells and the disease is histologically-characterized by the presence of epidermal and dermal infiltrates of atypical T-cells with irregular (cerebriform) nuclei. The early stages are characterized by persistent patches or plaques, but the disease can progress to tumor lesions and can transform to large T-cell lymphomas and disseminate to lymph nodes and internal organs (1).

Early recognition of MF has remained a major challenge owing to the fact that early MF and skin lesions in dermatitis are both characterized by infiltrating cluster of differentiation 4-positive ($CD4^+$) T-cells in an inflammatory environment dominated by T-helper cell 2 (Th2) cytokines. Several clinical and histological algorithms have been developed in an attempt to aid early diagnosis, but the specificity and sensitivity of these for early diagnosis in individual patients is limited (2). Similar limitations apply to phenotyping and T-cell receptor clonality testing. Unlike the advanced stages, the early lesions usually do not show phenotypic aberrances and often do not contain sufficient numbers of clonal T-cells (3, 4).

The pathogenesis of MF is largely unknown, but persistent antigen stimulation, aberrant expression of SRC kinases, abnormal regulation of cytokine receptor signaling, dysregulation of apoptotic pathways and epigenetic alterations have been implicated (1, 5-7). More recently, a central role for aberrant microRNA (miR) expression has also been suggested. miRs are small non-coding RNAs that regulate protein expression at the post-transcriptional level by mRNA degradation or translational repression. miRs are central to normal development and maintenance of homeostasis in adult tissues, and numerous investigations have shown that altered miR profiles are involved in the pathogenesis of both lymphomas and solid tumors (8, 9).

In CTCL, Valencak *et al*. reported that high expression of DICER is a negative prognostic factor (10). Furthermore, independent studies have shown that certain miRs are consistently differentially up-regulated (miR-155, miR-21, miR-199/214) or down-regulated (miR-223, miR-150) in CTCL and act as onco-miRs or tumor-suppressor miRs to promote proliferation, reduce apoptosis or enhance invasion and (10-17). miRs also appear to have potential as a diagnostic tool in CTCL (12, 18). We have previously used microarray technology to show that a classifier of five miRs (miR-326, miR-663b, miR-711, miR-203, miR-205) can discriminate between CTCL and benign inflammatory T-cell infiltrates with an accuracy of 97% (18). Furthermore, a quantitative real-time polymerase chain reaction (qPCR)-based minimal classifier of only three of these miRs (miR-155, miR-203, miR-205) discriminated between CTCL and benign inflammatory skin disorders with a classification accuracy above 90%, as shown in a subsequent study of an independent cohort of patients (19). Thus, qPCR-based miR profiling is a robust, reproducible technology for determining miR expression in skin samples, unlike miR microarrays, which are technically complicated, difficult to normalize, have a limited reproducibility and are hampered by false-positive and false-negative results (16, 18).

Taken together, these studies suggest that several miRs play key roles in CTCL pathogenesis. However, the available reports have, with very few exceptions (13,20), exclusively focused on advanced CTCL, *i.e*. tumor MF or Sezary syndrome (SS). No knowledge is available about the global miR expression profile in early MF. Similarly, it is unknown whether or how miR expression profiles differ between early MF and more advanced stages and whether miRs have any value as risk predictors in MF. In this study, we attempted to address these issues by using a global qPCR platform to compare the miR expression patterns in skin lesions from patients with early MF *vs*. atopic dermatitis (AD), early MF *vs*. more advanced disease, and progressive *vs*. stable MF plaque.

Materials and Methods

Patients and tissue samples. Formalin-fixed paraffin-embedded (FFPE) skin biopsies from a previously described cohort of 55 patients with CTCL sampled during the period 1985-2010 were retrieved from the archives of the Department of Pathology at the University Hospital of Copenhagen. The histological samples were revised and the clinical records were reviewed as described elsewhere (18, 19) to establish the diagnosis and stage in accordance with the WHO classification (21) and the EORTC/ISCL staging system (22). Thirteen patients had MF stage 1 with limited patches and plaques (<10% of the body surface area); 23 patients had MF stage 2 with more extensive (>10%) patch/plaque lesions; two patients had MF stage 3 with tumor lesions; and three patients had MF stage 4 with generalized erythroderma. Fourteen patients with primary cutaneous peripheral T-cell lymphoma, not otherwise specified (PTL, NOS), were also included because this disease resembles advanced/transformed MF with respect to the histological features and clinical behavior. FFPE tissue from 20 patients with AD was collected from the archives at Bispebjerg Hospital and as part of a clinical trial at Leo-Pharma.

