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Research Article

# Impact of Circulating miRNAs in Cutaneous T-cell Lymphoma Diagnosis: miR-146a and miR-26a as Promising Diagnostic Biomarkers

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## Abstract

**Background:** The diagnosis of early-stage Mycosis Fungoides (MF) can be very challenging due to overlapping clinicopathologic findings with reactive and inflammatory dermatoses. The aim of this study was to investigate whether selective peripheral blood microRNAs, are involved in CTCL pathogenesis and can be used as potential diagnostic biomarkers to differentiate inflammatory diseases from early MF.

**Methods:** The expression of miR-148a, miR-338-3p, miR-26a, miR-146a and miR-451 was evaluated in the serum of early MF patients (N = 15) using RT-PCR and also measured in skin biopsies (N = 10) and CTCL cell lines (My-La and Seax).

**Results:** Our data showed that miR-26a and miR-146a were significantly upregulated ($P < 0.001$) in early MF patients compared to controls and could be utilized to differentiate inflammatory skin diseases from early-stage MF. Of importance, miR-451 which has a tumor suppressor role in other hematologic malignancies, was overexpressed in MF.

**Conclusions:** Altogether these data indicate that liquid biopsy may detect upregulation of specific microRNAs (miR-26a and miR-146a) in early-stage MF and present a reliable non-invasive diagnostic technique. The correlation of liquid biopsy with histological data from the skin biopsy indicates that it may be further used as a supplementary tool to the established criteria for the differential diagnosis of early MF stages from inflammatory skin diseases.

**Keywords:** CTCL; Diagnosis; miRNA; MF; Biomarkers

## Abbreviations

CTCL: Cutaneous T-cell Lymphoma; MF: Mycosis Fungoides; miRs: MicroRNAs

## Introduction

Cutaneous T-cell lymphoma (CTCL) is a rare type of neoplasm which involves primarily the skin. The two well-characterized CTCL subgroups are Mycosis Fungoides (MF) and Sezary Syndrome (SS).

MF is the commonest type of CTCL, which mostly involves the skin [1]. It is characterized by lesions presented as skin plaques and patches, tumors or erythroderma [1]. MF is usually a slow-progressive disease with the ability to mask itself for an average period of 6-8 years as a typical skin infection due to its clinical and histopathological similarities with common dermatitis [1,2].

The histologic diagnosis of early MF is one of the most vexing problems in dermatopathology [3,4]. Importantly, some skin diseases such as atopic dermatitis may mimic MF histologically and result in the misdiagnosis of MF [4]. Differentiation of true MF from other dermatologic conditions is important to ensure proper management of the patient's symptoms and treatment, as well as to provide accurate prognostic information. At this point, it is obvious that skin biopsy has limitations, and it is of utmost importance to investigate new biomarkers in order to make a reliable diagnosis of MF patients in early stages.

A good alternative approach to skin biopsy is the liquid biopsy. While liquid biopsy techniques are extensively developed, most diagnostic studies are still performed on tissue samples. Liquid biopsy relies on peripheral blood sample evaluation, which is a less invasive technique that can provide information on the genetic and epigenetic landscape of all cancerous lesions (primary and metastatic) at an earlier stage than tissue biopsy [5]. Several biomarker molecules can be detected in liquid biopsies including microRNAs (miRNAs) which are small non-coding RNA molecules that function in RNA silencing and post-transcriptional regulation of gene expression [6]. In CTCL, several studies have been conducted in order to characterize the miRNA profiles (i.e., upregulation or downregulation of certain miRNAs) and their role in disease onset and pathogenesis.

The aim of this study was to evaluate the importance of "liquid biopsy" biomarkers in CTCL diagnosis by analyzing the differential expression profile of a panel of miRNAs (previously demonstrated to possess a significant role in CTCL) in serum of inflammatory dermatitis and early-stage MF samples compared to normal controls. The data were further tested in CTCL cell lines in comparison to normal PBMCs.

