MicroRNA-21 Expression in CD4+ T Cells Is Regulated by STAT3 and Is Pathologically Involved in Sézary Syndrome

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MicroRNAs (miRNAs) are small RNAs that control gene expression, and are involved in the regulation of fundamental biological processes including development, cell differentiation, proliferation, and apoptosis. miRNAs regulate gene expression in normal hematopoiesis, and aberrant miRNA expression might contribute to leukomogenesis. Specifically, miR-21 is abundantly expressed in various tumors including leukemia and lymphoma, and is functionally involved in oncogenic processes. We investigated a role for miR-21 in Sézary Syndrome (SS), a cutaneous T-cell lymphoma (CTCL) with CD4+ tumor cells (Sézary cells) present in the skin, lymph nodes, and peripheral blood. It was shown previously that SS is characterized by constitutively activated signal transducer and activator of transcription 3 (STAT3) signaling. In this study we show by chromatin immunoprecipitation that miR-21 is a direct STAT3 target in Sézary cells. Stimulation of Sézary cells or healthy CD4+ T cells with the common-γ chain cytokine IL-21 results in a strong activation of STAT3, and subsequent upregulation of miR-21 expression. Both pri- and mature miR-21 expression are increased in Sézary cells when compared with CD4+ T cells from healthy donors. Silencing of miR-21 in Sézary cells results in increased apoptosis, suggesting a functional role for miR-21 in the leukomogenic process. Consequently, miR-21 might represent a therapeutic target for the treatment of SS.

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INTRODUCTION

Sézary Syndrome (SS) is a rare leukemic cutaneous T-cell lymphoma (CTCL) with an aggressive clinical course. It is characterized by erythroderma, generalized lymphadenopathy, and neoplastic skin-homing CD4+ memory T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood (Willemze *et al.*, 2005). We previously performed a detailed genomic analysis of SS and demonstrated that deregulation of the common-γ chain cytokine/signal transducer and activator of transcription (STAT) signaling pathway might be one of the molecular mechanisms involved in its pathogenesis (Vermeer *et al.*, 2008). The transcription factor family of STAT proteins has a crucial regulatory role in numerous biological processes such as cell proliferation, differentiation, and survival, and is critical in malignant transformation and oncogenesis. STAT3 has been found to be activated inappropriately in a wide range of human cancers (Yu and Jove, 2004). Also, in SS, constitutive activation of STAT3 was demonstrated previously (Nielsen *et al.*, 1999; Eriksen *et al.*, 2001; van Kester *et al.*, 2008; Zhang *et al.*, 2008).

A functional role for STAT3 in oncogenic processes has been demonstrated extensively. In Sézary cells, inhibition of STAT3 results in rapid apoptosis (Eriksen *et al.*, 2001; van Kester *et al.*, 2008). STAT3 exerts its oncogenic effects by transcriptional regulation of its target genes, and identification of these targets is mandatory to understand fully the pathophysiological role of STAT3 in oncogenesis. Potential downstream STAT3 targets include genes that regulate cell cycle progression, cell survival/growth, and angiogenesis.

It was recently shown that STAT3 can also regulate the expression of the cancer-related microRNA-21 (miR-21) in multiple myeloma (Loffler *et al.*, 2007). miRNAs are non-protein coding small RNAs that regulate gene expression via post-transcriptional silencing of target genes. They are transcribed as primary transcripts (pri-miRNA) that are processed by Drosha in the nucleus to generate precursor miRNA. This pre-miRNA is transported to the cytoplasm, where it is subsequently processed by Dicer to yield the mature miRNA. MiRNAs have important regulatory roles in fundamental biological processes such as cell development, differentiation, proliferation, and apoptosis, and

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Abbreviations: BE, benign erythroderma; ChIP, chromatin immunoprecipitation; CTCL, cutaneous T-cell lymphoma; CucI, cucurbitacin I; miR, microRNA; PTEN, phosphatase and tensin homolog; SS, Sézary Syndrome; STAT, signal transducer and activator of transcription

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hematopoiesis is also extensively regulated by miRNAs (reviewed by Yendamuri and Calin, 2009). In addition, miRNAs are crucially involved in tumorigenesis by controlling gene expression of oncogenes and tumor suppressors. They have been found to be aberrantly expressed in many types of cancer, including hematopoietic malignancies such as leukemias and lymphomas (reviewed by Lawrie *et al.*, 2008; Yendamuri and Calin, 2009).