RNA extraction. RNA was extracted from six 10-μm sections of FFPE material using the RecoverAll kit (Ambion/Life Technologies, Carlsbad, CA, United States of America), following the manufacturer's guidelines. The RNA yield and quality was checked on a NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, United States of America). All samples displayed absorbance ratios of 260/280 above 1.6 and were included in the qPCR analysis.

qRT-PCR. Forty nanograms of total RNA from each patient was reverse transcribed to cDNA using the Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark). The cDNA was diluted ×80 and 4 μl was used in SYBR green mastermix in miR ready-to-use PCR panels I and II version 2R covering 742 human miRs (miRbase 16). The cDNA and mastermix were transferred to the panels using a pipetting robot. Amplification was carried out using a LightCycler 480 (Roche, Basel, Switzerland) in 2×384 well plates. The Roche LightCycler software was used to calculate the crossing point (Cp) value for each miR using the second derivative method. miRs detected at fewer than 5 Cps compared to negative control or at more than 37 Cps were excluded from the analysis. Post PCR, the individual PCR products were subjected to melting curve analysis to evaluate the specificity of amplification. Melting curves with a single peak in the expected range were accepted, discrepancies from these criteria resulted in exclusion from further analysis. Finally, the PCR efficiency was evaluated using an algorithm similar to the linereg software (23). Efficiencies below 1.6 were omitted from further analysis. On average, 144 miRs fulfilled the above mentioned criteria in each sample.

Statistics. Using the NormFinder software (24), the best way to normalize qPCR data is to use the mean value of all expressed miRs for a given panel. The expression level of each miR was normalized to this value. Relative miR expression was calculated using the $2^{-\Delta\Delta C_T}$ method (25). Fold-change was calculated as the difference between mean Cp values between different sub-diagnoses *e.g.* mean value of miR-155 for all MF1 samples *vs.* mean value of miR-155 for all AD samples. The significance of difference between expression levels were assessed by Student's *t*-test. *p*-Values of less than 0.05 were considered significant; *p*-values between 0.05 and 0.10 were considered of borderline significance.

Ethics. The project was approved by local ethical committees (H-B-2009-045 and H-1-2009-111) and the Data Protection Agency (Datatilsynet J.NR. 2010-41-4303). Biopsies from the patients with AD were collected after informed consent according to the Helsinki declaration.

Results

We used a global qPCR platform to compare miR expression profiles in skin lesions from 13 patients with early MF with limited patches/plaques *vs.* lesions from 20 patients with AD and 42 patients with more advanced CTCL. The results are summarized in Figure 1. Details of individual miRs are listed in Tables I-III together with selected previous investigations relating primarily to studies of CTCL and other lymphomas. Furthermore, we have compared miR expression in 16 patients with MF plaque with progressive *vs.* stable disease. These results are summarized in Table IV.

MF1 versus AD. The result from the qPCR analysis of skin lesions from 13 MF1 patients and 20 AD patients showed 21 differentially up-regulated (>2-fold) and 17 differentially down-regulated (>2-fold) miRs in MF1 compared to AD (Figure 1A). Three of these (miR-155, miR-203, miR-205) have previously been identified as a classifier for distinction between CTCL and benign inflammatory dermatoses (18, 19), and 16 have been described in advanced CTCL, including either SS or tumor MF (Tables I and II). The remaining miRs have been not previously been associated with CTCL or other lymphomas, but some have been identified in systemic lymphoma or acute leukemia as shown in Tables I and II. Others have been associated with solid tumors (mir-149, miR-186, miR-605, miR-663, miR-664, miR-940), angiogenesis (miR-19b) (57), or immune reactions of potential relevance to MF (miR-302c, miR-331-3p) (27,28).

MF1 versus advanced disease. As a next step in the investigation, we compared miR expression profiles in early MF1 with 42 patients with more advanced CTCL, including MF2, MF3, MF4 and cPTL, NOS. Fifteen miRs were differentially (>2-fold) expressed in MF2 compared to MF1 (Figure 1B) and 26 were differentially expressed (>2-fold) in more advanced disease with tumors or erythroderma (Figure 1C). Out of these, six were down-regulated, whereas the remaining miRs were up-regulated, suggesting that up-regulation of miRs is more commonly associated with advanced disease than down-regulation, in keeping with another investigation of tumor MF (12). As shown in Table III, five miRs were deregulated both in early MF and advanced stages (miR-155, miR-181a, miR-146b-5p, miR-766, miR-205). The remaining miRs listed in Table III were selectively deregulated in the more advanced stages, including several (miR-17, miR-25, miR-92a-1*, miR-93, miR-106a, and miR-106b*) encoded by the 17/92, 106b/25, and 106a/363 oncogenic miR clusters (9), as well as many previously associated with advanced CTCL, *e.g.* miR-15b, miR-17, miR-20a, miR-21, miR-25, miR-31, miR-92a-1*, miR-93, miR-106b*, miR-107, miR-191, miR-425, and miR-769-5p (11, 12). Others were novel in the context of CTCL, but have been associated with other subtypes of cutaneous or systemic lymphoma and leukemia as listed in Table III.

