Diagnostic microRNA profiling in cutaneous T-cell lymphoma (CTCL)

Ulrik Ralfkiaer,#1,2 Peter H. Hagedorn, #3 Nannie Bangsgaard,4 Marianne B. Løvendorf,3,4 Charlotte B. Ahler,3 Lars Svensson,3 Katharina L. Kopp,1,5 Marie T. Vennegaard,5 Britt Lauenborg,5 John R. Zibert,3 Thorbjørn Krejsgaard,5 Charlotte M. Bonefeld,5 Rolf Søkilde,1,5 Lise M. Gjerdrum,6 Tord Labuda,3 Anne-Merete Mathiesen,1,5 Kirsten Grønbæk,2 Mariusz A. Wasik,7 Malgorzata Sokolowska-Wojdylo,8 Catherine Queille-Roussel,9 Robert Gniadecki,6 Elisabeth Ralfkiaer,10 Carsten Geisler,5 Thomas Litman,1,5 Anders Woetmann,1,5 Christian Glue,11 Mads A. Røpke,3 Lone Skov,4 and Niels Odum,1,5

#these authors contributed equally to the study

1Department of Biology, University of Copenhagen (UoC), Copenhagen, Denmark; 2Department of Hematology, State University Hospital, Denmark; 3LEO Pharma A/S, Ballerup, Denmark; 4Department of Dermato-Allergology, Gentofte University Hospital, (UoC), Denmark; 5Department of International Health, Immunology and Microbiology, (UoC), Copenhagen, Denmark; 6Department of Dermatology, Bispebjerg University Hospital, Denmark; 7Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA; 8Department of Dermatology University of Gdansk, Gdansk, Poland; 9Centre de Pharmacologie Clinique Appliquée à la Dermatologie, Hopital l'Archet, Nice, France; 10Department of Pathology, State University Hospital, Copenhagen, Denmark; 11Exiqon A/S, Vedbæk, Denmark.

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Correspondence: Niels Odum; Immunology, ISIM, 22.5.34 Panum; Department of Biology and Department of International Health, Immunology and Microbiology, University of Copenhagen; Blegdamsvej 3c, DK-2200 Copenhagen N, Denmark, Telephone +45 35 32 78 68, Fax +45 35 32 78 76, E-mail: ndum@sund.ku.dk

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Abstract

Cutaneous T-cell lymphomas (CTCL) are the most frequent primary skin lymphomas. Nevertheless, diagnosis of early disease has proven difficult due to a clinical and histological resemblance to benign inflammatory skin diseases. To address if microRNA (miRNA) profiling can discriminate CTCL from benign inflammation, we studied miRNA expression levels in 198 patients with CTCL, peripheral T-cell lymphoma (PTL), and benign skin diseases (psoriasis and dermatitis). Using microarrays we show that the most induced- (miR-326, miR-663b, miR-711) and repressed- (miR-203, miR-205) miRNAs distinguish CTCL from benign skin diseases with > 90% accuracy in a training set of 90 samples and a test set of 58 blinded samples. These miRNAs also distinguish malignant and benign lesions in an independent set of 50 patients with PTL and skin inflammation and in experimental human xenograft mouse models of psoriasis and CTCL. QRT-PCR analysis of 103 patients with CTCL and benign skin disorders validates differential expression of 4 out of the 5 miRNAs and confirms previous reports on miR-155 in CTCL. A qRT-PCR-based classifier consisting of miR-155, miR-203, and miR-205 distinguishes CTCL from benign disorders with high specificity, sensitivity, and a classification accuracy of 95% indicating that miRNAs have a high diagnostic potential in CTCL.
Introduction

Cutaneous T-cell lymphomas (CTCL) are the most frequent primary lymphomas of the skin,\(^1\) with mycosis fungoides (MF) being the most prevalent clinical form accounting for around 60% of new cases.\(^2\) In early disease stages, which can last several years, MF presents as flat erythematous skin patches resembling inflammatory diseases such as dermatitis or psoriasis. In later stages, MF lesions gradually form plaques and overt tumors and may disseminate to lymph nodes and internal organs. The early skin lesions contain numerous inflammatory cells, including a large quantity of T cells with a normal phenotype as well as a small population of T cells with abnormal morphology and a malignant phenotype. The infiltrate primarily consists of non-malignant T helper 1 (Th1) cells, regulatory T cells (Treg), and cytotoxic CD8\(^+\) T cells, which to some degree seem to control the malignant T cells.\(^3,5\) The malignant T cells typically exhibit the phenotype of mature CD4\(^+\) memory T cells and are normally of clonal origin.\(^6\) T cells with a malignant phenotype are characterized by epidermotropism and are preferentially present in the upper parts of the skin, whereas T cells with a normal phenotype primarily are detected in the lower portions of the dermis. The epidermal T cells are sometimes found in patterns of Pautrier micro-abscesses, which are collections of T cells adherent to dendritic processes of Langerhans cells. During disease development, the epidermotropism is gradually lost concomitant with an increase in malignant, and a decrease in non-malignant, infiltrating T cells.