miR-21 is a miRNA that is frequently upregulated in human cancers such as breast cancer, glioma, colorectal cancer, and hepatocellular carcinoma, and in hematological malignancies such as chronic lymphatic leukemia, acute myeloid leukemia, B-cell lymphoma, and Hodgkin's lymphoma (Krichevsky and Gabriely, 2009). Previous observations demonstrate an oncogenic role for miR-21: ectopic overexpression of miR-21 increased tumor cell proliferation, migration, and invasion, whereas inhibition of miR-21 expression reduced proliferation, tumor growth, and invasion, while inducing apoptosis (reviewed by Krichevsky and Gabriely, 2009).

In this study we demonstrate that miR-21 is a direct STAT3 target in Sézary cells. In line with the previously described deregulated STAT3 activation in Sézary cells, we found that expression of miR-21 is increased in neoplastic CD4+ cells from Sézary patients, whereas inhibition of miR-21 results in increased apoptosis of Sézary cells. Together, our results suggest that miR-21 has a functional role in the pathogenesis of SS.

RESULTS

STAT3 binds to the promoter of the miR-21 gene in Sézary cells

Constitutive STAT3 activation in Sézary cells is demonstrated by various groups (Nielsen *et al.*, 1999; Eriksen *et al.*, 2001; van Kester *et al.*, 2008; Zhang *et al.*, 2008), whereas this activation is lost upon *in vitro* culturing (van Kester *et al.*, 2008). It was shown previously that STAT3 can be activated in CTCL cells cultured *in vitro* by the common-γ chain cytokine IL-21 (Marzec *et al.*, 2008). These results were validated in our experimental systems. Culturing of Sézary cells with recombinant IL-21 results in a rapid and transient increase in phosphorylated STAT3 levels, with a maximal STAT3 activation after 15–30 minutes (Figure 1a). Furthermore, pretreatment of cells with cucurbitacin I (CucI), an inhibitor of the JAK/STAT3 signaling pathway (Blaskovich *et al.*, 2003), resulted in a complete block of STAT3 activation by IL-21 (Figure 1b). Similar results were observed using CD4+ cells isolated from the peripheral blood from healthy donors, as well as in SeAx and HuT78, two continuous cell lines derived from patients with SS (data not shown).

A STAT3 target gene that was described previously is the gene encoding the primary transcript of miR-21 (Loffler *et al.*, 2007). We assayed whether miR-21 is also a direct STAT3 target in Sézary cells. SeAx cells were treated with IL-21 to activate STAT3, and analyzed by chromatin immunoprecipitation (ChIP). Chromatin that was precipitated with STAT3 antibodies was significantly enriched for the miR-21 promoter sequence when compared with chromatin precipitated with normal rabbit IgG. In contrast, in SeAx cells not treated

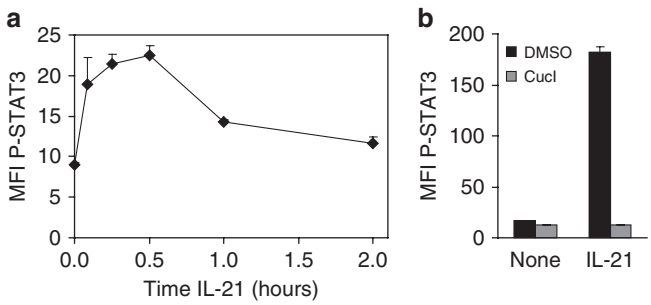

Figure 1. IL-21 transiently induces signal transducer and activator of transcription 3 (STAT3) activity in Sézary cells. CD4+ T cells were isolated from peripheral blood of Sézary Syndrome (SS) patients. STAT3 activation was assayed by flow cytometry using an antibody specific for phosphorylated STAT3 (P-STAT3), and depicted as mean fluorescent intensity (MFI) of duplicated measurements (mean ± SD). (**a**) Sézary cells were stimulated with IL-21 for the time points indicated. (**b**) Cells were pretreated *in vitro* with DMSO or cucurbitacin I (CucI) for 2 hours, and thereafter stimulated with IL-21 for 15 minutes.