MF1 and MF2, progressive vs. non-progressive disease. In an attempt to elucidate whether miR expression profiling has any predictive value, we selected 16 patients with MF with limited (n=7) or more advanced (n=9) plaque lesions based upon the availability of sufficiently detailed information about the disease course and behavior. Seven patients had stable, non-progressive disease including five with limited plaques and two with more extensive lesions. The remaining nine patients, including one with limited and eight with more extensive plaques at diagnosis had progressive disease. Comparison between these two groups showed significantly differential (>2-fold) expression of 72 miRs, 70 of which were up-regulated (Table IV). Twenty-six of these have previously been identified as risk predictors in an investigation of 22 patients with SS (Table IV) and 10 (miR-21, miR-155, miR-17, miR-20a, miR-25, miR-106b, miR-107, miR-142-5p, miR-191, and miR-769-5p) have been associated with advanced disease in this and previous studies, as shown in Table III.

Discussion

miRs are small non-coding RNAs involved in the regulation of protein expression at the post-transcriptional level. miRs are central to many cellular functions altered in transformed cells, *e.g.* proliferation, apoptosis, differentiation, migration and angiogenesis, and altered miR expression patterns have been identified in a vast number of cancer types, including CTCL. miR studies in CTCL have, with very few exceptions (13, 18, 20), exclusively focused on advanced MF or SS (a disseminated form of CTCL with malignant cells in the skin, lymph nodes and blood). No knowledge is available about the global miR expression pattern in early MF. Similarly, it is not known whether or how the global miR profile in early MF differs from that of more advanced lesions, nor whether miR profiling has any value as a predictive tool in MF.