The etiology of CTCL remains poorly understood, and occupational exposures, infectious agents, and genetic mutations have been proposed as etiological factors, but no evidence of causation has been provided.\(^7\) Instead, an aberrant expression and function of transcription factors and regulators of signal transduction is a characteristic feature of CTCL. Accordingly, it has been hypothesized that a dysfunctional regulation of signal molecules and cytokines plays a key role in the malignant transformation and epigenetic modifications such as aberrant gene methylation and histone de-acetylation are clearly involved in the pathogenesis of CTCL.\(^8-11\) Recent data indicate that several small, non-coding RNA molecules, microRNAs (miRNAs), are differentially expressed and
possibly also involved in the pathogenesis of this disease. Thus, miR-21 expression is upregulated and appears to play a role in the regulation of apoptosis in malignant T cells obtained from patients with Sézary Syndrome (SS), a leukemic variant of CTCL. These findings are in keeping with studies in other cancers where miRNAs have been ascribed a key role in cancer development and metastasis. Indeed, specific miRNAs are directly involved in the malignant transformation as they can function as oncogenes and tumor suppressors.

Early diagnosis of CTCL has important consequences concerning therapeutic options and determination of prognosis. Currently, the diagnosis is primarily based on clinical observations and histological examinations of skin biopsies as well as additional laboratory tests such as analysis of T-cell receptor (TCR) clonality by polymerase chain reaction (PCR). Unfortunately, early diagnosis of CTCL has proven difficult because of the great clinical, pathological, and histological resemblance to benign inflammatory skin diseases and because inflammatory skin disorders can be associated with clonal TCR rearrangements. From the initial appearance of skin lesions to a certain MF diagnosis is established, it takes a median of almost 6 years. Because miRNA expression profiling can distinguish other cancers according to diagnosis and developmental stage of the tumor to a greater degree of accuracy than traditional mRNA analysis, the present investigation was undertaken to address whether miRNA expression profiling has a diagnostic potential in relation to CTCL.
Materials and Methods

Selection of patients

The study includes formalin-fixed and paraffin-embedded biopsies from 63 patients with cutaneous T-cell lymphoma, 39 patients with nodal PTL (Not otherwise specified = NOS) and 96 patients with benign skin diseases including psoriasis, atopic dermatitis, contact dermatitis, and unspecified dermatitis as well as skin from two healthy volunteers. Biopsies from the lymphoma patients were sampled during the period 1979-2004 and were collected from the archives at the Departments of Pathology at Rigshospitalet, Bispebjerg Hospital, Aalborg Sygehus and Herlev Hospital. From all lymphoma cases, tissue samples were reviewed by histology and immunohistochemistry, using as a minimum CD3, CD4, CD8, CD30, CD56, TIA-1 and Granzyme B stains. The samples were then classified in accordance with the WHO-EORTC17,20-22 guidelines and the clinical characteristics of the cohort were reviewed to establish the final diagnoses. Biopsies from the patients with benign skin diseases and healthy controls were collected after informed consent in accordance with the Declaration of Helsinki at the Department of Dermato-Allergology, Gentofte Hospital Department of Dermatology, Bispebjerg Hospital, Department of Pathology, Rigshospitalet, and as part of clinical trials at LEO Pharma A/S and approved by the local ethical committees (H-B-2009-045 and H-1-2009-111) and the Data Protection Agency (Datatilsynet J.NR. 2010-41-4303).

Human psoriasis xenograft mouse models

Three patients with chronic plaque-psoriasis were used as donors for the psoriatic keratome biopsies (thickness 0.5 mm). Keratome biopsies were taken from infiltrated red plaques located on the anterior or lateral aspect of the femoral region.23 Each biopsy was divided into four pieces of 1.5 × 1.5 cm. As recipients, female CB.17 SCID mice (M&B Taconic, Denmark) aged 6 weeks were used. The split human keratome biopsies were then grafted onto the back of the anesthetized animals and the grafts were protected by a bandage during the following two weeks in a-pathogen-free (SPF) environment as described elsewhere.24 After two weeks, the animals were randomized into two groups. The groups were treated with betamethasone (0.5 mg/g (as dipropionate; BDP) in
ointment \((n = 6)\) or ointment vehicle \((n = 6)\) twice daily. After 4 weeks of treatment, the animals were bled and sacrificed, and a 4-mm punch biopsy was taken from each xenograft. Biopsies were fixed in 10% neutral buffered formalin for a maximum of 48 hours and were processed according to standard histological procedures and embedded in paraffin.

**CTCL xenograft model**

The murine xenograft model of CTCL is based on the immunodeficient NOD.Cg-Prkdc<sup>scid</sup>B2m<sup>tm1Unc/J</sup> strain \((\text{NOD/SCID-B}2\text{m}^{-/-})\) (The Jackson Laboratory, Bar Harbor, ME, USA) as described in detail elsewhere.\(^{25}\) In brief, NOD/SCID-B2m<sup>−/−</sup> mice were inoculated subcutaneously into each flank with \(1 \times 10^6\) MyLa2059 cells and when a mouse had established palpable tumors it was allocated alternately to a group receiving vehicle or a group receiving Celecoxib. The mice received 10 µg/g Celecoxib \((\text{Selleck Chemicals, Houston, Texas, USA})\) or vehicle intra-peritoneally daily and the tumor growth was monitored continuously by slide caliper measurements. All animal experiments were carried out in accordance with the local ethics committee and with animal welfare guidelines provided by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

**RNA extraction**

Total RNA was isolated from six 10 µm tissue sections using the RecoverAll Total Nucleic Acid Isolation Kit \((\text{Applied Biosystems/Ambion, USA})\) according to manufacturer guidelines. Total RNA quantity and quality were checked by spectrophotometer \((\text{Nanodrop ND-1000})\).