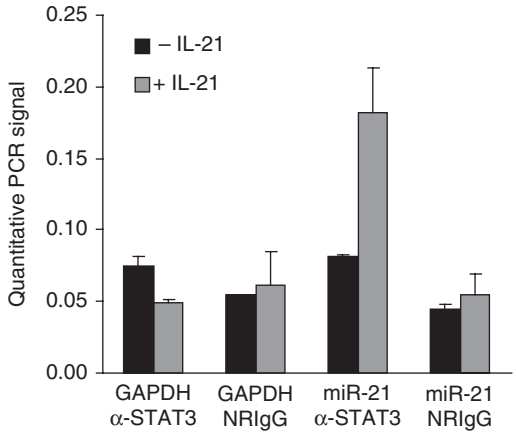

Figure 2. Signal transducer and activator of transcription 3 (STAT3) directly binds the microRNA-21 (miR-21) promoter in Sézary cells. SeAx cells were cultured with or without IL-21 for 30 minutes, and chromatin immunoprecipitation (ChIP) was performed using antibodies against STAT3 or normal rabbit IgG (NRIgG) as a negative control. Immunoprecipitated DNA was used in quantitative PCR reactions with primers specific for the promoter region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or miR-21. Bars represent mean ± SD of duplicate measurements.

with IL-21, hardly miR-21 promoter sequences were detected in chromatin precipitated with STAT3 antibodies (Figure 2). As a control, PCR was performed on the precipitated chromatin for the promoter of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a promoter that lacks STAT3 binding sites. No precipitation of the GAPDH promoter was observed in SeAx cells cultured with or without IL-21 (Figure 2), demonstrating the specificity of the ChIP.

Expression of primary miR-21 is rapidly and transiently increased upon STAT3 activation

Quantitative reverse transcriptase PCR (RT-PCR) analysis of RNA isolated from freshly isolated Sézary cells stimulated with IL-21 showed that the expression of the primary miR-21 transcript (pri-miR-21) is rapidly and transiently induced,

with a maximum induction after 1 to 2 hours (Figure 3a). The kinetics of this induction follows the transient induction of STAT3 activation upon IL-21 stimulation (Figure 1a). A similar induction of pri-miR-21 was observed after stimulation of CD4+ T cells isolated from healthy controls (Figure 3b). To assay the STAT3 dependency of the pri-miR-21 induction by IL-21, cells were pretreated with CucI for 2 hours. Surprisingly, CucI treatment by itself consistently induced pri-miR-21 expression (Figure 3c). Nevertheless, induction of miR-21 expression by IL-21 was significantly diminished after pretreatment of CD4+ T cells with CucI when compared with pretreatment with the solvent DMSO (Figure 3d). Similar results were obtained using isolated CD4+ T cells from SS patients and healthy controls (Figure 3d). Collectively, our results suggest that the induction of pri-miR-21 expression by IL-21 stimulation of CD4+ T cells is dependent on the activation of STAT3.