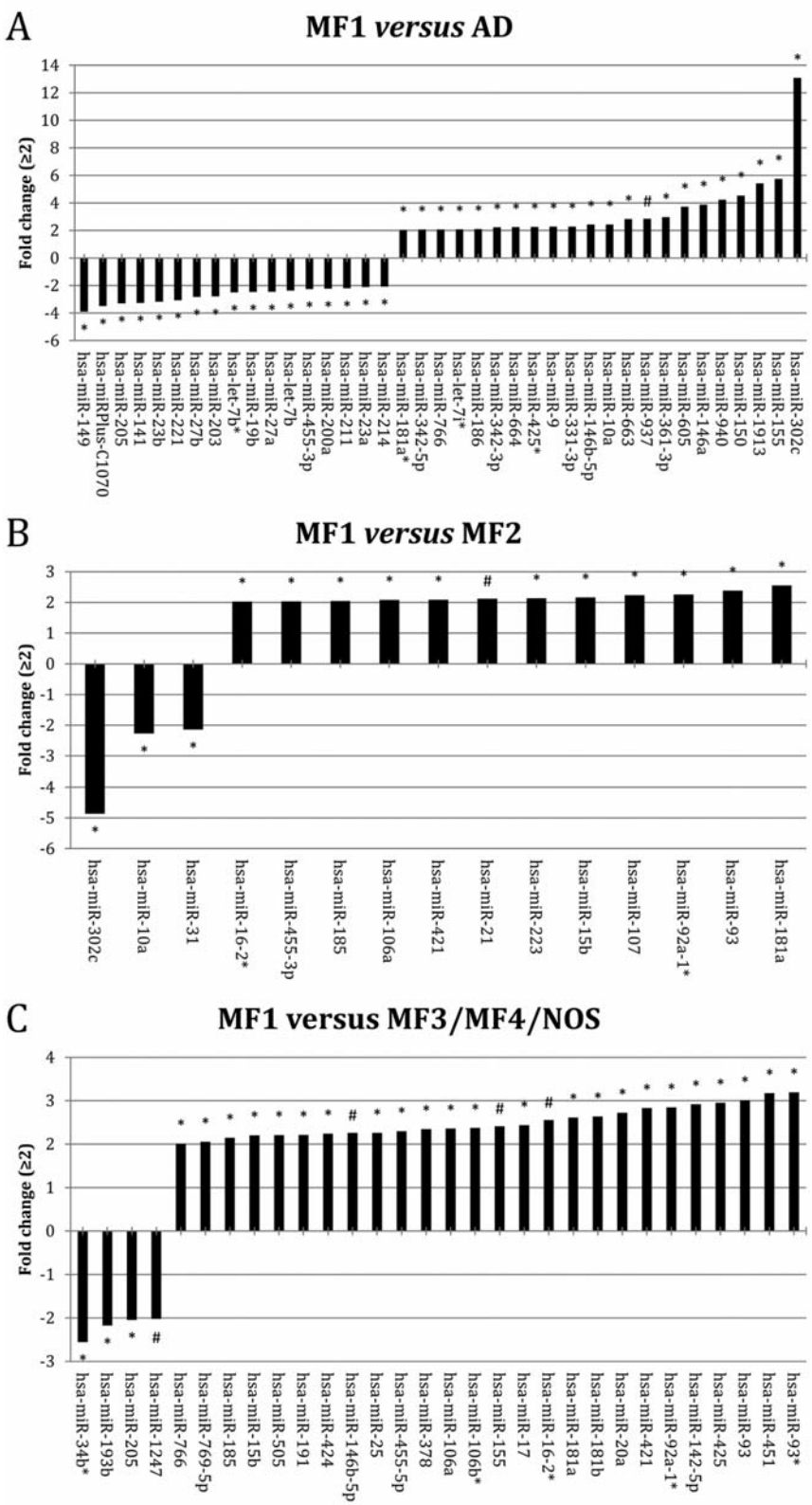

Figure 1. *miRs de-regulated when comparing: A: early-stage mycosis fungoides (MF1) vs. atopic dermatitis (AD); B: MF1 vs. more advanced (stage 2) MF (MF2); C: MF1 vs. advanced (stages 3 and 4) MF (MF3, MF4) and primary cutaneous peripheral T-cell lymphoma, not otherwise specified (PTL, NOS). All miRs displayed ≥2-fold change; #borderline significance, 0.10>p≥0.05; *significant, p<0.05.*

Table I. *Selected miRs differentially up-regulated (≥-fold) in early MF (MF1) vs. atopic dermatitis (AD) in this study compared with other investigations.*

miR, this study	Previously associated with	Previous reported expression level	Selected validated functions, effects/targets	Ref.
↑miR-155	MF, SS, NHL, HL	Up-regulated	Promotes proliferation, targets SHIP1-PIP3-AKT, associated with poor outcome in SS	(8, 11, 18)
↑miR-146a	MF, SS	Up-regulated		(11, 12)
↑miR-342-3p	MF, cALCL	Up-regulated	miR-342 inhibits apoptosis in SS	(12, 49)
↑let-7i*	MF, SS	Up-regulated	Associated with poor outcome in SS	(11, 12)
↑miR-181a*	MF, T-ALL	Up-regulated	Enhances NOTCH induced transformation in T-ALL, targets negative regulators downstream of NOTCH	(12, 45)
↑miR-425*	MF (miR-425-5p)	Up-regulated		(12)
↑miR-146b-5p	MF (miR-146b)	Up-regulated		(12)
↑miR-342-5p	MF, SS	Up-regulated (MF) Down-regulated (SS)	miR-342 inhibits apoptosis in SS	(11, 12, 14)
↑miR-150	SS	Down-regulated	Inhibits invasion and metastasis, targets CCR6	(14, 17)
↑miR-9	HL, activated CD4 cells	Up-regulated	Increases growth of HL, enhances IL-2 production in T-cells, targets DICER, PRDM1, BCL-6	(35, 36)
↑miR-10a	AML	Up-regulated	Decreases apoptosis	(50)
↑miR-766	AML	Up-regulated	Decreases apoptosis, targets BAX	(51)

AKT: AKT kinase; AML: Acute myeloid leukemia; BAX: BCL2-associated X protein; BCL-6: B-cell lymphoma 6 protein; cALCL: cutaneous anaplastic large cell lymphoma; CCR6: Chemokine receptor 6; HL: Hodgkin lymphoma; IL-2: interleukin 2; MF: mycosis fungoides; NHL: non-Hodgkin lymphoma; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate; PRDM1: PR domain zinc finger protein 1; SHIP1: Inositol polyphosphate-5-phosphatase; SS: Sézary syndrome; T-ALL: T-cell acute lymphoblastic leukemia; *Significant at *$p<0.05$.

Table II. *Selected miRs differentially down-regulated (≥2-fold) in early MF (MF1) vs. atopic dermatitis (AD) in this study compared with other investigations.*

miR, this study	Previously associated with	Previous reported expression level	Selected validated functions, effects/targets	Ref.
↓miR-203	CTCL, NHL, MPN, MM, carcinomas	Down-regulated	Epigenetically inactivated, enhances proliferation, inhibits apoptosis	(18, 33)
↓miR-205	CTCL Carcinomas	Down-regulated Down-/up-regulated	Tumor suppressor or onco-miR in carcinomas, targets TF in EMT	(18, 34)
↓miR-23b	SS	Down-regulated		(11)
↓let-7b/7b*	SS	Down-regulated		(11)
↓miR-214	SS	Up-regulated	Promotes cell survival and inhibits apoptosis in SS	(11, 14, 16)
↓miR-221	MF	Up-regulated		(12)
↓miR-211	MF	Up-regulated		(12)
↓miR-27b	cALCL	Up-regulated (*vs.* MF tumor)		(49)
↓miR-200a	MALT-lymphoma	Down-regulated	Cyclin E2	(52)
↓miR-455-3p	sALCL, ALK–neg	Down-regulated (*vs.* PTL, NOS)		(53)
↓miR-27a	ALL	Down-regulated		(54)
↓miR-23a	ALL	Down-regulated		(54)

