MicroRNA-106b Regulates Expression of the Tumour Suppressors p21 and TXNIP and Promotes Tumour Cell Proliferation in Mycosis Fungoides

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A prognostic 3-miRNA classifier for early-stage mycosis fungoides has been developed recently, with miR-106b providing the strongest prognostic power. The aim of this study was to investigate the molecular function of miR-106b in mycosis fungoides disease progression. The cellular localization of miR-106b in mycosis fungoides skin biopsies was determined by in situ hybridization. The regulatory role of miR-106b was assessed by transient miR-106b inhibitor/mimic transfection of 2 mycosis fungoides derived cell lines, followed by quantitative real-time PCR (RT-qPCR), western blotting and a proliferation assay. MiR-106b was found to be expressed by dermal T-lymphocytes in mycosis fungoides skin lesions, and miR-106b expression increased with advancing mycosis fungoides stage. Transfection of miR-106b in 2 mycosis fungoides derived cell lines showed that miR-106b represses the tumour suppressors cyclin-dependent kinase inhibitor 1 (p21) and thioredoxin-interacting protein (TXNIP) and promotes mycosis fungoides tumour cell proliferation. In conclusion, these results substantiate that miR-106b has both a functional and prognostic role in progression of mycosis fungoides.

Key words: mycosis fungoides; cutaneous T-cell lymphoma; microRNA; progression; tumour suppressors; p21; TXNIP.

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Cutaneous T-cell lymphoma (CTCL) is a group of non-Hodgkin’s lymphomas, with mycosis fungoides (MF) representing the most common subtype (1). MF is characterized by proliferation of malignant T cells in a chronic inflammatory microenvironment in the skin (2). Epigenetic changes (3–5), gene expression alterations (6), in addition to genetic (7) and environmental factors (8, 9) have been linked to the aetiology of MF, which is still far from understood.

MicroRNAs (miRNAs) have gained attention, both as markers and as substantial functional players in the pathogenesis of CTCL. MiRNAs are processed from larger pri-miRNA precursors to mature non-coding single-stranded RNA molecules of approximately 22 nucleotides. MiRNAs elicit their effects by regulating post-transcriptional gene expression through mRNA degradation or blockage of protein translation (10). The functional role of miRNAs has been explored for a number of miRNAs in CTCL, including miR-21, miR-214 and miR-155. MiR-21 targets STAT3, STAT5 and HDAC1 (4, 11, 12) and supports growth of malignant T cells by repression of PTEN (13). MiR-214 is induced by TWIST1 and BRD4, and promotes increased tumour growth, and an in vivo CTCL mouse model indicates that treatment with a miR-214 inhibitor provides clinical improvement (3). In addition, miR-155 downregulates SATB1 and supports survival and proliferation of malignant T cells in CTCL (14). Recently, promising results were obtained in a first-in-man study in MF using a drug targeting miR-155, emphasizing the importance of miRNAs in the pathogenesis of CTCL (15).

MiRNAs also serve as diagnostic and prognostic markers (16, 17) and can discriminate between clinically similar subtypes of CTCL, e.g. Sézary syndrome (SS), a rare leukaemic, aggressive variant of CTCL, and erythrodermic MF (18).

At the onset of early-stage MF, prediction of the prognosis is a clinical challenge. One-third of patients will progress to advanced disease stages and have a signifi-
cantly worsened prognosis, but all patients are treated equally until first sign of progression, due to challenging prognostic stratification (19). Recently, we developed and validated a prognostic 3-miRNA classifier, which at the time of the early-stage MF diagnosis can predict the risk of progression at an individual level (16). The strongest predictive power was provided by miR-106b in this classifier (16).

miR-106b is an oncomiR clustering with miR-93 and miR-25 in intron 13 of the MCM7 gene on chromosome 7 (20). MiR-106b is aberrantly expressed in both MF and SS and is reported to have both prognostic and oncogenic functions in SS (13, 21); and may also have an oncogenic function in other haematological and solid cancers (22). However, the molecular function of miR-106b in MF remains to be elucidated.