**miRNA microarray**

From each sample 100 ng of total RNA was labeled with Hy3 fluorescent dye using the miRCURY LNA Array power labeling kit \((\text{Exiqon, Denmark})\). All samples were labeled the same day with the same master mix, in order to minimize technical variation. The Hy3-labelled samples were hybridized to miRCURY LNA arrays \((\text{v11.0})\) \((\text{Exiqon, Denmark})\), containing capture probes targeting all human miRNAs registered in the miRBASE version 15.0 at the Sanger Institute. The hybridization was performed overnight at 56°C according to manufacturer specifications using a Tecan HS4800 hybridization station \((\text{Tecan, Austria})\). Since it was not possible to hybridize all
arrays in one go, samples were randomly split into 5 batches as to minimize day-to-day variation in
the hybridization process. After hybridization the microarray slides were scanned using an Agilent
G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) at 5µm resolution, and
the resulting TIFF images were analyzed using the ImaGene 8.0 software on standard settings
(BioDiscovery, Inc., USA). All microarray data are available on the Gene Expression Omnibus
(GEO) under accession number GSE31408.

miRNA real-time qRT-PCR

Ten ng RNA was reverse transcribed in triplicate 10µl reactions using the miRCURY LNA™
Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon, Denmark). cDNA
was diluted 50 x and assayed in 10 µl PCR reactions according to the protocol for miRCURY
LNA™ Universal RT miRNA PCR; each miRNA was assayed once by qPCR. Negative controls
excluding template from the reverse transcription reaction were performed and profiled in parallel.
The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384
well plates. The amplification curves were analyzed using the Roche LC software, both for
determination of Cp (by the 2nd derivative method) and for melting curve analysis. All assays were
inspected for distinct melting curves and the Tm was checked to be within known specifications for
the assay. Furthermore, assays must be detected with 3 Cp’s less than the negative control, and with
Cp<39 to be included in the data analysis. Data that did not pass these criteria were omitted from
any further analysis. MiR-103 and miR-423-5p were stably expressed and the average of their Cp’s
in each sample was used as normalization factor.

Microarray data preprocessing

Probe signals were background corrected by fitting a convolution of normal and exponential
distributions to the foreground intensities using the background intensities as a covariate. Four
technical replicate spots for each probe were combined to produce one signal by taking the
logarithmic base-2 mean of reliable spots. If all four replicates for a given probe were judged
unreliable that probe was removed from further analysis. A reference data vector R was calculated
as the median signal of each probe across all samples. For all probe signals in a given sample, represented by the sample data vector $S$, a curve $F$ was determined by locally weighted polynomial regression so as to provide the best fit between $S$ and $R$.\textsuperscript{26} A normalized sample vector $M$ was calculated from this by transforming it with the function $F$, so that $M = F(S)$. In this manner, all samples were normalized to the reference $R$. This normalization procedure largely follows that outlined in.\textsuperscript{27} Remote data points (probes in sparsely sampled intensity regions with less than 15 probes per signal unit) were considered unreliably adjusted by this method and removed before further analysis.

**Classifier statistics**

Significance of differences in expression levels was assessed by a two-sided unpaired $t$-test. Class prediction was done using nearest shrunken centroid classification.\textsuperscript{28} Briefly, a standardized centroid is computed for each class as the average expression of each miRNA in each class divided by the within-class standard deviation for that miRNA. The miRNA expression profile of a new sample is then compared to each of these class centroids, and the class, whose centroid is closest in Euclidean distance, is the predicted class for that new sample. The algorithm is trained by shrinking class centroids towards the overall centroid for all classes by a threshold amount that minimizes the misclassification error as determined through 10-fold cross validation on the training set.
Results

miRNA expression profiling using microarrays

To address whether miRNA expression profiling can differentiate CTCL from benign chronic skin disorders such as psoriasis and dermatitis, as well as from normal skin, we initially used microarrays to perform miRNA profiling of 148 FFPE samples. Of these samples, 63 were from patients with various forms of CTCL and 85 were from patients with benign inflammatory skin diseases or healthy individuals (BDN) (Table 1). The samples were divided into 3/5 for training (n=90) and 2/5 for testing (n=58) with approximately equal proportion of CTCL to BDN samples in both sets. This division follows the five microarray production batches used in the study (Table 1). Out of the 688 miRNAs that passed preprocessing filtering criteria, initial statistical analysis of the training set identified 27 miRNAs showing strong (at least 50% change) and highly significant (Bonferroni corrected \( P \)-values <0.001 from \( t \)-test) differences between CTCL and benign skin diseases and normal skin (Fig. 1). Thus, the expression levels of a large number of miRNAs differ considerably between patients with CTCL and patients with BDN. Essentially similar results were obtained using unsupervised hierarchical clustering based on the 209 most variable miRNAs (Suppl Fig. 1S).

