Targeted Delivery of microRNA-29b by Transferrin-Conjugated Anionic Lipopolyplex Nanoparticles: A Novel Therapeutic Strategy in Acute Myeloid Leukemia

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Abstract

**Purpose:** miR-29b directly or indirectly targets genes involved in acute myeloid leukemia (AML), namely, DNMTs, CDK6, SP1, KIT, and FLT3. Higher miR-29b pretreatment expression is associated with improved response to decitabine and better outcome in AML. Thus, designing a strategy to increase miR-29b levels in AML blasts may be of therapeutic value. However, free synthetic miRs are easily degraded in bio-fluids and have limited cellular uptake. To overcome these limitations, we developed a novel transferrin-conjugated nanoparticle delivery system for synthetic miR-29b (Tf-NP-miR-29b).

**Experimental Design:** Delivery efficiency was investigated by flow cytometry, confocal microscopy, and quantitative PCR. The expression of miR-29b targets was measured by immunoblotting. The antileukemic activity of TF-NP-miR-29b was evaluated by measuring cell proliferation and colony formation ability and in a leukemia mouse model.

**Results:** TF-NP-miR-29b treatment resulted in more than 200-fold increase of mature miR-29b compared with free miR-29b and was approximately twice as efficient as treatment with non-transferin-conjugated NP-miR-29b. TF-NP-miR-29b treatment significantly downregulated DNMTs, CDK6, SP1, KIT, and FLT3 and decreased AML cell growth by 30% to 50% and impaired colony formation by approximately 50%. Mice engrafted with AML cells and then treated with TF-NP-miR-29b had significantly longer survival compared with TF-NP-scramble (P = 0.015) or free miR-29b (P = 0.003). Furthermore, priming AML cell with TF-NP-miR-29b before treatment with decitabine resulted in marked decrease in cell viability in vitro and showed improved antileukemic activity compared with decitabine alone (P = 0.001) in vivo.

**Conclusions:** TF-NP effectively delivered functional miR-29b, resulting in target downregulation and antileukemic activity and warrants further investigation as a novel therapeutic approach in AML. Clin Cancer Res; 19(9); 2355–67. ©2013 AACR.

Introduction

Acute myeloid leukemia (AML) is one of the most common leukemias and is characterized by a differentiation arrest and an uncontrolled proliferation of malignant blasts. Despite advances in our understanding of disease mechanisms, the outcome of most patients with AML remains poor (1–3). Thus, novel therapeutic strategies are needed.

MicroRNAs (miR) are short noncoding RNAs that regulate the expression of their target mRNA-encoded proteins. The aberrant expression of some miRs has been shown to be involved in AML leukemogenesis and to have prognostic significance (4–7). miR-29b has been shown to be downregulated in AML (8). This miR directly or indirectly targets a panel of genes that, when deregulated, contribute to myeloid leukemogenesis. These genes are involved in DNA
methylation (i.e., DNMT1, DNMT3A, and DNMT3B), cell-cycle progression (i.e., CDK6), and apoptosis (i.e., MCL1; refs. 8, 9). Furthermore, we recently showed that increasing miR-29b levels resulted in decreased expression of the receptor tyrosine kinases FLT3 and KIT, which are frequently mutated in AML. This resulted in decreased leukemia growth and improved survival in an AML-mouse model. Furthermore, we showed that pretreatment with miR-29b nanoparticles improved the antileukemic activity of decitabine, a hypomethylating agent often administered in elderly patients. These promising results warrant further development of nanoparticle-based miR-29b treatment as a novel approach in AML.

**Translational Relevance**

Patients with AML frequently have a poor outcome. The microRNA miR-29b has been shown to have tumor suppressor activity by targeting genes involved in myeloid leukemogenesis and to be associated with better outcome in patients with AML. Thus, increasing miR-29b in AML blasts may be beneficial. Here, we developed a targeted, nanoparticle-based system to deliver microRNAs to AML blasts. Following miR-29b-nanoparticle treatment, we showed an efficient increase of mature and functional intracellular miR-29b levels. The treatment downregulated the miR-29b targets DNMTs, CDK6, SP1, and the receptor tyrosine kinases FLT3 and KIT, which are frequently mutated in AML. This resulted in decreased leukemia growth and improved survival in an AML-mouse model. Furthermore, we showed that pretreatment with miR-29b nanoparticles improved the antileukemic activity of decitabine, a hypomethylating agent often administered in elderly patients. These promising results warrant further development of nanoparticle-based miR-29b treatment as a novel approach in AML.

**Materials and Methods**

**Preparation of nanoparticles**

The synthetic miR-29b, miR-scramble control (scramble miR molecules), and scramble control labeled with the fluorescent dye FAM (FAM-miR) were purchased from Ambion. The lipid components were 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol (MW/C242000; DMG-PEG; Avanti Polar Lipids), and linoleic acid (Sigma-Aldrich). The molar ratio of DOPE/linoleic acid/DMG-PEG was 50/48/2.