ALK: Anaplastic lymphoma kinase; ALL: acute lymphoblastic leukemia; cALCL: cutaneous anaplastic large cell lymphoma; CTCL: cutaneous T-cell lymphoma; EMT: epithelial–mesenchymal transition; NHL: non-Hodgkin lymphoma; MALT: mucosa associated lymphoid tissue; MF: mycosis fungoides; MM: multiple myeloma; MPN: myeloproliferative neoplasm; PTL, NOS: peripheral T-cell lymphoma, not otherwise specified; sALCL: systemic anaplastic large cell lymphoma; SS: Sézary syndrome; TF: transcription factors. **Significant at $p<0.05$.

In the present study, we attempted to address these issues. This was approached by using a global qPCR platform to compare miR profiles in early MF with these in limited patches/plaques (MF1) *vs.* AD and *vs.* more advanced CTCL, including MF with extensive plaques (MF2), tumors (MF3), or erythroderma (MF4) and primary cutaneous PTL, NOS, which resembles MF tumor histologically and clinically.

Table III. *Selected miRs differentially expressed (≥2-fold change) in advanced mycosis fungoides (MF) with extensive plaques (MF2), tumors (MF3), erythroderma (MF4), primary cutaneous peripheral T-cell lymphoma, not otherwise specified (cPTL, NOS) vs. early MF (MF1) with limited patches/plaques.*

This study		Previous studies			
Differentially expressed in	MiR	Previously associated with	Expression	Selected validated functions and/or targets	Refs
MF1, MF3, MF4, PTL, NOS	↑miR-155#	Advanced MF, HL, NHL	Up-regulated	Promotes proliferation, targets SHIP1-PIP3-AKT, associated with poor outcome in SS	(8, 11, 18)
	↑miR-181a				
	↑miR-181a/b	MF, T-ALL	Up-regulated	Enhances NOTCH induced transformation in T-ALL, targets negative regulators downstream of NOTCH	(12, 45)
	↑miR-146b-5p#	Advanced MF	Up-regulated		(12)
	↑miR-766	AML	Up-regulated	Decreases apoptosis, targets BAX,	(51)
	↓miR-205	CTCL Carcinomas	Down-regulated Up-/down-regulated	Tumor suppressor or onco-miR in carcinomas, targets TF in EMT	(18, 34)
MF2, MF3, MF4, cPTL, NOS	↑miR-15b	Advanced MF	Up-regulated		(12)
	↑miR-92a-1*	Advanced MF	Up-regulated		(12)
	↑miR-93	Advanced MF	Up-regulated		(12)
	↑miR-106a	Murine T-cell lymphomas	Up-regulated		(55)
MF2	↑miR-21#	Advanced MF, SS, HL, NHL	Up-regulated	Decreases apoptosis, targets PTEN, associated with poor outcome in SS	(8, 11, 12, 41)
	↑miR-107	Advanced MF	Up-regulated	Associated with poor outcome in SS	(11,12)
	↑miR-223	MF, SS	Down-regulated	E2F, TOX	(13, 14)
	↓miR-31	SS	Down-regulated	Promotes invasion/metastases	(11)
MF3, MF4, PTL, NOS	↑miR-25	Advanced MF	Up-regulated	Associated with poor outcome in SS	(11, 12)
	↑miR-20a	SS	Up-regulated	Associated with poor outcome in SS	(11)
	↑miR-17	Advanced MF, SS, NHL, ALL, AML	Up-regulated	Associated with poor outcome in SS	(9, 11, 12)
	↑miR-93*	Advanced MF	Up-regulated		(12)
	↑miR-106b*	SS	Up-regulated	Targets PTEN, associated with poor outcome in SS	(11, 41)
	↑miR-142-5p	SS	Up-regulated	Associated with poor outcome in SS	(11)
	↑miR-191	Advanced MF, SS	Up-regulated	Associated with poor outcome in SS	(11, 12)
	↑miR-378	AML	Up-regulated		(56)
	↑miR-424	T-ALL	Up-regulated		(57)
	↑miR-425	Advanced MF, cALCL	Up-regulated		(12,49)
	↑miR-451	sALCL, ALK-neg	Down-regulated (*vs.* PTL, NOS)		(53)
	↑miR-455-5p	sALCL, ALK-neg	Down-regulated (*vs.* PTL, NOS)		(53)
	↑miR-769-5p	SS	Up-regulated	Associated with poor outcome in SS	(11)
	↓miR-34b*	BL (miR-34b)	Down-regulated	cMYC	(58)
	↓miR-193b	SS	Down-regulated		(11)