Identification of a CTCL- specific miRNA signature

To find a CTCL-specific signature we analyzed the training set with a nearest shrunken centroid algorithm,\(^ {28}\) using only the top 3 most induced (miR-326, miR-663b, miR-711) and repressed (miR-203, miR-205, miR-718) miRNAs among the 27 highly significant miRNAs as identified in Fig. 1. All six miRNAs had Bonferroni corrected \( P \)-values < \(10^{-8}\) by \( t \)-test. After shrinking of centroids (Suppl. Fig. 2S), samples in the training set could be classified with 93% accuracy (84% sensitivity and 100% specificity, \( P < 0.001\) by Fisher’s exact test, (Fig. 2A and B)). Shrinkage of centroids identified an optimal threshold value at 5.135 (dashed vertical line in Suppl. Fig. 2S, A). At this threshold value, the relative contribution of individual miRNAs in the classifier is shown in Suppl. Fig. 2S, B. As shown in Suppl. Fig. 2S, B, miR-718 was shrunk to zero, indicating that it did not
contribute to the classification and, accordingly, removed from the classifier. The final classifier consequently consisted of five miRNAs. To assess the performance of the five miRNAs in the classification of unknown samples, we used the already trained classifier on the 58 test set samples, and here achieved 97% classification accuracy (92% sensitivity and 100% specificity, P < 0.001 by Fisher’s exact test(Fig. 2C and D)). Fig. 3A shows the expression of the individual miRNAs in the classifier. For each of the five miRNAs, the normalized log2 expression values are grouped according to patient type (CTCL and BDN, respectively) (Fig. 3A).

Next, we evaluated the robustness of the classifier by ten-fold cross-validation, each time selecting different batches as training and test set (but keeping the ratio 3/5 to 2/5). The above approach identified miR-203, miR-663b, miR-205, and miR-711 in almost all cases (Suppl. Table 1). One miRNA, miR-326, was only selected in 2 out of 10 divisions. Importantly, no matter which division was chosen, the classification accuracy in the test set was consistently above 90% (93.1% in average with a 99% confidence interval between 90.5% and 95.6%) (Suppl. Table 1).

As shown in Fig. 1, several other miRNAs (besides those included in the classifier) discriminate to some degree between CTCL and BDN. To evaluate the potential of these miRNAs as classifiers we repeated the selection and training procedure as outlined above, but excluding the 5 miRNAs from the input set. This identified a new set of miRNAs for which we recorded the classification accuracies in the training and test set. We repeated this 4 more times, each time excluding the miRNAs selected for the classifier in the last round. As shown in Suppl. Fig. 3S, the accuracy in the test set decreased rapidly: When the best 23 miRNAs were excluded, the 4 miRNAs selected only achieve 67% accuracy in the test set. Even the second-best set of classifier miRNAs (those identified in round 2) clearly performed worse in the test set than the 5-miRNA classifier actually used (Suppl. Fig. 3S). Also, combining the miRNAs identified in round 1 and 2 into one 9-miRNA classifier did not improve the performance either (data not shown). Taken together, these findings show that the 5-miRNA classifier consisting of miR-326, miR-663b, miR-711, miR-203, and miR-205 performed best when compared to the other classifiers examined.
Extension and validation of the miRNA classifier

CTCL is characterized by primary involvement of the skin but other PTLs may secondarily disseminate to the skin and give rise to skin lesions with resemblance to CTCL. To address whether the miRNA classifier also distinguishes between PTLs and benign inflammatory skin disorders, microarray analysis was performed on lesions from patients with PTL (NOS) (39 patients), and 11 patients with benign disorders (dermatitis (6 patients) and psoriasis (5 patients)). As shown in Fig. 4, using the 5-miRNA classifier, 10 out of 11 patients with benign skin disorders were classified as benign whereas 39 out of 39 PTL patients were classified as malignant resulting in an overall classification accuracy of 98% (p < 0.001) (Fig. 4). Fig. 5 shows the expression levels of each individual classifier miRNA in all examined patients divided into clinical subgroups. The overall dichotomy between malignant and benign disorders also applied to each clinical subgroup of patients. However, the expression of individual miRNAs clearly varied within each subgroup and there was also a considerable overlap in expression levels of each individual miRNA in malignant and benign disorders (Fig. 5). Recently, miR-21 was identified as differentially expressed and involved in anti-apoptosis in malignant T cells from SS patients as compared to peripheral CD4+ T cells in healthy individuals.29 We also observed an increased expression of miR-21 in CTCL when compared to most benign disorders (Suppl. Fig. 4S). However, miR-21 was also highly increased in psoriatic lesions and in some dermatitis lesions (Suppl Fig. 4S) confirming other studies30 and explaining why miR-21 is not identified as a classifier miRNA.

Classification of treated and untreated CTCL and psoriasis from xenograft models and patients

As xenograft mouse models are momentous for the development of novel therapies, we examined the expression of the classifier miRNAs in tumors and skin lesions from human xenograft mouse models of CTCL and psoriasis, respectively.25 A principal component analysis (PCA) plot of samples from 19 mice with human xenograft CTCL lesions and 12 mice with human psoriatic skin xenograft lesions based on the 5-miRNA profile (Fig. 6A) shows similar organization and
separation of samples as with the clinical samples (Figs 2A, 2C and 4A). Accordingly, when the 5-miRNA classifier is applied, 17 out of 19 samples from the xenograft CTCL model and 12 out of 12 samples from the xenograft psoriasis model were classified as malignant and benign, respectively, i.e. with a 94% overall accuracy (p < 0.001 (Fig. 6B)). Interestingly, the most differentially expressed of the 5 miRNAs, miRNA 203 and miR-205, in the xenografts were also the most differentially expressed miRNAs is the clinical samples (Fig. 6C versus Fig. 5).