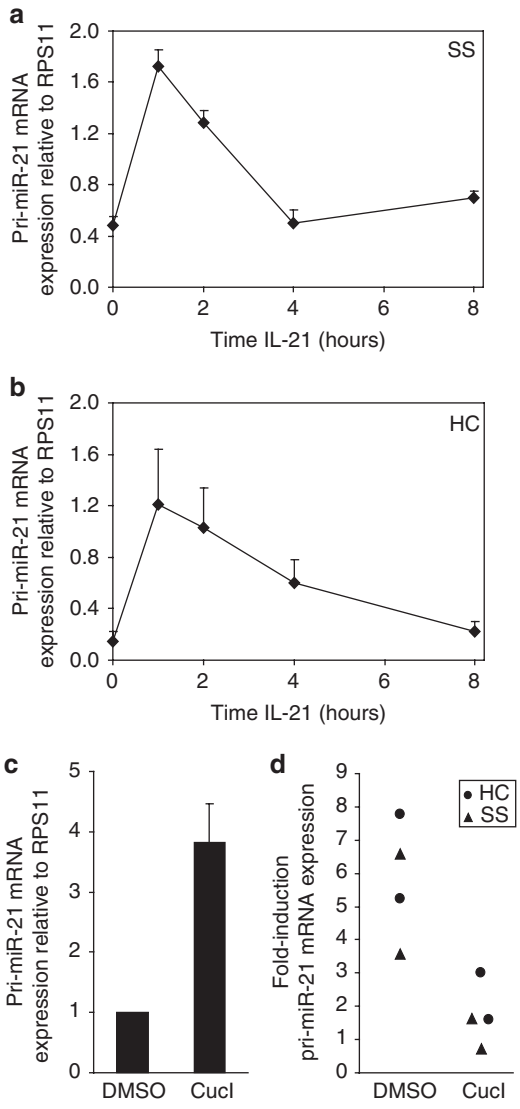

Figure 3. Primary microRNA-21 (Pri-miR-21) expression is induced by IL-21, dependent on STAT3 activation. CD4+ T cells were *in vitro* stimulated by IL-21. Pri-miR-21 expression was assayed by quantitative reverse transcriptase PCR (RT-PCR), and normalized for expression of RPS11. Pri-miR-21 expression (mean ± SD) from duplicate measurements is shown. Representative examples of (**a**) two Sézary Syndrome (SS) patients and (**b**) two healthy controls are shown. (**c**) CD4+ T cells from SS patients ($n=2$) and healthy controls ($n=2$) were treated with DMSO or cucurbitacin I (CucI) for 2 hours. Bars represent pri-miR-21 expression (mean ± SEM) relative to levels in DMSO-treated cells. (**d**) CD4+ T cells from SS patients or healthy controls were pretreated with DMSO or CucI for 2 hours, and subsequently cultured with or without IL-21 for 1 hour. Pri-miR-21 expression was assayed by RT-PCR and normalized for RPS11 expression. Symbols represent fold-induction of pri-miR-21 expression by IL-21 for individual healthy controls (HC, circles) or SS patients (triangles).

Increased expression of miR-21 in Sézary cells

MiR-21 expression has been found upregulated in many different malignancies. We quantitatively compared miR-21 expression in CD4+ T cells isolated from SS patients and healthy controls. As an additional control, we used CD4+ cells that were isolated from patients with benign erythroderma (BE) secondary to atopic dermatitis. This cell population consists of activated T cells, as evidenced by a 9-fold increased expression of the T-cell activation marker CD25 when compared with CD25 expression in CD4+ T cells from healthy donors (data not shown). Significant increased pri-miR-21 expression was observed in SS patients when compared with healthy controls and patients with BE (Figure 4a). Interestingly, levels of mature miR-21 were significantly increased in both patients with SS and BE when compared with healthy controls (Figure 4b). Correlation analysis (Pearson) revealed no correlation between the expression levels of pri-miR-21 and mature miR-21 in the individual patients and controls (data not shown).

Correlation of miR-21 expression with clinical characteristics

Careful examination of the pri-miR-21 expression levels in the individual SS patients suggests the existence of two

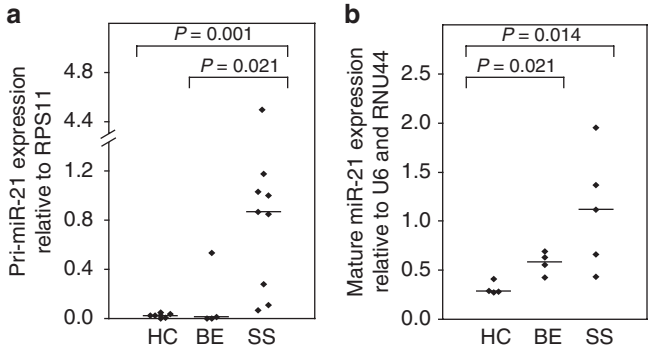

Figure 4. MicroRNA-21 (miR-21) levels are elevated in Sézary cells. RNA and miRNA were extracted from CD4+ T cells isolated from peripheral blood from healthy controls (HC), patients with benign erythroderma (BE), or patients with Sézary Syndrome (SS). (**a**) Pri-miR-21 expression was determined by quantitative reverse transcriptase PCR (RT-PCR) and normalized for expression of a stably expressed reference gene (RPS11). (**b**) Mature miR-21 expression was determined by TaqMan, and normalized for U6 and RNU44 expression. Each symbol represents the expression in an individual patient, and the median is indicated with a horizontal line. Statistically significant differences ($P<0.05$; Mann–Whitney) are indicated.

subgroups of patients, that is, a group with slightly elevated pri-miR-21 expression when compared with healthy controls and patients with BE, and a group with extremely elevated pri-miR-21 levels (Figure 4a). There was no obvious correlation of the pri-miR-21 levels in these SS patients with clinical parameters such as total leukocyte count, absolute numbers of Sézary cells in the peripheral blood, or CD4/CD8 ratio (data not shown). Remarkably, the 3 patients with relative low pri-miR-21 expression are all alive (median follow-up 10 months, range 10–14), whereas 4 out of 6 patients with relative high pri-miR-21 expression died of their disease (median follow-up 14 months, range 10–48), while 2 are still alive (follow-up 7–54 months).