## Materials and Methods

### Patient cohorts

Fifteen (15) CTCL patients were registered to the study along with 12 patients with benign inflammatory condition (psoriasis and atopic dermatitis), and 4 healthy controls. We subdivided these patients into independent groups. The first group was consisted of 15 patients with early MF (7 of them in stage IA and 3 in stage IB), the second was composed of patients with psoriasis (from the psoriasis clinic), following with a group of 8 patients with atopic dermatitis (from atopic dermatitis clinic), and the last one consisting of 4 normal controls. The diagnosis performed with dermatopathologic assessments as well as by histology and immunohistochemistry. Regarding therapy, patients have used skin-directed therapies (topical corticosteroids, phototherapy, or nitrogen mustard) upon diagnosis. No patients received systemic therapies. The clinical characteristics of the MF and control (psoriasis and atopic dermatitis) cohort are shown in tables 1- 3. Informed consent was obtained from all patients and the study was authorized by the ethics committee of Attikon University hospital and the Medical School of the National and Kapodistrian University of Athens.

| Patient No | Gender | Age | Onset | Diagnosis | mSwat | BSA % | Stage |
|---|---|---|---|---|---|---|---|
| 1 | Female | 40 | 2000 | 2012 | 10 | 9.5 | IA |
| 2 | Male | 82 | 2012 | 2018 | 4 | 4 | IA |
| 3 | Male | 74 | 1988 | 1990 | 13 | 14.5 | IB |
| 4 | Male | 71 | 2006 | 2019 | 7 | 7 | IA |
| 5 | Male | 56 | 2012 | 2017 | 15.45 | 14.35 | IB |
| 6 | Male | 80 | 2018 | 2019 | 22 | 14.2 | IB |
| 7 | Male | 57 | 2013 | 2017 | 0,2 | 5 | IA |
| 8 | Male | 61 | 2015 | 2018 | 9 | 0.2 | IA |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 9 | Female | 54 | 2016 | 2017 | 4.15 | 5 | IA |
| 10 | Female | 52 | 2019 | 2019 | 5.5 | 5.5 | IA |
| 11 | Female | 66 | 2020 | 2020 | 25.5 | 17 | IB |
| 12 | Female | 38 | 2020 | 2020 | 6 | 6 | IA |
| 13 | Male | 68 | 2016 | 2016 | 16.5 | 16.5 | IB |
| 14 | Male | 18 | 2005 | 2020 | 7.5 | 7.5 | IA |
| 15 | Male | 73 | 2019 | 2019 | 5.5 | 5.5 | IA |

**Table 1:** Demographic and clinical data of MF-Patient cohort.

| Patient No | Gender | Age | Onset | Diagnosis | PASI |
|---|---|---|---|---|---|
| 16 | Female | 58 | 2006 | 2006 | 5.6 |
| 17 | Female | 37 | 1990 | 1994 | 21 |
| 18 | Female | 36 | 2009 | 2009 | 2 |
| 19 | Male | 14 | 2009 | 2009 | 6 |

**Table 2:** Demographic and clinical data of Psoriasis -Patient cohort.

| Patient No | Gender | Age | Onset | Diagnosis AD | EASI |
|---|---|---|---|---|---|
| 20 | Male | 21 | 2003 | 2003 | 25.5 |
| 21 | Male | 52 | 2006 | 2008 | 41 |
| 22 | Female | 24 | 2019 | 2019 | 25 |
| 23 | Male | 58 | 2014 | 2016 | 18.2 |
| 24 | Male | 73 | 2018 | 2019 | 11 |
| 25 | Male | 92 | 2013 | 2018 | 7.1 |
| 26 | Female | 75 | 2018 | 2019 | 12.1 |
| 27 | MALE | 68 | 2013 | 2019 | 8 |

**Table 3:** Demographic and clinical data of Atopic Dermatitis -Patient cohort.

## Cell lines and PBMCs

The My-La (mycosis fungoides) and Seax (SeÂzary's syndrome) cell lines were a generous gift from Dr Jan P. Nikolay (Klinik für Dermatologie, Venerologie und Allergologie, Universitätsmedizin Mannheim, Ruprecht-Karls-Universität Heidelberg, Mannheim, Deutschland), previously tested and authenticated. Moreover, peripheral blood mononuclear cells (PBMCs) were collected from 4 normal volunteers by density-gradient centrifugation using the Ficoll-Hypaque technique (Stem cell technology, Canada) and used as controls.

## Measurement of gene expression

Total RNA, containing small size RNA>200nt was extracted from samples using the miRNeasy Serum/Plasma kit and miRNeasy kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The Bioanalyzer (2100 Agilent Bioanalyzer, Agilent Technologies, USA) was used to determine RNA integrity and purified total RNA. RNA was next used for cDNA synthesis using miScript II RT kit (Qiagen, Venlo, Netherlands). Mature microRNAs were polyadenylated by poly-A polymerase followed by cDNA synthesis using oligo-dTs.