We prepared the transferrin-conjugated nanoparticle as shown in Fig. 1. Mimic miRs were mixed with polyethyleneimine (MW, ~2000; Sigma-Aldrich) at room temperature (Step 1). The N/P ratio (the ratio of moles of the amine group of PEI to those of the phosphate groups of DNA) was 10:1. To form empty nanoparticles, lipid ethanol solvent was mixed with the PEI at a 20 mmol/L HEPES buffer. Step 3: the miR-PEI were mixed with the empty nanoparticles and sonicated to load the miR-PEI core into the nanoparticles. Step 4: NP-miR were modified to incorporate Tf-PEG-DSPE micelles to form the Tf-NP-miRs.

**Figure 1.** Preparation of miR-loaded transferrin-conjugated nanoparticles (Tf-NP-miR). The preparation of Tf-NP-miR is schematically illustrated. Step 1: negatively charged miR molecules were mixed with positively charged polyethyleneimine (PEI) to form a miR-PEI core structure. Step 2: empty nanoparticles were formed by injection of a lipid ethanol solvent into 20 mmol/L HEPES buffer. Step 3: the miR-PEI were mixed with the empty nanoparticles and sonicated to load the miR-PEI core into the nanoparticles. Step 4: NP-miR were modified to incorporate Tf-PEG-DSPE micelles to form the Tf-NP-miRs.
was injected into 20 mmol/L HEPES buffer, pH 7.4 (Step 2). The percentage of ethanol was less than 5%. The previously prepared empty nanoparticles were then added (Step 3). The mass ratio of lipid to miR was 10:1. Using vortexing and sonication, lipopolyplex nanoparticle-containing the mimic miRs were produced. Finally, a post-insertion method was adopted to incorporate transferrin ligand onto the miR-loaded nanoparticles, as previously described (Step 4; ref. 15).

Characterization of nanoparticles
The size of the nanoparticles was analyzed on a NICOMP Particle Sizer Model 370 (Particle Sizing Systems). The ζ-potential was determined on a ZetaPALS, Zeta Potential Analyzer (Brookhaven Instruments Corp.).

The miR entrapment efficiency was assessed by gel electrophoresis. 0.5% SDS was used to dissolve the nanoparticles. The amount of miR in solution was compared before and after dissolution by SDS by agarose gel electrophoresis of RNA using empty nanoparticles and free miR as controls.

Cell lines, patient samples, and cell culture
Kasumi-1, MV4-11, THP-1, KG1, and KG1a cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA); OCI-AML3 cells were obtained from DSMZ (Braunschweig, Germany). Cell lines were not authenticated by authors after purchase. AML patient blasts were obtained from the Ohio State University (OSU) Leukemia Tissue Bank. All patients provided written informed consent in accordance with the Declaration of Helsinki under an Institutional Review Board-approved protocol for discovery studies according to OSU institutional guidelines for tissue collection and the use of the tissue in research.

Delivery studies
Kasumi-1, OCI-AML3, and MV4-11 cells at a concentration of $3 \times 10^5$/mL were treated with miR-29b-loaded nanoparticles and controls. For Kasumi-1, OCI-AML3 cells together with the patients’ blasts to a final concentration of 100 nmol/L of miR-29b mimic molecules were used in all experiments. Because the high treatment sensitivity of MV4-11 cells, a final concentration of 30 nmol/L was used for this cell line in all experiments. After 24 and 48 hours, cells were collected and analyzed by quantitative reverse transcription-PCR (qRT-PCR) and Western blotting as described later.

Laser-scanning confocal microscopy
Cells were incubated with TF-NP-FAM-miR or NP-FAM-miR at a final concentration of 100 nmol/L for 4 hours at 37°C and washed twice with PBS followed by fixation with 4% paraformaldehyde. Nuclei were stained with 5 μg/mL of Hoechst (Biostatus Limited) for 5 minutes at room temperature. The cells were attached to a poly-L-lysine-coated cover glass slide (Sigma-Aldrich). Green fluorescence of FAM-miR and blue fluorescence of Hoechst were acquired by confocal microscopy (Olympus FV1000).

Flow cytometry
The transferrin receptor cell surface expression was detected using antibodies from BD Bioscience. Flow cytometry was carried out on a FACS-Calibur (BD Biosciences). A minimum of 10,000 events were collected and analyzed using FlowJo software (Tree Star Inc.).