AKT: protein kinase B; AML: Acute myeloid leukemia; ALK: anaplastic lymphoma kinase; BAX: BCL2-associated X protein; BL: Burkitt lymphoma; CTCL: cutaneous T-cell lymphoma; EMT: epithelial–mesenchymal transition; HL: Hodgkin lymphoma; MF: mycosis fungoides; NHL: non-Hodgkin lymphoma; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate; PTL, NOS: peripheral T-cell lymphoma, not otherwise specified; sALCL: systemic anaplastic large cell lymphoma; SHIP1: Inositol polyphosphate-5-phosphatase; T-ALL: T-cell acute lymphoblastic leukemia; TF: transcription factors; TOX: Thymocyte selection-associated high mobility group box protein. #Borderline significance, $0.10>p\geq0.05$;*significant at $p<0.05$.

The results indicate that early MF exhibits differential up- or down-regulation of many miRs compared to AD and, importantly, the majority of deregulated miRs are of potential relevance to the pathogenesis (Tables I and II). We have already reported on increased expression of miR-155 and decreased expression of miR-203 and miR-205 in these patients (18, 19) and, as expected, the present study using a qPCR platform for global miR expression confirms this observation, supporting the notion that up-regulation of miR-155 combined with down-regulation of miR-203 and miR-205 is a useful diagnostic marker, even in the early MF stages with very subtle clinical and histological findings.

Table IV. *miRs significantly (p<0.05) deregulated when comparing progressive vs. non-progressive early-stage mycosis fungoides (MF1 and MF2). miRs shown in bold have also been associated with poor outcome in a previous investigation of Sezary Syndrome (40).*

miR	Progressive/ non-progressive	dCp	FC
hsa-miR-125a-3p	Non-progressive	–1.270	–2.411
hsa-miR-1247	Non-progressive	–1.229	–2.343
hsa-miR-30b	Progressive	1.009	2.013
hsa-miR-505	Progressive	1.016	2.023
hsa-miR-24	Progressive	1.020	2.028
hsa-miR-21*	Progressive	1.032	2.046
hsa-miR-374a	Progressive	1.057	2.081
hsa-miR-320a	Progressive	1.068	2.096
hsa-miR-29a*	Progressive	1.087	2.125
hsa-miR-484	Progressive	1.095	2.137
hsa-miR-125b	Progressive	1.104	2.149
hsa-miR-497	Progressive	1.124	2.180
hsa-miR-140-5p	Progressive	1.130	2.188
hsa-miR-199a-5p	Progressive	1.146	2.213
hsa-miR-22	Progressive	1.150	2.219
hsa-miR-154	Progressive	1.158	2.231
hsa-let-7g	Progressive	1.166	2.243
hsa-let-7c	Progressive	1.191	2.283
hsa-miR-342-3p	Progressive	1.224	2.335
hsa-miR-15a	Progressive	1.250	2.378
hsa-miR-191	Progressive	1.263	2.400
hsa-miR-17	Progressive	1.341	2.533
hsa-miR-143	Progressive	1.346	2.542
hsa-let-7a	Progressive	1.350	2.549
hsa-miR-28-5p	Progressive	1.369	2.582
hsa-miR-23a	Progressive	1.379	2.600
hsa-miR-142-5p	Progressive	1.380	2.603
hsa-miR-221	Progressive	1.380	2.603
hsa-miRPlus-C1070	Progressive	1.384	2.609
hsa-miR-15b	Progressive	1.385	2.611
hsa-miR-493*	Progressive	1.403	2.644
hsa-miR-10b	Progressive	1.411	2.659
hsa-miR-30c	Progressive	1.421	2.677
hsa-miR-29c	Progressive	1.423	2.682
hsa-miR-223	Progressive	1.428	2.690
hsa-miR-501-5p	Progressive	1.441	2.716
hsa-miR-103	Progressive	1.446	2.724
hsa-miR-222	Progressive	1.471	2.771
hsa-miR-331-3p	Progressive	1.489	2.808
hsa-miR-1979	Progressive	1.541	2.909
hsa-miR-29b	Progressive	1.541	2.911
hsa-miR-106a	Progressive	1.543	2.914
hsa-miR-339-5p	Progressive	1.558	2.944
hsa-miR-26a	Progressive	1.622	3.078
hsa-miR-195	Progressive	1.623	3.079
hsa-miR-423-3p	Progressive	1.658	3.155
hsa-miR-455-3p	Progressive	1.666	3.173
hsa-miR-29a	Progressive	1.670	3.182
hsa-miR-126	Progressive	1.723	3.302
hsa-miR-425	Progressive	1.733	3.323
hsa-miR-199a-3p	Progressive	1.818	3.525
hsa-miR-101	Progressive	1.828	3.552
hsa-miR-150	Progressive	1.839	3.578
hsa-miR-30e*	Progressive	1.843	3.588
hsa-miR-326	Progressive	1.847	3.597
hsa-miR-26b	Progressive	1.869	3.653
hsa-miR-107	Progressive	1.928	3.804
hsa-let-7f	Progressive	1.978	3.940
hsa-miR-93	Progressive	1.988	3.968
hsa-miR-708	Progressive	1.991	3.975
hsa-miR-196b	Progressive	2.138	4.401
hsa-miR-766	Progressive	2.151	4.441
hsa-miR-146b-5p	Progressive	2.265	4.808
hsa-miR-25	Progressive	2.267	4.813
hsa-miR-196a	Progressive	2.302	4.931
hsa-miR-142-3p	Progressive	2.348	5.092
hsa-let-7i	Progressive	2.354	5.114
hsa-miR-155	Progressive	2.624	6.166
hsa-miR-34a	Progressive	2.793	6.930
hsa-miR-185	Progressive	2.834	7.133
hsa-miR-16	Progressive	2.846	7.188
hsa-miR-21	Progressive	3.053	8.302