Nine out of the 19 mice in the CTCL xenograft group had been treated with daily i.p. injections of 10 µg/g Celecoxib®, an inhibitor of the cyclooxygenase-2 (COX-2) which significantly inhibited tumor growth.31 Likewise, six out of the 12 mice in the psoriasis xenograft group were treated daily with topical glucocorticoid (betamethasone dipropionate). As shown in Fig. 6C, the expression levels of three of the miRNAs (miR-203, miR-663b, and miR-711) were not influenced by the Celecoxib or betamethasone whereas the expression of miR-205 and miR-326 was inhibited by betamethasone but unaffected by Celecoxib. However, the changes were relatively small and did not interfere with the malignant/benign classification (Fig. 6B) indicating that the 5-miRNA classifier was relatively insensitive to treatment-induced changes in miRNA expression.

To address whether betamethasone treatment influenced the expression of the five miRNAs in psoriasis patients, we compared the expression in 5 patients treated with vehicle and 3 patients treated daily with topical betamethasone. As shown in Suppl. Fig. 5S, four weeks of topical steroid treatment improved the clinical score but had little influence on the miRNA expression levels and miRNA-based classification in psoriasis patients confirming the observations in xenograft human psoriatic skin (Fig. 6).

**Identification of a qRT-PCR-based miRNA classifier**

For diagnostic purposes, qRT-PCR is often considered more sensitive, specific, and applicable than microarrays. To confirm the microarray results above, expression levels were measured for the 5-miRNA signature on a subset of 103 samples by qRT-PCR. Samples were selected based on high RNA content and covered both training and test set samples. MiR-103 and miR-423-5p were
identified as the most stably expressed references across samples and their average Cp was used as normalization factor when calculating ΔCp. The differential expression was clearly confirmed for miR-203, miR-205, and miR-326 with $P$-values below $10^{-13}$ and for miR-663b with a $P$-value below $10^{-7}$ (Fig. 3B) whereas the last miRNA, miR-711 could not be measured reliably above background fluorescence (Suppl. Fig. 6S). Essentially similar results were obtained in an independent series of qRT-PCR experiments on 44 patient samples using a different qRT-PCR platform (TaqMan, data not shown). Recent studies on skin lesions from tumor stage MF and blood samples from SS patients reported on a differential expression of miR-155, miR-21, miR-24, miR-34b, miR-191, miR-486, miR-214, Let-7b, and other miRNAs. Accordingly, we performed qRT-PCR for these miRNAs and confirmed a differential expression of miR-155, miR-24, miR-191, and Let-7b (Fig. 7, and data not shown). In contrast, miR-34b did not achieve significance in the qRT-PCR measurements whereas miR-21 was increased in CTCL but also in a fraction of psoriasis patients (Suppl. Fig. 4S, and data not shown). The nearest shrunken centroid algorithm identified miR-155, miR-203, and miR-205 as the most discriminative set of miRNAs. By rewriting the equations for nearest centroid classification as an equivalent linear combination, and simplifying, we write the discriminant function, or sample score, as: $S = Cp(\text{miR-155}) - Cp(\text{miR-203})/2 - Cp(\text{miR-205})/2$.

We chose thresholds by inspecting the distribution of samples scores (Fig. 7A and B) and the ROC curve (Fig. 7C), and introduced a low-confidence region around the threshold (Fig. 7A and B). As miR-203 and miR-205 expression was decreased in CTCL (Fig. 7D) and miR-155 expression increased in CTCL (Fig. 7D), the score $S$ was smaller in CTCL when compared to benign skin disorders (Fig. 7A and B). In 103 samples, this qRT-PCR-based “minimal” miRNA classifier (miR-155, miR-203, and miR-205) distinguished patients with CTCL from benign skin diseases with 95% classification accuracy ($P<0.001$, Fig. 7B) and high sensitivity/specificity as illustrated by the ROC graph in Fig. 7C (dot indicates 91% sensitivity at 97% specificity). Importantly, MF patients were classified as malignant independently of the disease stage (Suppl Fig. 7S).
Discussion

In the present study we used microarrays for an initial screening of miRNAs with a potential ability to distinguish between malignant (CTCL) and benign inflammatory disorders such as psoriasis, atopic dermatitis, and contact dermatitis. Five miRNAs (miR-203, miR-205, miR-326, miR-663b, and miR-711) were identified, which discriminated with high accuracy (>90%) between malignant and benign conditions in a total of 198 patients including an initial training set of 90 patients, a test set of 58 patients and an independent cohort of 50 patients. Importantly, the expression pattern of four out of five miRNAs was verified using qRT-PCR on 103 patients. Likewise, 31 samples from human xenograft mouse models of CTCL and psoriasis were also classified with more than 90% accuracy. Moreover, the classification was relatively resistant to topical treatment of psoriatic skin with steroids. Taken together these findings indicate that miRNAs can distinguish between CTCL and benign skin disorder with a high accuracy and a high level of robustness.