In CTCL, tumour suppressors play a central role in the process of malignant proliferation, and are epigenetically regulated by miRNAs (23). The current study investigated the miR-106b-dependent regulation of the tumour suppressors cyclin-dependent kinase inhibitor 1 (p21) and thioredoxin-interacting protein (TXNIP) in MF. p21 is involved in CTCL progression (24) and TXNIP was recently linked to regulation of T-cell proliferation (25). The current study demonstrates that miR-106b, in addition to its prognostic function, may promote progression of MF by repression of the tumour suppressors p21 and TXNIP, and induce tumour cell proliferation.

MATERIALS AND METHODS

Patients

A cohort of 198 patients with MF was included to assess miR-106b expression in the initial diagnostic MF biopsy. Identification of patients and collection of their initial diagnostic formalin-fixed paraffin-embedded (FFPE) skin biopsies were performed as described previously (16). The 2 previous early-stage (stage IA–IIA) MF patient cohorts (16) were pooled and a cohort of 44 patients with advanced stage (stage IB–IVB) MF added. The patients were subdivided according to T-stage (T1–T4) for subsequent analysis.

For assessment of p21 and TXNIP expression, snap-frozen skin biopsies from a cohort of 11 MF patients and 6 healthy controls, were collected.

The study was approved by The Central Denmark Region Committees on Research Ethics (1-10-72-91-13 and 1-10-72-151-16) and the Data Protection Agency (1-16-02-478-15).

In situ hybridization

In situ hybridization analyses were performed on 10–12-μm thick cryosections obtained from snap-frozen skin biopsies, as described previously, using a chromogenic detection (26) or fluorescence detection (27) in cooperation with BS Nielsen at Bioneer, Hørsholm, Denmark. In brief, in situ hybridization was performed with double digoxigenin (DIG)-labelled locked nucleic acid (LNA) probes for miR-106b-5p (ATCTGCACTGT-CAGCACTTTA, RNA Tm=82°C, %LNA= 24), reference probes to miR-203-3p (CTAGTGTCCTAACAATTTCAC, RNA Tm=83°C, %LNA=33, positive control), and scramble probe (TGTAACCGCTCTATAGCCTC, RNA Tm=87°C, %LNA=33, negative control), obtained from Qiagen (Hilden, Germany). Probes were diluted in Exiqon hybridization buffer (Qiagen, Vedbaek, Denmark) and hybridized at 55°C (miR-106b at 20nM) or 58°C (miR-203 at 20nM and scramble at 20 nM). The probes were detected with alkaline phosphatase or peroxidase-conjugated anti-DIG antibodies (Roche, Basel, Switzerland) for chromogenic staining (NBT-BCIP substrate) or fluorescence staining (Cy5 TSA substrate), respectively. Chromogenic-stained sections were counterstained with Nuclear Fast Red (Vector Laboratories, Stonesfield, UK), dehydrated and mounted with Eukitt medium (VWR, Radnor, Pennsylvania, USA). Combined with fluorescence staining, sections were subsequently incubated with anti-CD4 antibody (rabbit mAb, Roche) followed by Cy3-conjugated anti-Rabbit (Jackson Immunoresearch, Ely, UK). Sections were then mounted with DAPI-containing mounting medium.

Myco s fungoides cell lines

For in vitro studies the MF-derived cell lines MyLa2059 and MyLa3675 were used (28, 29). Both cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 1% penicillin/streptomycin. The medium for MyLa2059 was supplemented with 10% heat-inactivated foetal bovine serum, and 10% human serum was added to the medium for MyLa3675. MyLa3675 cells were also supplemented with 5 ng/ml IL-2. The cells were grown in 5% CO2, and 95% humidified air at 37°C. To assess whether the function of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) is mediated through miR-106b, 0–20 mM SAHA was added.