The miRNA expression profile was analyzed using the miScript SYBR® Green PCR kit (Qiagen, Venlo, Netherlands). The reaction was performed using the iQTm 5 Cycler system (Bio-Rad Laboratories, Hercules, USA) and each sample was analyzed in triplicate. MiRNA primers were commercially synthesized (Qiagen, Venlo, Netherlands). The level of miRNA expression was measured using the C q (quantification cycle) value. The fold change was generated using the equation 2−ΔΔCq. The 2−ΔΔCq method for relative quantification of gene expression was used to determine the level of miRNA expression. ΔC q was calculated by subtracting the C q value of RNU6B (RNU6-2) RNA from the C q value of the miRNA of interest. All data were expressed as mean ± standard deviation (SD). Statistical significances were evaluated using Student's t-test or nonparametric Mann-Whitney U test. Differences with p-value <0.05 were considered to be statistically significant. All analyses were performed using IBM SPSS Statistics Software (version 17.0).

## Results and Discussion

### Results

#### miRNAs were detected in serum of MF patients and may be used as "liquid biopsy"

Based on literature data, we proceeded to investigate the presence of circulating miRNAs in serum of MF patients. Using polymerase chain reaction (PCR), we detected the expression of miR-148a, miR-338-3p, miR-26a, miR-146a and miR-451 in the peripheral blood of MF patients and controls.

#### miR-146a, miR-26a, miR-148a, miR-338-3p and miR-451 were highly overexpressed in MF patients in comparison with patients with inflammatory diseases

Furthermore, we quantitatively analyzed miR-146a, miR-26a, miR-148a, miR-338-3p and miR-451 expression by qRT-PCR and compared their levels in serum samples taken from 15 CTCL patients, 12 patients with benign inflammatory condition (psoriasis and atopic dermatitis) as well as 4 healthy controls.

All miRNAs were found to be differentially expressed in MF patients compared to control groups (Figure 1- 5). The mean expression of these miRs was higher compared to control groups and each one presented a characteristic expression pattern ($p < 0.05$).

**Figure 1:** A) Expression levels of miR-146a in serum of MF patients compared to patients with inflammatory diseases. Increased expression of miR-146a was detected in the serum of MF patients with qRT-PCR compared to patients with inflammatory diseases ($p < 0.001$). B) Individual expression levels of miR-146a in the serum of each MF patient compared to every patient with inflammatory disease and normal controls.

**Figure 2:** A) Expression levels of miR-26a in serum of MF patients compared to patients with inflammatory diseases. Increased expression of miR-26a was detected in the serum of MF patients with qRT-PCR compared to patients with inflammatory diseases ($p < 0.001$). B) Individual expression levels of miR-26a in the serum of each MF patient compared to every patient with inflammatory disease and normal controls.

**Figure 3:** A) Expression levels of miR-338-3p in serum of MF patients compared to patients with inflammatory diseases. Increased expression of miR-338-3p was detected in the serum of MF patients with qRT-PCR compared to patients with inflammatory diseases ($p = 0.006$). B) Individual expression levels of miR-338-3p in the serum of each MF patient compared to every patient with inflammatory disease and normal controls.

**Figure 4:** A) Expression levels of miR-148a in serum of MF patients compared to patients with inflammatory diseases. Increased expression of miR-148a was detected in the serum of MF patients with qRT-PCR compared to patients with inflammatory diseases ($p = 0.01$). B) Individual expression levels of miR-148a in the serum of each MF patient compared to every patient with inflammatory disease and normal controls.

**Figure 5:** A) Expression levels of miR-451 in serum of MF patients compared to patients with inflammatory diseases. Increased expression of miR-451 was detected in the serum of MF patients with qRT-PCR compared to patients with inflammatory diseases ($p < 0.001$). B) Individual expression levels of miR-451 in the serum of each MF patient compared to every patient with inflammatory disease and normal controls.

In particular, miR-146a was the most highly upregulated miRNA in all groups (MF vs. controls). It was overexpressed in MF patients in comparison to patients with psoriasis, atopic dermatitis and to normal controls (Figure 1). All MF patients presented a higher expression of miR-146a in their blood in comparison to all other control groups. Therefore, miR-146a was determined as a good diagnostic biomarker which can differentiate early MF from inflammatory diseases.