Recent studies on subpopulations of CTCL patients identified miR-21 and miR-155 as differentially expressed in SS patients and advanced (tumor-stage) MF, respectively.\textsuperscript{29,37} The aberrant expression of miR-21 expression was driven by Signal Transducer and Activator of Transcription-3 (STAT3), which is aberrantly activated in CTCL and involved in anti-apoptosis.\textsuperscript{38} Accordingly, it was a surprise that we did not identify miR-21 as differentially expressed in CTCL. However, miR-21 expression was indeed significantly increased in CTCL when compared to dermatitis and non-lesional skin from psoriasis patients. In contrast, miR-21 was not increased in CTCL when compared to lesional skin from psoriasis patients. On the contrary, miR-21 expression was equal to and often higher in psoriatic lesions than in CTCL. The increased miR-21 expression in lesional psoriasis might easily explain why we did not pick-up miR-21 as one of the highly differentially expressed miRNAs in CTCL. Our observation of a high miR-21 expression in psoriatic lesions is also in agreement with recent reports on an increased miR-21 expression in psoriatic skin lesions.\textsuperscript{30} Our findings do not, however, argue against an important role of miR-21 in the pathogenesis in CTCL but suggest that miR-21 might not be suited for this diagnostic purpose.
In advanced MF, van Kester et al.\textsuperscript{37} identified several miRNAs as differentially expressed in tumor stage MF from 19 patients as compared to 12 patients with benign skin diseases. The majority of miRNAs were also identified as differentially expressed in our array analysis. Since they did not report on miR-203, miR-205, miR-326, miR-663b, and miR-711 (and raw data were not presented) we have not been able to verify whether these miRNAs were also able to distinguish between CTCL and benign disorders in their patient cohort. Interestingly, miR-155 was the most significantly up-regulated in the microarray data by van Kester et al. and a series of studies have previously identified miR-155 as differentially expressed and linked to the pathogenesis of other lymphomas.

We were therefore puzzled that we did not identify miR-155 as differentially expressed in CTCL in our microarray analysis. A possible explanation could relate to differences in patient subgroups and control populations as observed for miR-21. This was, however, not the case. Accordingly, we performed qRT-PCR for miR-155 and were able to confirm and extend the findings of van Kester et al. that miR-155 was aberrantly expressed in all subgroups of CTCL. Importantly, our qRT-PCR results also showed that miR-155 was one of the most significantly differentially expressed miRNAs in CTCL. At present, it is unclear why miR-155 was not identified as differentially expressed in our microarray analysis. As the LNA-based microarray platform used here has previously been used to identify miR-155 as differentially expressed in the NCI-60 cell line panel\textsuperscript{39}, it is unlikely that the difference is due to platform related issues. We therefore speculate that cross-hybridization or presence of pre-miR-155 might have masked the signal of the mature miR-155 because the hybridization probes do not discriminate between mature and pre-miRNAs in the microarray platform. Our data strongly underline the importance of qRT-PCR validation of microarray data and support the notion that in case of miR-155, qRT-PCR assays are more sensitive than microarrays.

For diagnostic purposes, qRT-PCR is not only more sensitive and specific but also more applicable than microarrays. Using the nearest shrunken centroid algorithm, miR-155, miR-203, and miR-205 were identified as the most discriminative set of miRNAs, and a score formulated as the difference
between the Cp of miR-155 and the average Cp of miR-203 and miR-205 found to distinguish patients with CTCL from benign skin diseases with very high sensitivity, specificity, and classification accuracy (95%). Our observation that the classifier miRNAs were differentially expressed in almost all CTCL patients suggests that they might play a key role in the pathogenesis of CTCL. As mentioned above, miR-155 is differentially expressed in other lymphomas and hematological malignancies and linked to the malignant transformation. Accordingly, it was not unexpected that miR-155 was differentially expressed in CTCL. Several miR-155 targets have been identified and miR-155 modifies oncogenesis at multiple levels including down regulation of negative regulators such as Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) and increased expression of autocrine growth factors. Studies are in progress to unravel the function of miR-155 in the malignant transformation in CTCL.

Our finding of an increased expression of miR-203 in benign disorders is in line with previous reports on miR-203 expression in psoriasis and atopic dermatitis. MiR-203 is of particular interest to skin diseases as it plays a key role in keratinocyte biology. As mentioned, miR-203 is highly up-regulated in keratinocytes in psoriatic lesions and involved in the repression of Suppressor of Cytokine Signaling-3 (SOCS3). Among other cytokine signaling pathways, SOCS3 inhibits signaling from the interferon-alpha (IFNa) receptor. As psoriasis patients exhibit hypersensitivity to IFNa which is partly caused by a deficient SOCS3 expression, and IFNa triggers or exuberates psoriasis in cancer patients, studies are in progress to address whether the aberrant miR-203 expression plays a role in IFNa hypersensitivity in psoriatic cells. A decreased expression of miR-203 has not previously been reported in CTCL, but Bueno et al. provided evidence that miR-203 is a tumor suppressor and miR-203 silencing promotes lymphoma development via increased expression of oncogenes such as ABL. Although these oncogenes are not expressed in CTCL it is possible that miR-203 silencing also plays a direct role in malignant transformation in CTCL. Malignant T cells often display an aberrant expression of SOCS3 which protects against IFNa-mediated growth inhibition. Therefore, it is possible that miR-203 silencing (via an increased
SOCS3 expression) plays a role in resistance to IFNa treatment in CTCL patients. A few verified targets of miR-205 have recently been reported the most interesting of which (in a CTCL context) is VEGF which is aberrantly expressed in all stages of CTCL. Accordingly, it is possible that silencing of miR-205 could play a role in the spontaneous VEGF production in CTCL lesions. Thus, it seems likely that all of the three miRNAs in the “minimal” qRT-PCR classifier play important roles as oncogenic (miR-155) and tumor suppressor (miR-203 and miR-205) miRNAs. We speculate that such features explain why the miRNA classifier is so robust and conserved across all stages of CTCL.