Transient transfection

A total of 4×10⁴ cells per sample were transfected to a NucleoTect (Lonza, Basel, Switzerland) and transfected in SF 4D-Nucleofector Solution (Lonza) with miR-106b-5p inhibitor or mimic, or a non-targeting control (mirVana™ miRNA inhibitor or mimic, Life Technologies, Carlsbad, California, USA) using an Amaxa 4D-Nucleofector (Lonza). Immediately after transfection 500 µl pre-warmed medium was added and incubated for 10 min at 37°C and then transferred carefully to the culturing medium and cultured for 24 h before harvesting.

RNA purification and quantitative real-time PCR

RNA extraction was performed using the RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City California, USA) for FFPE skin biopsies and mirNeasy Mini Kit (Qiagen, Hilden, Germany) for fresh frozen skin samples in accordance with the manufacturer’s instructions.

For RNA purification in in vitro studies, the miRNAeasy Mini Kit (Qiagen) was used.

Quantitative real-time PCR (RT-qPCR) for assessment of miRNA expression in FFPE skin biopsies was performed as described previously (16). For gene expression, cDNA was synthesized using Taqman Reverse Transcription Reagents (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) was used for the PCR reaction, with GAPDH as reference gene. The miRNA expression was assessed using TaqMan™ miRNA Reverse Transcription Kit (Applied Biosystems) for cDNA synthesis and the PCR reaction was performed using TaqMan™ miRNA assays and TaqMan Universal Master Mix II, no UNG (Applied Biosystems), with U6 as reference. The pri-miR-106b expression was assessed using High Capacity cDNA Reverse Transcription Kit
and TaqMan® Gene Expression Assays (Applied Biosystems) using GAPDH as reference. For PCR amplification TaqMan Gene Expression Master Mix was used and performed on StepOnePlus (Applied Biosystems).

[3H]-thymidine proliferation assay

Tumour cell proliferation was measured by incorporation of radioactive labelled [3H]-thymidine (Perkin Elmer, Waltham, MA, USA) into the cells. The cells were incubated for 24 h with [3H]-thymidine before cell proliferation was analysed by measurement of counts per min.

Protein extraction and western blotting

Protein was extracted from cell pellets, and concentrations were determined by Bradford assay. Western blotting was performed by gel electrophoresis separation of proteins, blotted into a nitrocellulose membrane, which was blocked and incubated with primary antibody (p21, Santa Cruz #6246 and Cell Signaling #2947; TXNIP, Cell Signaling #14774 and Abcam #188865). The antibody was detected using antirabbit IgG antibody (Cell Signaling #7074).

Gene array analyses

Global gene expression 24 h post-transfection of miR-106b inhibitor in the MF tumour cell line MyLa2059 was assessed on extracted RNA using Affymetrix GeneChip Human Gene 2.0 ST Array covering >33,500 coding transcripts and >1,100 long non-coding transcripts (conducted at the Center for Genomic Medicine, Rigshospitalet, Copenhagen, Denmark). 100 ng RNA was amplified and labelled using the WT Plus Expression Kit (Affymetrix, Santa Clara, CA, USA). Next, the labelled RNA samples were hybridized to Human Gene 2.0 ST GeneChip arrays (Affymetrix), and subsequently washed and stained with phycoerythrin-conjugated streptavidin (SAPE) and scanned to generate probe cell intensity (CEL) files. Probe intensities were converted to gene expression values using the RMA (Robust Multichip Average) and quantile normalization algorithms in Bioconductor (https://www.bioconductor.org/). Analysis of variance (ANOVA) was used to assess differentially expressed genes, with significance adjusted for multiple testing by estimation of false discovery rate (FDR). Visualization of the data was performed in Quilcore Omics Explorer v.3.6 (Qlucore AB, Lund, Sweden).