FC: Fold change. * All miRs in the table were significant at $p<0.05$.

The function of miR-155 has been studied in considerable detail. By contrast, knowledge of the functions of miR-203 and miR-205 for CTCL is limited. It is well-known that miR-155 is central to normal T-cell differentiation and can act as an onco-miR both in lymphoma induction in mice and in human lymphoma, including in addition to CTCL, Hodgkin lymphoma and different types of B-cell lymphoma (8, 12). Since miR-155 in CTCL promotes proliferation, is expressed *in situ*, and is driven by activated signal transducer and activator of transcription 5 (STAT5) (15, 29), it is conceivable that miR-155 is indeed involved in progression in all stages of the disease (30, 31). miR-203 is important for normal keratinocyte differentiation (32) and is silenced by promoter hypermethylation in many tumors including B- and T-cell lymphomas (33). miR-203 has several interesting validated targets, *e.g.* survivin, p63, and cAMP response element-binding protein , and preliminary observations indicate that it is also a tumor suppressor in the context of CTCL (Ralfkiaer U, unpublished observations). miR-205 is important for differentiation of both squamous and glandular epithelium and can act as both an onco-miR and a tumor suppressor in solid tumors, presumably by targeting important transcription factors involved in epithelial–mesenchymal transition (34). No knowledge is available about the functional significance of miR-205 in lymphomagenesis.

Other significantly up- or down-regulated miRs in early MF *vs*. AD in the current study (miR-146a, miR-342-3p, miR-342-5p, let-7i*, miR-181a*, miR-425, miR-23b, let-7b, and miR-221) have been reported to be deregulated in advanced MF or SS (11, 12, 20), suggesting a putative role of these miRs both in early malignant transformation and disease progression. Interestingly, we also identified a series of differentially expressed miRs in early MF, which are novel in the context of MF. Some of these have been linked to other malignancies or to immune regulation of potential relevance to MF. For example, miR-9 is expressed in Hodgkin lymphoma (35) and can promote production of interleukin-2 (36), a cytokine central to MF pathogenesis (37) that has been linked to growth and survival of malignant T-cells (38). Indeed, interleukin-2 receptors are highly expressed by malignant T-cells and constitute a potential target for treatment with toxin-coupled antibodies (Ontak, Eisai Inc., Woodcliff Lake, NJ, United States of America) (39). Other miRs up-regulated in early MF have been implicated in natural killer cell activation (miR-331-3p) (28), resistance to killing by natural killer cells (miR-302c) (27) and angiogenesis (miR-19b) (26), key features of MF indicating a putative oncogenic role of these miRs in early MF (40).

Many additional deregulated miRs have been associated with advanced MF or SS compared to benign controls in other reports, including miR-15b, miR-17, miR-21, miR-25, miR-31, miR-92a-1*, miR-93, miR-106b*, miR-107, miR-191, miR-425, and miR-769-5p (11, 12). The present study confirms and extends these observations by showing enhanced expression in advanced disease compared with early MF, indicating that these miRs are likely to be involved in disease progression. Interesting, several of these miRs are encoded by the 17/92, 106a/363, 106b/25 oncogenic miR clusters that are up-regulated in many types of systemic lymphoma, leukemia and solid tumors (9). Very limited information is available about the function of these miRs in CTCL, and conflicting results have been obtained in different studies. Narducci *et al*. (11) and Cristofoletti *et al*. (41) showed that miR-106b is up-regulated in SS and targets Phosphatase and tensin homolog (PTEN), consistent with a potential oncogenic function. By contrast, Ballabio *et al*. found that miR-17-5p, miR-19a, miR-92 and miR-106a are down-regulated and act to decrease apoptosis and enhance proliferation in SS cells (14). The reasons for these discrepancies are unclear.