In conclusion, we provide the first evidence that a miRNA classifier can distinguish CTCL from benign inflammatory skin disorders with very high accuracy suggesting a diagnostic potential in CTCL.
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Authorship

Contribution: U.R. designed and performed research (qRT-PCR), sample handling (malignant biopsies), analyzed data, wrote the paper; P.H.H. designed research (statistical models), handled and analyzed data, wrote the paper; N.B. performed research (qRT-PCR); M.B.L. sample handling (benign skin disease biopsies), performed research; C.B.A. performed research (qRT-PCR); L.Sv. patient and sample handling, performed research (animal model); K.L.K. designed and performed research (animal models); M.T.V Performed research (qRT-PCR) (animal model CTCL); B.L. designed and performed research (animal models); J.R.Z. designed research (RNA extraction), analyzed data; T.K. designed and performed research (animal models) analyzed; C.M.B. Performed research and analyzed data, wrote paper; L.M.G. supplied and analyzed clinical data for the malignant biopsies; T.L. research design (qRT-PCR); A.M.M. designed and performed research (animal models); K.G. overall research design; M.A.W. overall research design, analyzed data, wrote paper; T.Li. overall research design, array analysis, research on miR-targets; M.S-W. supplied the malignant biopsies; C.Q-R. supplied and analyzed clinical data for the biopsies from benign skin disorders; R.G. supplied and analyzed clinical data for the malignant biopsies; E.R. overall research design, supplied malignant biopsies, analyzed data, histological classification of malignant biopsies; R.S. data analysis of qRT-PCR, array analysis, annotation, and submission to GEO; C.G. overall research design (arrays and qRT-PCR), analyzed data; A.W. overall research design, analysis of data (animal models), wrote paper; C.G. research design (arrays and qRT-PCR) analyzed data; M.A.R. overall research design, supplied benign skin disease biopsies, analyzed data, wrote paper; L.Sk. overall research design, supplied benign skin disease biopsies, histological classification of benign biopsies, analyzed data, wrote paper; N.O. overall research design, analyzed data, primus motor and chairman, wrote the paper.

Conflict-of-interest disclosure: C.G., R.S., and T.Li. are or have been employed by Exiqon A/S and M.A.R., P.H.H., M.B.L., TL, LaSv, J.R.Z. are or have been employed by LEO Pharma A/S. Some
of the information presented is part of a patent application filed by the institutions and companies engaged in the project.

Correspondence: Niels Odum; Immunology, ISIM, 22.5.34 Panum; Department of Biology and Department of International Health, Immunology and Microbiology, University of Copenhagen; Blegdamsvej 3c, DK-2200 Copenhagen N, Denmark, Telephone +45 35 32 78 68, Fax +45 35 32 78 76, E-mail: ndum@sund.ku.dk
List of References


Table 1

<table>
<thead>
<tr>
<th>Age (years) *</th>
<th>Cutaneous Lymphoma (n=63)</th>
<th>Benign skin disease or normal skin (n=85)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MF</td>
<td>SS</td>
<td>CALCL</td>
</tr>
<tr>
<td>&lt;30</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>30-44</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45-59</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>60-74</td>
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<td>6</td>
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<tr>
<td>≥75</td>
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<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
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<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Microarray batch</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>8</td>
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<td>1</td>
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</tr>
<tr>
<td>5</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1. Clinical characteristics of patients in the study. Indications are stratified according to age, gender, and the microarray production batch. P-values were calculated using Fisher’s exact test on sums across sub-indications (the Σ columns). MF, mycosis fungoides; SS, Sézary syndrome; CALCL, cutaneous anaplastic large cell lymphoma; NOS, cutaneous T-cell lymphoma – not otherwise specified; AD, atopic dermatitis; ND, unspecified dermatosis; PP, lesional skin from psoriasis patients; PN, non-lesional skin from psoriasis patients; NN, normal skin from healthy controls. *Gender and age of 2 CALC and one MF sample are unknown.
Legends

**Figure 1. Expression profiles in training set for highly significant miRNAs.** We analyzed microarray measurements of 688 miRNAs in the training set of 90 samples with t-test to discover differences in expression between samples of subjects with CTCL and those of BDN subjects. The 27 miRNAs that displayed highly significant (Bonferroni corrected $P < 0.001$) and strong differences (at least 50% change) are presented in the heat map. Samples are arranged in columns, miRNAs in rows, and both are hierarchically clustered using Euclidean distance with average linkage of nodes. Red-to-yellow shades indicate increased relative expression; blue shades indicate reduced expression; green indicates median expression. Top 5 most significantly induced or repressed miRNAs are shown in bold.

**Figure 2. Classification of CTCL and BDN.** (A) Principal component analysis (PCA) plot of samples from subjects with CTCL (green) and those of BDN subjects (orange) in the training set based on the 5-miRNA profile identified by the nearest shrunken centroids (NSC) algorithm. Percentages indicate percent variance explained by that component. (B) Classification performance in the training set using the NSC algorithm. $P$-values were calculated using Fisher’s exact test. (C) PCA plot of samples in the test set based on the 5-miRNA profile identified from the training set. (D) Classification performance in the test set using the trained NSC algorithm.