Statistical analysis

Graphs and statistical analysis were performed using GraphPad Prism 7. Comparisons were performed using a 2-tailed Student’s t-test and error bars were shown as standard error of the mean (SEM). Statistical significance was considered as p < 0.05.

RESULTS

MiR-106b expression increases with advancing mycosis fungoides stage

Expression of miR-106b was determined in a cohort of 198 patients with MF compared with 20 healthy controls. Significantly higher expression of miR-106b was found in lesional MF skin and increased miR-106b expression with advancing T-stage was demonstrated, indicating that miR-106b could be a molecular driver of MF disease progression (Table 1).

Table 1. Stage-dependent miR-106b expression in lesional skin from patients with mycosis fungoides (MF) compared with healthy controls (HC)

| Group       | Fold change | p-value   |
|-------------|-------------|-----------|
| T1 vs HC    | 1.5         | <0.0001   |
| T2 vs HC    | 2.8         | <0.0001   |
| T3+T4 vs HC | 3.3         | <0.0001   |
| T3+T4 vs T1+T2 | 1.8     | <0.0001   |

The number of patients according to T-stage was: T1 n = 71; T2 n = 86; T3 n = 27; T4 n = 14.

In situ localization of miR-106b in lesional mycosis fungoides skin

Next, in situ hybridization of miR-106b in MF skin lesions revealed that miR-106b is primarily expressed in dermal lymphocytes, while a stromal background staining may either reflect local release of miR-106b or expression of miR-106b in stromal cells (Fig. 1). Thus, miR-106b stains positive in the dermal microenvironment, whereas epidermal miR-106b expression is less pronounced (Fig. 1b, c) compared with the scramble probe (Fig. 1a). In addition, double immunofluorescence staining of miR-106b and CD4 substantiates that miR-106b is primarily expressed in dermal T cells (Fig. 1d–i), whereas control double staining with miR-203 and CD4 confirms that miR-203 is expressed in keratinocytes and CD4 is expressed in the dermal infiltrate that stains positive for miR-106b (Fig. S1).

Tumour suppressors p21 and TXNIP are down-regulated in mycosis fungoides skin lesions

This study also explored the molecular function of miR-106b in MF disease progression. Global gene expression analysis of MyLa2059 cells transfected with miR-106b inhibitor revealed that the tumour suppressor TXNIP was regulated by miR-106b (Fig. S2). In addition, the tumour suppressor p21 is described to be involved in CTCL progression (24) and miR-106b is an epigenetic modulator of its expression in cancer (30, 31). It was found that p21 and TXNIP expression was lower in lesional MF skin compared with healthy skin (Fig. 2), indicating that the tumour suppressors p21 and TXNIP may be involved in MF pathogenesis. In addition, the p21 expression was suppressed in MF compared with lesional psoriasis skin, whereas the TXNIP expression was downregulated in both MF and psoriasis, thus indicating that p21 is specifically downregulated in MF, whereas TXNIP may also have a pathogenic role in inflammatory processes.

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Fig. 1. In situ hybridization of miR-106b in skin biopsies from patients with mycosis fungoides. (a–c) In situ hybridization of miR-106b stained positive in the dermal lymphocytes and surrounding stroma (b) (original magnification ×10) and (c) (original magnification ×40) compared with the scramble control (a) (original magnification ×10). (d–f) Double immunofluorescence staining of miR-106b and CD4 revealed that miR-106b is expressed in T-lymphocytes (original magnification ×10). (d) Double miR-106b and CD4 staining. (e, f) Single immunofluorescence staining of miR-106b and CD4, respectively. (g–i) Double miR-106b and CD4 staining (g) and single miR-106b (h) and CD4 (i) (original magnification ×40).