From the group of patients in whom mature miR-21 levels were studied (Figure 4b), 4 of 5 patients are alive (median follow-up 12 months, range 10–54), whereas 1 patient died of disease (follow-up 11 months). Interestingly, this latter patient displayed the highest level of mature miR-21 expression.

Summarizing, these initial observations suggest that miR-21 expression levels might have a prognostic value. Obviously, this should be verified in a larger group of SS patients before conclusive statements can be made.

miR-21 is involved in regulation of apoptosis in Sézary cells

The functional relevance of the increased miR-21 expression for the survival of Sézary cells was studied. MiR-21 expression was knocked down in SeAx cells by transfecting the cells with anti-miR-21 oligonucleotides, and subsequently analyzed for apoptosis. Quantitative PCR analysis of the transfected cells showed that miR-21 levels were decreased by 80% when compared with untransfected control cells (data not shown). Transfection of anti-miR-21 resulted in decreased numbers of viable cells, and increased numbers of dead and apoptotic cells, when compared with cells transfected with fluorescently labeled oligonucleotides only, or with a negative control oligonucleotide (Figure 5).

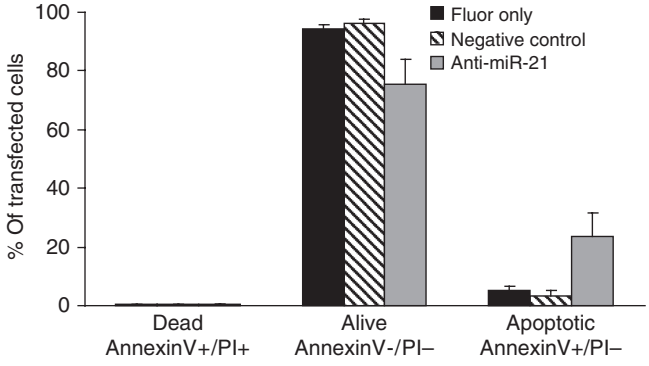

Figure 5. Inhibition of microRNA-21 (miR-21) in Sézary cells results in increased apoptosis. SeAx cells were transfected with fluorescently labeled oligonucleotides, in combination with anti-miR-21 oligonucleotides, or negative control oligonucleotides. After 24 hours, apoptosis was assayed by flow cytometric analysis for Annexin V and propidium iodide (PI). The number of dead, living, and apoptotic cells was calculated as percentage of the total number of transfected (=fluorescently labeled) cells. Bars denote mean ± SD of duplicate measurements in a representative experiment of four.

These results clearly demonstrate a functional role for miR-21 in the regulation of apoptosis in Sézary cells.

The tumor-suppressor gene phoshatase and tensin homolog (*PTEN*), is a validated miR-21 target in various malignancies (Meng *et al.*, 2006, 2007; Yamanaka *et al.*, 2009). Loss of PTEN was shown to be relevant in the pathogenesis of T-cell malignancies, including CTCL (Scarisbrick *et al.*, 2000). We therefore assessed whether PTEN is a miR-21 target in Sézary cells. Knockdown of miR-21 in Sézary cells did not alter PTEN protein or mRNA expression (data not shown). In addition, the signaling pathway downstream of PTEN was not affected by miR-21 knockdown as phosphorylated AKT levels were unaltered (data not shown). Combined, these data argue against a role for PTEN as miR-21 target in the regulation of Sézary cell survival. Thus, the molecular pathway by which miR-21 regulates apoptosis in Sézary cells is still elusive.

DISCUSSION

In this paper we demonstrate that the expression of miR-21 is induced by IL-21 in CD4+ T cells, mediated via the activation of STAT3. MiR-21 expression levels are significantly elevated in CD4+ tumor cells from the peripheral blood of patients with SS, and inhibition of miR-21 results in apoptosis of Sézary cells. Our results are schematically summarized in Figure 6.