**Figure 3. Classifier miRNA expressions measured by microarray and qRT-PCR.** For each miRNA, expressions are grouped according to patient type (CTCL and BDN respectively), with a small scatter on the x-axis within each group to allow better visualization of all measurements. $P$-values were calculated using t-test. (A) Expressions measured by microarray. (B) Expressions measured by qRT-PCR.
Figure 4. **Classification of PTL and dermatitis.** (A) PCA plot of samples from subjects with PTL (green), dermatitis (light orange), and psoriasis (yellow) for comparison, in the explorative set, based on the 5-miRNA profile. (B) Classification performance in the explorative set using the trained NSC algorithm.

Figure 5. **Overview of miRNA expressions measured by microarray for the miRNAs in the classifier for all sub indications included in the study.** For each miRNA, expressions are grouped according to sub indication (same abbreviations as in table 1).

Figure 6. **Classification of treated and untreated CTCL and psoriasis from mouse xenograft models.** (A) PCA plot of all samples from mouse xenograft CTCL model (green), and psoriasis model (orange), based on the 5-miRNA profile. Solid bars indicate vehicle controls whereas hatched green bars indicate systemic treatment with a Cox-2 inhibitor (Celecoxib) and hatched orange bars indicate topical treatment with steroid (betamethasone). (B) Classification performance in the mouse xenograft set using the trained 5-miRNA classifier. (C) Bar plots showing normalized log2 expression levels relative to average of lowest expressions measured across all samples. Error bars denote ±1 standard deviation. For each miRNA, $P$-values were calculated using $t$-test between treated and untreated samples from each model respectively.

Figure 7. **qRT-PCR-based classification of samples from patients with CTCL and benign skin disease.** (A) A Cp based sample score ($S$) were calculated for each sample. Patients are ordered by increasing values of this score. The solid line shows the cutoff between patients with CTCL (green) and patients with benign skin disease (orange). The dotted lines show the cutoffs for the low confidence region. (B) Classification performance using the cutoffs defined in (A). $P$-values were calculated using Fisher’s exact test. (C) Receiver operator characteristic curve (ROC) showing the sensitivity and specificity for various cutoff values on the sample score of the samples. (D) Relative
expression of the three miRNAs used in the classification in samples from patients with CTCL and benign skin disease. Error bars indicate ±1 standard deviation.
Figure 2

A

2nd principal comp. (14%)

1st principal comp. (80%)

B

Training set (n = 90)

CTCL Benign

n =

38
52

Classified as CTCL

32
0

Classified as Benign

6
52

93% overall (P < 0.001)

C

2nd principal comp. (17%)

1st principal comp. (74%)

D

Test set (n = 58)

CTCL Benign

n =

25
33

Classified as CTCL

23
0

Classified as Benign

2
33

97% overall (P < 0.001)
Figure 3

A

miR-326 1.5-fold up

$P < 10^{-14}$

miR-663b 1.6-fold up

$P < 10^{-16}$

miR-711 1.6-fold up

$P < 10^{-14}$

miR-203 1.7-fold down

$P < 10^{-21}$

miR-205 2.1-fold down

$P < 10^{-12}$

Normalized $\log_2$ expression from microarray

CTCL  Benign

B

miR-326 3.6-fold up

$P < 10^{-11}$

miR-203 6.6-fold down

$P < 10^{-14}$

miR-663b 3.2-fold up

$P < 10^{-7}$

miR-205 11.2-fold down

$P < 10^{-15}$

Relative $\log_2$ expression from qRT-PCR

CTCL  Benign

(n=41)  (n=49)

(n=43)  (n=61)
Figure 4

A

2nd principal comp. (6%)

1st principal comp. (89%)

B

Exploratory set (n = 50)

PTL PP Dermatitis

n = 39 5 6

Classified as malign

39 0 1

Classified as benign

0 5 5

98% overall (P < 0.001)
Figure 5

miR-326

miR-203

miR-663b

miR-205

miR-711

Normalized log₂ expression

CTCL-MF  CTCL-SS  CTCL-NOS  CTCL-ALCL  NTCL-NOS  AD  Dermatitis  PP  PN  NN
Figure 7

A

$S = \frac{-C_{p(miR-205)}}{2} - \frac{C_{p(miR-203)}}{2} + \frac{C_{p(miR-155)}}{2}$

B

qPCR set ($n = 103$)

CTCL Benign

$\begin{array}{cc}
43 & 60 \\
\end{array}$

n =

$\begin{array}{ccc}
2 & 56 & \\
1 & 2 & \\
2 & 2 & \\
38 & 0 & \\
\end{array}$

93% 97%

95% overall ($P < 0.001$)

C

D

Relative log$_2$ expression (dCp)

$\begin{array}{ccc}
\text{miR-155} & \text{miR-203} & \text{miR-205} \\
\text{CTCL} & \text{Benign} & \text{CTCL} & \text{Benign} & \text{CTCL} & \text{Benign} \\
\end{array}$

$\begin{array}{cc}
P < 10^{-12} & P < 10^{-13} \\
\end{array}$
Diagnostic microRNA profiling in cutaneous T-cell lymphoma (CTCL)