Fig. 2. MicroRNA expression of the tumour suppressors cyclin-dependent kinase inhibitor 1 (p21) and thioredoxin-interacting protein (TXNIP) in skin lesions from patients with mycosis fungoides (MF). p21 and TXNIP expression were assessed by quantitative real-time PCR (RT-qPCR) in lesional skin biopsies from 11 patients with MF and compared with the expression in skin biopsies collected from 6 healthy controls (HC) and 6 patients with psoriasis. *p < 0.05; **p < 0.01.
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A P21 mRNA and protein expression

B TXNIP mRNA and protein expression

C Proliferation

miR-106b down-regulates p21 and TXNIP expression and inhibits proliferation of mycosis fungoides derived malignant T cells

To investigate the mechanism of miR-106b in MF disease progression, the MF-derived cell lines MyLa2059 and MyLa3675 were transfected with a miR-106b inhibitor or mimic to examine whether reduced or increased miR-106b expression changes p21 and TXNIP expression. Transfection with a miR-106b inhibitor increased p21 mRNA expression by 25% (Fig. 3a, left-hand part) and profoundly increased p21 protein expression (Fig. 3a, right-hand part) in both MF cell lines. As miR-106b is highly expressed in malignant MF cells, transfection with miR-106b mimic provided minor changes in the p21 expression, at both the mRNA and protein level (Fig. 3a). TXNIP mRNA expression was increased 2-fold (Fig. 3b, left-hand part) and the TXNIP protein expression was profoundly increased (Fig. 3b, right-hand part) following transfection with miR-106b inhibitor in both the MyLa2059 and MyLa3675 cell line. Transfection with miR-106b mimic caused only minor changes in the TXNIP mRNA and protein expression. Next, proliferation following transfection with miR-106b inhibitor, proliferation of both the MyLa2059 and MyLa3675 cell lines was reduced 30–35% (Fig. 3c). Taken together these findings could indicate that miR-106b promotes malignant cell proliferation by down-regulation of the tumour suppressors p21 and TXNIP.

Suberoylanilide hydroxamic acid inhibits pri-miR-106b and induces p21 and TXNIP in malignant T cells derived from patients with mycosis fungoides

SAHA is a histone deacetylase inhibitor (HDACi) causing epigenetic modulations in malignant T cells and is an effective therapy for patients with CTCL (32, 33). The current study investigated whether SAHA acts through...
Fig. 4. Expression of pri-miR-106b, cyclin-dependent kinase inhibitor 1 (p21) and thioredoxin-interacting protein (TXNIP) following treatment with suberoylanilide hydroxamic acid (SAHA) in mycosis fungoides (MF)-derived cell lines. (a) SAHA induces a dose-dependent inhibition of the pri-miR-106b expression in 2 MF-derived cell lines. (b) The p21 mRNA (left-hand part) and protein (right-hand part) expression are induced by SAHA in both MF cell lines. (c) Similarly, the TXNIP mRNA (left-hand part) and protein (right-hand part) expression are increased following treatment with SAHA in both cell lines. $n = 3$ in all experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Fig. 5. Cyclin-dependent kinase inhibitor 1 (p21) and thioredoxin-interacting protein (TXNIP) protein expression following miR-106b transfection and suberoylanilide hydroxamic acid (SAHA) treatment. (a) Combined miR-106b inhibition and SAHA treatment provides an additive effect on the p21 protein induction in both mycosis fungoides (MF) cell lines. (b) In contrast, miR-106b mimic inhibits the SAHA-induced p21 expression, which was most pronounced in the MyLa3675 cell line. (c) Similarly, an additive effect of combined miR-106b inhibition and SAHA treatment was observed for TXNIP protein expression in both cell lines. (d) miR-106b mimic also inhibited SAHA-induced TXNIP protein expression in both cell lines. $n = 3$ in all experiments.
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modulation of miR-106b, and found a dose-dependent reduction in pri-miR-106b expression in both the MyLa2059 and MyLa3675 cell line upon treatment with SAHA (Fig. 4a). In addition, SAHA induced increased mRNA and protein expression of p21 and TXNIP in both MF cell lines in a dose-dependent manner (Fig. 4b,c).