Previous microarray analysis of CTCL cells identified a limited number of genes modulated by IL-21 stimulation (Marzec *et al.*, 2008). The *miR-21* gene, shown in this study to be a STAT3 target and induced upon IL-21 stimulation, was not among these genes. This seemingly discrepancy is

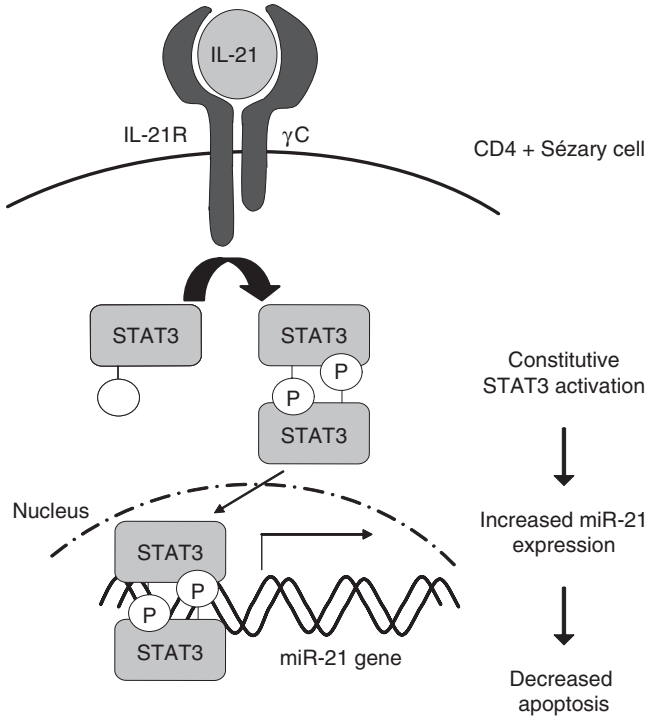

Figure 6. Schematic model summarizing our results.

probably caused by the rapid and transient nature of miR-21 induction as shown in this paper (Figure 3a and b), whereas the microarray analysis to identify IL-21-responsive genes was only conducted after 4 hours of IL-21 stimulation (Marzec *et al.*, 2008).

Various mechanisms can attribute to aberrant expression of primary transcripts for miRNAs in cancer cells (reviewed by Calin and Croce, 2007). Transcription of pri-miR-21 is controlled by a promoter region that contains several conserved elements, including binding sites for AP-1 (activator protein 1), Ets/PU.1, C/EBP-α (CCAAT/enhancer-binding protein-α), p53, and STAT3 (Fujita *et al.*, 2008). In this study we demonstrate that in SS, miR-21 expression is positively regulated by direct binding of STAT3. Similarly, it was shown previously that in multiple myeloma cells, induction of miR-21 expression by IL-6 is strictly STAT3 dependent (Loffler *et al.*, 2007). Thus, increased miR-21 expression, as shown in this study, might well be a direct consequence of constitutive activation of STAT3 in Sézary cells (Nielsen *et al.*, 1999; Eriksen *et al.*, 2001; van Kester *et al.*, 2008). In addition, chromosomal abnormalities can contribute to deregulated gene expression in cancer. The *miR-21* gene is mapped to chromosome 17q23.2, and amplification of this region is associated with a number of cancers, including breast (Wu *et al.*, 2000) and prostate cancer (Kasahara *et al.*, 2002), as well as Hodgkin's lymphoma (Chui *et al.*, 2003). Our previous research showed gain of the 17q23 region containing the *miR-21* gene in 16/20 of SS patients (Vermeer *et al.*, 2008), as well as in the SeAx cell line (data not shown). However, in the group of seven patients for whom both pri-miR-21 expression and genomic data are available, no clear correlation between these parameters was observed (data not shown), indicating that other transcription regulatory mechanisms are equally or more important.

An additional level of regulation of miRNA expression is at the level of processing primary miRNA transcripts into mature miRNAs. Systematic evaluation showed that levels of pre- and mature miR-21 correlated very well in numerous tissues, cell lines, and tumors (Lee *et al.*, 2008), suggesting that expression of miR-21 is not dominantly regulated at the level of processing by Dicer. In contrast, Drosha-mediated cleavage of pri-miR-21 into pre-miR-21 is heavily regulated (Davis *et al.*, 2008). Thus, the lack of correlation between pri- and mature miR-21 levels in SS, as described in this paper, might well be caused by regulation at the level of Drosha activity. To test this, it would be interesting to assay pre-miR-21 expression in tumor cells from SS patients.