Furthermore, miR-26a was the second most highly upregulated miRNA in all groups (MF vs. controls) (Figure 2). It was overexpressed in MF patients in comparison to patients with psoriasis, atopic dermatitis, and to normal controls. Only one patient with MF stage IA had lower expression of miR- 26a in comparison to patients of the control group of atopic dermatitis and psoriasis. All the other MF patients presented higher expression of miR-26a in their blood in comparison to all other control groups. Thus, miR-26a is a good diagnostic biomarker which can differentiate early MF from psoriasis but needs further investigation in respect to atopic dermatitis.

In addition, miR-338-3p was overexpressed in MF patients in comparison to psoriasis, atopic dermatitis and to normal controls (Figure 3). However, a high expression of miR-338-3p was also detected in some patients of atopic dermatitis group suggesting that miR-338-3p does not present a good diagnostic biomarker between MF patients and controls.

Moreover, miR-148a was overexpressed in MF patients in comparison to patients with psoriasis, atopic dermatitis and normal controls (Figure 4). However, some patients in the group of atopic dermatitis exhibited an approximately equal or a little higher expression of miR-148a in their blood compared to MF patients. These data revealed that miR-148a is a good diagnostic biomarker of differentiation between MF patients and patients with psoriasis, but it cannot be used effectively to differentiate all MF patients from those with atopic dermatitis.

Finally, miR-451 was overexpressed in MF patients compared to the majority of patients with inflammatory diseases and normal controls (Figure 5). However, there were some patients in the group of atopic dermatitis and psoriasis that exhibited an approximately equal or a little higher expression of miR-148a in their blood compared to MF patients. Therefore, miR-451 cannot serve as potential biomarker to differentiate inflammatory diseases from early MF.

### Increased expression of miR-146a, miR-26a, miR-148a, miR-338-3p and miR-451 in CTCL cell lines in comparison to normal PBMCs

To further validate our results, we measured the expression of miR-146a, miR-26a, miR-148a, miR- 338-3p and miR-451 in cell lines and confirmed that they were expressed at significantly ($p < 0.05$) higher levels in malignant CTCL cells than in non-malignant peripheral blood mononuclear cells (Figure 6). In particular, miR-146a and miR-26 expression was significantly upregulated in both malignant T cell lines (MyLa2059 and SeAx) in comparison to the non-malignant cells. Likewise, miR-148 expression in the malignant T cell lines MyLa2059, was higher than in a non-malignant PBMCs. Also, the SS cell line, SeAx showed 2 times higher expression than the non-malignant cells (Figure 6). On the other hand, miR-338-3p and miR-451 were expressed in lower levels in comparison to the other miRs tested. Their expression was not significantly elevated compared to controls.

**Figure 6:** The expression levels of miR-148a, miR-451, miR-146a, miR-26a and miR-338-3p in CTCL cell lines compared to normal PBMCs (*$p < 0.05$, **$p < 0.01$).

## Discussion

The genetic and epigenetic profile of solid tumors is currently based on tissue biopsy, an invasive technique which cannot be performed routinely [7,8]. On the other hand, liquid biopsy represents a reliable alternative approach of sampling and analysis of non-solid biological tissue, primarily blood with many advantages over tissue biopsy, being less invasive, less risky and easily repeated [9].

Circulating miRNAs possess many advantages as biomarkers including a non-invasive and feasible methodology. Moreover, their expression levels are not affected by gender, age, smoking status, body mass index (BMI) or other basic characteristics when evaluating their pathogenic potential [10]. Hence, the altered expression levels might be distinguishable between normal and disease states. Furthermore, despite the presence of ribonucleases, miRNAs were found to be highly stable in blood and in other body fluids [11]. Another important advantage of miRNAs is that their expression may be detected before the presence of clinical symptoms or clear biopsy and image examination evidence [10].

In this study we first identified a subset of miRNAs (miR-148a, miR-451, miR-146a, miR-26a and miR-338-3p) that are detected in the serum of MF patients by convectional PCR method and can be used as a "liquid biopsy" and second, we showed that this set of miRNAs is differentially expressed in inflammatory conditions and early MF stages, which could be used to differentiate MF from benign conditions in the future.