RNA extraction and qRT-PCR
Total RNA extraction was carried out as previously described by using Trizol (Invitrogen; ref. 7). Total RNA from leukemic mice was isolated using the MirVana miRNA Isolation kit (Ambion) according to the manufacturer’s instructions. Then, cDNA was synthesized using Superscript III (Invitrogen) or the TaqMan miR Reverse Transcription kit (Applied Biosystems) for miR-29b, miR-140, and U44. In addition, qRT-PCR was carried out with TaqMan gene expression assays (Applied Biosystems) following the manufacturer’s protocols. Expression of pri-miR-29b-1, pri-miR-29b-2, DNM1T1, DNM1T3A, DNM1T3B, SP1, CDK6, FLT3, and KIT was normalized to 18S. miR-29b and miR-140 expression was normalized to U44. The comparative cycle threshold ($C_t$) method was used for the relative quantification of gene expression as previously described (8).

Western blot analysis
Western blot analysis was conducted as described previously (7, 16). Anti-DNMT1 (ab87656) and -DNMT3B (52A1018) antibodies were from Abcam. Anti-KIT (SC-17806) and -DNMT3A (SC-20703) antibodies were from Santa Cruz Biotechnology. Anti-SP1 (CS200631) antibodies were from Millipore. Anti-CDK6 (DCS83) and -FLT3 (8F2) antibodies were from Cell Signaling Technology. Equivalent loading was confirmed by Actin (SC-1616; Santa Cruz). The intensity of the resulting bands was measured by ImageJ 1.45s [http://imagej.nih.gov/ij]. The intensity ratio of each band respective to the corresponding actin intensity was used for relative quantification and is displayed in the figures.

Growth analysis
OCI-AML3, Kasumi-1 and MV4-11 cells (3 × 10^4/mL), and AML patient blasts were treated as described earlier. For cell lines, cells were counted at 24-hour intervals using a ViCell counter (Beckman Coulter). Growth curves were generated by MATLAB 7.9.0.529 (R2009b; The Mathworks, Inc.). For patient blasts, after 96-hour incubation, cell viability was measured by MTS assay. CellTiter 96 AQueous One Solution Reagent (Promega) was used according to the manufacturer’s instructions. Absorbance was read in a microplate reader Germini XS (Molecular devices). Each sample was run in triplicates.

Decitabine treatment and cytotoxicity studies by MTS assay
Kasumi-1, MV4-11, and OCI-AML3 cells were pretreated with TF-NP-miR-29b, TF-NP-scramble, or mock (buffer only) 48 hours before decitabine exposure. The
decitabine doses were based on our previous studies (17). After 48 hours incubation, cell viability was measured as described earlier.

**Colony formation assays**

Methylcellulose colony formation assays were carried out as previously described (18) and counted after 14 days.

**Nanoparticle toxicity profiling**

Immunocompetent B6.SJL-Ptprc<sup>−/−</sup> Pepc<sup>−/−</sup> mice (BoyJ; The Jackson Laboratory) were used for in vivo nanoparticle toxicity studies. In a first group, 7-week-old male mice were injected with saline, empty nanoparticles (15 mg/kg/d of lipids), or TF-NP-miR-29b (1.5 mg/kg/d of miR). Blood was collected 24 hours after the injection. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamatic transferase (GGT), blood urea nitrogen (BUN), and creatinine were assessed by the Clinical Pathology Services at OSU. A second group of mice were treated with either saline or TF-NP (1.5 mg/kg/d of miR) with 3 doses, every other day. During the treatment, body weight was monitored every other day and for 1 additional week after the treatment. Blood counts were assessed weekly.

**In vivo studies**

To test the antileukemic activity of TF-NP-miR-29b, we used a leukemic nonobese diabetic/severe combined immunodeficient mouse (NOD/SCID-gamma; NSG) mouse model. Six-week-old male NSG mice (The Jackson Laboratory) were intravenously injected through a tail vein with MV4-11 cells (0.3 x 10<sup>8</sup>) as described previously (19). The treatment started 10 days after the engraftment. In the first trial, mice were treated with miR-29b mimic (n = 3), TF-NP-scramble (n = 6), and TF-NP-miR-29b (n = 6; 1 mg/kg/d of miR molecule intravenously on Monday, Wednesday, and Friday for 2 weeks). Mice survival was monitored and recorded. Spleens from the same two mice were weighed. In the second trial, mice were treated with saline (n = 5), TF-NP-scramble alone (n = 7), 1.5 mg/kg/d miR intravenously, TF-NP-miR-29b alone (n = 7), decitabine alone (n = 7; 0.4 mg/kg/d, intraperitoneally as previously described; ref. 20), TF-NP-scramble followed by (→) decitabine, and TF-NP-miR-29b→decitabine as depicted in Fig. 6B. At day 24, blood was collected for gene expression analysis. These studies were carried out in accordance with the OSU institutional guidelines for animal care and under protocols approved by the OSU Institutional Animal Care and Use Committee.