In this paper we demonstrate that inhibition of miR-21 induces apoptosis in Sézary cells, similarly as demonstrated for cell lines from various other malignancies (Chan *et al.*, 2005; Meng *et al.*, 2006, 2007; Si *et al.*, 2007; Zhu *et al.*, 2007; Fei *et al.*, 2008; Frankel *et al.*, 2008; Li *et al.*, 2009). mIR-21 mediates these effects by silencing its target genes. mIR-21 targets validated independently by several research groups include tumor suppressors such as programmed cell death protein 4 (PDCD4), tropomyosin 1 (TPM1), and PTEN (reviewed by Krichevsky and Gabriely, 2009). Knockdown of miR-21 in Sézary cells does not alter the expression of PTEN or its downstream signaling component phospho-AKT (data not shown), arguing against a role for PTEN as miR-21 target in the regulation of Sézary cell survival. Thus, the miR-21 target genes important in regulating apoptosis of Sézary cells remain to be identified.

Inhibition of miR-21 resulted in induction of apoptosis of ~20–25% of transfected Sézary cells. In contrast, inhibition of STAT3 using CucI resulted in apoptosis in 73–91% of Sézary cells (van Kester *et al.*, 2008). This apparent discrepancy might be caused by the contribution of other STAT3 target genes besides miR-21. Among the STAT3 target genes that were described previously, several function as inhibitors of apoptosis (e.g., Bcl-xL (B-cell lymphoma-extra large), Mcl-1 (myeloid cell leukemia sequence 1), and survivin), and cell-cycle regulators (e.g., cyclin D1 and c-myc).

In summary, we demonstrate that miR-21 is a direct STAT3 target in SS, and that constitutive STAT3 activation in Sézary cells, as shown previously (Nielsen *et al.*, 1999; Eriksen *et al.*, 2001; van Kester *et al.*, 2008; Zhang *et al.*, 2008), is accompanied by increased miR-21 expression. In numerous cancers, overexpression of miR-21 has been reported (Krichevsky and Gabriely, 2009), often associated with constitutively activated STAT3 (Jing and Tweardy, 2005). Therefore, the oncogenic properties of STAT3 are (at least partly) mediated via miR-21. Although STAT3 has been regarded as a promising therapeutic target for many years, its clinical development has been hampered, generating a need for reassessment of the ongoing strategies on the targeting of STAT3 (Jing and Tweardy, 2005). The identification of miR-21 as one of the oncogenic STAT3 downstream targets might offer a selective and promising therapeutic option for SS and other malignancies.

MATERIALS AND METHODS

Patient selection

Patients with SS (2 males and 8 females, median age 67 years) were diagnosed based on criteria of the WHO/EORTC 2005 classification (Willemze *et al.*, 2005). All patients presented with erythroderma, and showed T-cell clonality in the peripheral blood with a CD4/CD8 ratio of >10. As control groups, patients with erythroderma secondary to atopic dermatitis (BE), and healthy donors were included.

Approval for these studies was obtained from the Leiden University Medical Center review board, and informed consent was provided according to the Declaration of Helsinki Principles.

Cell culturing and stimulation experiments

SeAx, a cell line derived from the peripheral blood of a patient with SS (kindly provided by Dr K Kaltoft) (Kaltoft *et al.*, 1987) was cultured in RPMI-1640 (Invitrogen, Breda, The Netherlands), supplemented with 10% fetal calf serum (HyClone/Greiner, Nürtingen, Germany), 2 mM L-glutamine (Invitrogen), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Invitrogen), and 200 U ml^{-1} IL-2 (PeproTech, Rocky Hill, NJ). HuT78, a cell line derived from peripheral blood of a patient with SS (ATCC number TIB-161) was cultured in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

Heparinized blood was drawn from patients and controls, and peripheral blood mononuclear cells were isolated by Ficoll density centrifugation. Subsequently, CD4+ T cells were isolated by negative selection with magnetic beads (CD4+ T-cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the cell population was determined by flow cytometry for CD3, CD4, and CD8 expression, and was generally >85%. In addition, earlier experiments demonstrated that >90% of the CD4+ cells comprise malignant cells characterized by expression of a clonally rearranged T-cell receptor.

Cells were cultured in RPMI-1640 supplemented with 10% human AB serum (Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 200 U ml^{-1} IL-2, and 5 ng ml^{-1} IL-7 (PeproTech).

Cells used for stimulation experiments were grown overnight in cytokine-deprived medium, and subsequently stimulated with IL-21 (100 ng ml^{-1}; ImmunoTools, Friesoythe, Germany) for the times indicated. CucI (Indofin, Hillsborough, NJ) was dissolved in DMSO at a concentration of 50 mM, and diluted in culture medium to a final concentration of 30 μM.