More consistent results have been obtained respecting the functions of other dysregulated miRs associated with advanced CTCL in this and other reports. For example, it has been shown that miR-21 is up-regulated in both advanced MF and SS and is induced by STAT3 (42), which is constitutively activated in MF (43). miR-21 targets a known tumor-suppressor (PTEN) that is down-regulated in SS and to a lesser extent in advanced MF and acts to inhibit Phosphatidylinositol-4,5-bisphosphate 3-kinase/ AKT kinase-induced apoptosis (41). Likewise, the miR-199/214 cluster has been shown to be up-regulated in blood cells in patients with SS compared to circulating T-cells from patients with benign, inflammatory erythroderma, and to promote cell survival and inhibit apoptosis in CTCL cell lines (11, 14, 16). By contrast up-regulation of miR-199/214 was not observed in this study nor another of MF skin biopsies (12), indicating that miR-199/214 is primarily associated with leukemic CTCL. Similarly, down-regulation of miR-150 has been identified in an animal model of advanced, metastatic CTCL, and in primary samples of blood from two patients with SS and involved lymph nodes from 10 patients with disseminated MF (17). In the present investigation, miR-150 was up- rather than down-regulated, suggesting that the tumor-suppressor function of miR-150 is not relevant to the pathogenesis of the early lesions. This is consistent with the observation that miR-150 targets a chemokine receptor (CCR6) that is involved in invasion and metastasis (17) and is not expressed in MF biopsies (44).

An oncogenic function is also suggested for miR-181a/b which acts in synergy with NOTCH to induce T-cell acute lymphoblastic leukemia in a murine model by inhibiting negative feedback regulators downstream of NOTCH1 (45). NOTCH1 is ectopically expressed *in situ* in advanced MF skin lesions, and inhibition of NOTCH activation by gamma-secretase inhibitors induces apoptosis and reduces both NOTCH and miR-181a in CTCL-derived cell lines (46, 47). By contrast, miR-223 has been shown to be down-regulated in both SS, advanced MF and to a lesser extend in early MF (13, 14). miR-223 targets known oncogenes such as *E2F1* and its down-regulation results in enhanced proliferation, suggesting that miR-223 has tumor-suppressor properties in CTCL. However, in this study, miR-223 was up- rather than down-regulated. Up-regulation of miR-223 has been identified in T-cell acute lymphoblastic leukemia in which miR-223 has oncogenic functions and targets PTEN to reduce apoptosis (48). However, whether this observation has any relevance in the context of CTCL is unknown.

Very few studies have addressed the potential value of miR profiling for risk prediction in CTCL. The present investigation indicates that many miRs are deregulated in progressive *vs*. stable plaque stage MF, several of which have also been identified as predictors of poor outcome in a study of 22 patients with SS, including for example miR-21, miR-155, miR-17, miR-25 and miR-106b (11). Thus, it is possible that miR profiling may have an impact in the clinical setting, although more comprehensive studies addressing this issue are clearly required.

In conclusion, the present study provides first evidence for a unique global miR expression profile in early MF skin lesions *vs*. AD and the first evidence for a unique miR

profile in advanced CTCL *vs*. early disease, suggesting a role for dysregulated miRs in both early malignant transformation and disease progression. Our results also suggest that miR profiling may help improve risk prediction in MF, although further studies are obviously required to validate this possibility in more comprehensive patient series.

Acknowledgements

This work was supported by research funding from The Danish Foundation for Advanced Technology (Højteknologifonden), The Carlsberg Foundation (Carlsbergfondet), The Danish Research Councils, The Lundbeck Foundation, The Danish Cancer Society (Kræftens Bekæmpelse), Dansk Kræftforsknings Fond, The Novo Nordic Foundation, Fabrikant Vilhelm Pedersen og Hustrus Mindelegat, The A. P. Moeller Foundation, The University of Copenhagen, the National Cancer Institute (grant CA89194, M.A.W.), LEO Pharma A/S and Exiqon A/S.

Conflicts of Interest

CB. Ahler, T. Litman and C. Glue are or have been employed by Exiqon A/S. T. Litman, CB. Ahler, T. Marstrand and MA. Røpke are or have been employed by LEO Pharma A/S. The remaining Authors declare no competing financial interests.

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Received August 12, 2014
Revised September 7, 2014
Accepted September 8, 2014