Suberoylanilide hydroxamic acid and miR-106b regulate the p21 and TXNIP expression in mycosis fungoides

Next, western blotting analysis assessed the regulatory effect of both SAHA and miR-106b on the protein expression of p21 and TXNIP. Of note, addition of both SAHA and miR-106b inhibitor provided an additive induction of both p21 (Fig. 5a) and TXNIP (Fig. 5c) in the MyLa2059 and MyLa3675 cell lines. Conversely, the protein induction of p21 and TXNIP by SAHA was reduced or neutralized by addition of miR-106b mimic in both MF cell lines (Fig. 5b,d), all indicating that miR-106b has a key regulatory role on the tumour suppressors p21 and TXNIP and that SAHA may act through miR-106b in the regulation of p21 and TXNIP in MF.

DISCUSSION

MiR-106b is a strong prognostic marker of disease progression in MF (16). The current study provides evidence, for the first time, for its molecular function in MF disease progression.

The results of this study substantiate that miR-106b is expressed in situ in dermal T-lymphocytes in MF and that a dermal stromal staining either reflects miR-106b release from the T cells or a miR-106b expression in stromal cells, indicating that miR-106b may impact the entire dermal tumour microenvironment in MF. MiR-106b expression increases with advancing T-stage and promotes malignant T-cell proliferation, indicating that miR-106b is involved in molecular changes leading to progression of MF. In skin biopsies from patients with MF, the tumour suppressors p21 and TXNIP were repressed compared with healthy control skin samples. Furthermore, transfection of 2 MF-derived cell lines with miR-106b inhibitor or mimic revealed that miR-106b is responsible for or at least contributes to the p21 and TXNIP repression, which may promote tumour cell proliferation. This points to miR-106b as a potential driver of MF progression.

The molecular mechanism for tumour cell expansion in MF needs to be elucidated for development of new effective targeted therapies. MiRNAs have the potential to serve as therapeutic targets in CTCL, demonstrated by the current clinical trial, testing a miR-155 inhibitor in the treatment of CTCL (15).

MiR-106b may be a possible new therapeutic target. MiR-106b is also upregulated in several other cancer types, including breast cancer (34), colorectal cancer (35), prostate cancer (36) and non-small cell lung cancer (37). In most of these cancers miR-106b is involved in tumour cell proliferation by targeting p21 (38), as demonstrated for MF. Also miR-16 may contribute to p21 gene expression regulation in CTCL (23). p21 has a key regulatory role in the tumourigenesis of CTCL and is induced during treatment with SAHA, but the exact mode of action is elusive (39). The current study demonstrates that SAHA treatment inhibits miR-106b, which may increase the expression of p21, supporting that miR-106b has a crucial role in tumour cell proliferation in MF.

In addition, for the first time, this study demonstrates that the tumour suppressor TXNIP is regulated by miRNAs in CTCL. TXNIP inhibits cellular glucose uptake (40). Activated T cells and malignant T cells require high glucose uptake to generate the energy required for cell proliferation. Thus, limited glucose uptake induced by TXNIP provides decreased T-cell proliferation (40). MiR-106b represses expression of TXNIP, and SAHA may induce TXNIP expression through miR-106b down-regulation. Because SAHA has significant clinical effect in the treatment of CTCL, miR-106b may be a putative therapeutic target for treatment of MF. As miR-106b is highly expressed also in the early stages of MF, a miR-106b inhibiting drug may prevent or postpone progression when initiated at an early disease stage, in particular in patients at high risk of disease progression.

In conclusion, this study demonstrates, for the first time, that miR-106b is expressed in situ in dermal T-lymphocytes in MF, its expression increases with advancing T-stage and miR-106b promotes tumour cell proliferation by inhibiting expression of the tumour suppressors p21 and TXNIP, substantiating that miR-106b is both a prognostic marker and a functional promoter of tumour progression in MF, and thus may serve as a potential future therapeutic target for MF.

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