Flow cytometry

STAT3 activation was assayed by flow cytometry. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at room temperature. After washing with phosphate-buffered saline/0.2% bovine serum albumin, cells were permeabilized in 90% ice-cold methanol for 30 minutes at 4 °C. After washing, cells were stained with Alexa647-conjugated antibodies against phosphorylated STAT3 (BD Biosciences, Franklin Lakes, NY) for 20 minutes at 4 °C.

Flow cytometric analysis for apoptotic cells was performed by staining cells with allophycocyanin-conjugated antibodies against Annexin V and propidium iodide, using the apoptosis detection kit (eBioscience, San Diego, CA).

Samples were acquired on a flow cytometer (FacsCalibur, BD Biosciences), and analyzed using CellQuest software (BD Biosciences).

Quantitative reverse transcriptase PCR

Expression levels of pri-miR-21 were assayed in total RNA samples isolated from 1 to 10×10^6 cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA (1 μg) was treated with RQ1 DNase I (Promega, Madison, WI) and converted into complementary DNA using IScript reverse transcriptase (Bio-Rad, Hemel Hempstead, UK), oligo$(dT)_{12-18}$, and random hexamer primers (Bio-Rad). Real-time PCR was performed using the IQ5 instrument and SYBR Green Supermix (Bio-Rad), using the following cycle parameters: 5 minutes at 95 °C, followed by 45 cycles of 15 seconds at 97 °C and 30 seconds at 60 °C. Primer sequences for pri-miR-21 and RPS11 have been described previously (Loffler *et al.*, 2007; Vermeer *et al.*, 2008). Data were analyzed using IQ5 software using the ΔΔCt method (Bio-Rad), and expressed relative to RPS11 expression levels. Specificity of the PCR products was confirmed by melting curve analysis.

For determining mature miR-21 expression levels, total RNA was isolated from 1 to 10×10^6 cells using the mirVana RNA isolation kit (Ambion, Austin, TX). Total RNA (150 ng) was used for reverse transcriptase and subsequent pre-amplification reaction using Megaplex primer pool A, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The miRNA real-time PCR was performed for miR-21, U6, and RNU44 using the TaqMan miRNA assays and Universal PCR mastermix (Applied Biosystems) on a IQ5 instrument (Bio-Rad), according to the manufacturer's protocol (Applied Biosystems). The cycle parameters were as follows: 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 60 seconds at 60 °C. miR-21 expression was analyzed using the ΔΔCt method in the IQ5 software (Bio-Rad), and expressed relative to U6 and RNU44 expression levels.

Chromatin immunoprecipitation

ChIP was performed using the EZ-ChIP kit (Upstate, Temecula, CA), according to the manufacturer's instruction. In short, SeAx cells were grown overnight in medium deprived of cytokines. Cells were incubated with or without IL-21 (50 ng ml^{-1}) for 30 minutes at 37 °C, immediately fixed in formaldehyde, and chromatin was sonicated using the Bioruptor (Diagenode, Liege, Belgium). Immunoprecipitation was performed using anti-STAT3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), or with control antibodies supplied in the ChIP kit (anti-acetyl-Histone H3, or normal rabbit IgG). Co-immunoprecipitated DNA was amplified using primers for the GAPDH promoter, as supplied in the ChIP kit, or primers specific for the upstream region of the *miR-21* gene (Loffler *et al.*, 2007). Real-time PCR was performed using the IQ5 instrument and SYBR Green Supermix (Bio-Rad), using the following cycle parameters: 6 minutes at 95 °C, followed by 45 cycles of 15 seconds at 96 °C, 30 seconds at 57 °C, and 30 seconds at 72 °C. Data were analyzed using IQ5 software using the ΔCt method (Bio-Rad). Specificity of the PCR products was confirmed by melting curve analysis.

Transfection experiments

SeAx cells were transferred to medium without penicillin and streptomycin. Next day, cells were transfected by Lipofectamine 2000 (Invitrogen), with 100 nM fluorescently labeled oligonucleotides (BLOCK-iT, Invitrogen) combined with 40 nM anti-miR-21 miRNA inhibitor (no. AM10206; Ambion) or negative control (negative control no. 1: AM17010; Ambion). At 24 hours after transfection, cells were analyzed for apoptosis by flow cytometry.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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