Many studies have been conducted regarding these miRs which were reported to play a role in CTCL pathogenesis, apart from miRNA-451 which is widely dysregulated in human cancer but it has not yet been related to CTCL [12]. In particular, Lindahl et al. showed that miR-106b-5p, miR-148a-3p, and miR-338-3p extracted from MF tissues are highly predictive of disease progression and they can be used to successfully separate patients into high- and low-risk groups [13]. Moreover, the study of Manso et al. reported that a restricted number of miRNAs (26a, 222, 181a and 146a) could differentiate tumoral from reactive CTCL conditions [14]. The only study demonstrating the use of liquid biopsy in CTCL was performed by Dusílková et al. indicating that plasma miR-155, miR-203, and miR-205 can be used as biomarkers for CTCL diagnosis and therapy response monitoring [15].

In the present study, we have detected elevated miR-146a and miR-26a levels in both CTCL cell lines and patients' blood suggesting that they could present promising biomarkers. Our results are also consistent with the study of Manso et al. where these miRs were also overexpressed in CTCL skin biopsies [12].

Studies have shown that miR-146a plays an important role in tumorigenesis since it was found dysregulated in several tumor types and was associated with the Jak/STAT pathway. Specifically, in hepatocellular carcinoma (HCC), miR-146a was shown to promote the expression of STAT3 activation– associated cytokines [16]. Sun et al. showed that blockade of STAT3 downregulated miR- 146 expression which led to the upregulation of known miR-146a targets, STAT1 and TRAF6, as well as their downstream signaling pathways, including Jak/STAT [16]. Taking altogether, it seems that miR-146a may participate in CTCL pathogenesis by reducing the production of Th1 cytokines in human T cells and may lead to the switch from Th1 to Th2 phenotype through JAK/STAT and NF-κB signaling pathways.

Regarding miR-26, it is implicated in many biological processes such as the growth and development of normal tissues, apoptosis, cell proliferation as well as in tumorigenesis [17]. Notably, Kim et al. showed that miR-26a induces glioblastoma formation by decreasing PTEN, RB1, and MAP3K2/MEKK2 protein expression, thus increasing AKT activation, promoting proliferation and inhibiting JNK-dependent apoptosis [18]. Genetic analysis in CTCL detected mutations in the TP53, RB1, PTEN, DNMT3A and CDKN1B tumor suppressors and in RAS/RAF/MEK/ERK pathway revealing their importance in the pathogenesis of the disease [19–21]. From the aforementioned results, it is evident that miR-26 may participate in the CTCL pathogenesis by downregulating PTEN and increasing the PI3/AKT-mediated pathway of cell growth and survival. Another proposed mechanism may involve miR-26a reduction of JNK-dependent apoptosis by inhibiting MAP3K2/MEKK2 and resulting in propagation of the malignant T-cell clone, acting as an oncomir in CTCL.

Regarding miR-451, it is markedly reduced in various types of cancer such as colorectal cancer [22], NSCLC [23], renal cell carcinoma [24] and Pre-B-ALL [25], indicating that miR-451 exerts its biologic role as a tumor-suppressive gene. In our study, we showed elevated miR-451 levels in CTCL in contrast to the majority of other cancers. In addition, the study of Godlewski has shown that in glioblastoma, miR-451 expression is overexpressed under conditions of abundant energy (when glucose is high) [26]. The overexpression of miR-451 allows cells to keep their proliferation rates elevated via mTOR activation [27]. This pathway may also be implicated in CTCL pathogenesis, but this hypothesis needs further investigation. Thus, miR-451 may allow cells to survive the metabolic stress and seek out favorable growth conditions, facilitating tumor growth

in CTCL. A better understanding of the regulation of miR-451 expression and its function will enable to elucidate the mechanisms underlying the development and progression of human cancers and especially CTCL.

## Conclusion

The early stages of MF diagnosis are difficult to be clinically and morphologically differentiated from other inflammatory conditions. A major and fundamental advance in recent years has been the identification of miRNAs as highly conserved regulators of gene expression. Liquid biopsy using miRNAs may be a very useful non-invasive technique for CTCL diagnosis, presenting an additional tool to the established criteria for the differential diagnosis of early MF stages from inflammatory skin diseases.

Herein, we propose that the analysis of two miRNAs (26a and 146a) could help differentiate inflammatory skin diseases from early-stage MF and may serve as promising diagnostic biomarkers. Finally, based on our primary results, miRNA-451 is a potential CTCL-related miRNA which needs to be further investigated in larger sample sizes and clinical context.

## Conflict of Interest

The authors declare no conflict of interest.

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