**Statistical analysis**

Data were represented as mean ± SD of at least 3 independent experiments and analyzed by the 2-tailed Student t test. The mean and SD were calculated and displayed in bar graphs as the height and the corresponding error bar, respectively. Mouse survival was calculated using the Kaplan–Meier method, and survival curves were compared by log-rank test. A P < 0.05 was considered statistically significant.

**Results**

**Preparation and characterization of the nanoparticle delivery system**

To provide a nonviral delivery system for miRs to AML cells, NP-miR (NP-miR-29b and NP-scramble) were synthesized. Consistent with a previous report in AML patient blasts (21), a high transferrin receptor surface expression was observed in Kasumi-1, OCI-AML3, and MV4-11 cells (Fig. 2A) and in AML patient blasts (Supplementary Fig. S1). Thus, to facilitate an efficient, targeted delivery, we conjugated the nanoparticles with transferrin (TF-NP).

Particle size and ζ-potential values are presented in Supplementary Table S1. The average size and ζ-potential of empty nanoparticles were 129.6 ± 1.0 nm (±SD) and −9.8 ± 1.5 mV (±SD) and NP-miR were 137.6 ± 1.0 nm and 22.5 ± 1.4 mV, respectively. After the transferrin conjugation, the size of the miR-loaded TF-NP was increased to 147.3 ± 4.7 nm and the ζ-potential was 5.8 ± 1.9 mV. The achieved size and charge of the nanoparticles has been previously shown to be optimal for a long-lasting in vivo circulation time (22, 23). The mimic miR-29b entrapment efficiency of nanoparticles was evaluated by agarose gel electrophoresis. Analysis of the TF-NP encapsulating miR before the SDS treatment showed no visible band, whereas a clear band comparable with the size and intensity of free miR was observed after dissolving the TF-NP and releasing the entrapped miR molecules, thereby supporting a high miR entrapment efficiency (Supplementary Fig. S2).

**Intracellular uptake of TF-NP-miR-29b**

To assess the efficiency of cellular uptake of the miR molecules, we treated 3 AML cell lines with relatively low endogenous miR-29b expression (i.e., Kasumi-1, OCI-AML3, and MV4-11; Supplementary Fig. S3) with free FAM-labeled miR (FAM-miR), non–transferrin–conjugated FAM-miR-loaded nanoparticles (NP-FAM-miR), or transferrin–conjugated FAM-miR-loaded nanoparticles (TF-NP-FAM-miR). Four hours after the treatment, the FAM-label fluorescence was measured by flow cytometry. The mean fluorescence intensity (MFI) levels for TF-NP–treated Kasumi-1, OCI-AML3, and MV4-11 were, 2.5-, 7.4-, and 4.7-fold higher than the non-transferrin–conjugated nanoparticle-treated cells, whereas free FAM-labeled-miR was barely detectable in the cells (Fig. 2B). This indicated an enhancement of miR uptake using TF-NP. The qualitative intracellular FAM-miR uptake by AML cells following TF-NP treatment was confirmed by confocal microscopy that showed an accumulation of FAM-miR mostly in the cytoplasm (Fig. 2C).

**Intracellular increase of mature miR-29b**

The delivery efficiency of the TF-NP was tested by measuring intracellular levels of mature miR-29b (Fig. 3A). Treatment with NP-miR-29b and TF-NP-miR-29b, respectively, increased levels of mature miR-29b approximately 240- versus 420-fold (P = 0.009) in Kasumi-1, 130- versus 240-
fold ($P = 0.008$) in OCI-AML3, and 150- versus 220-fold ($P = 0.01$) in MV4-11 compared with mock treatment. Thus, Tf-NP were approximately 2 times more efficient than unconjugated nanoparticles in increasing the $\text{miR-29b}$ levels. These results also indicated an efficient processing of the delivered $\text{miR-29b}$ mimic molecules into mature $\text{miR-29b}$. No significant change of the expression of an unrelated miR, that is, $\text{miR-140}$, was observed (Fig. 3B), thereby supporting the specificity of our delivery system and the lack of interference with the expression of other endogenous miRs in the targeted cells.

### Downregulation of $\text{miR-29b}$ target genes by Tf-NP-$\text{miR-29b}$ in AML cells

Next, we tested the $\text{miR-29b}$ targeting activity. We previously reported that $\text{miR-29b}$ directly downregulates the DNA methyltransferases $\text{DNMT3A}$ and $\text{DNMT3B}$ and indirectly downregulates $\text{DNMT1}$ by targeting the transcription factor $\text{SP1}$ that drives $\text{DNMT1}$ expression (9). Furthermore, $\text{miR-29b}$ has been shown to target the cell cycle regulator $\text{CDK6}$ (8). Indeed, we observed a marked downregulation of $\text{DNMT1}$ by 18.5-, 2.5-, and 5.1-fold, $\text{DNMT3A}$ by 4.8-, 15.7-, and 3.4-fold, $\text{DNMT3B}$ by 3.6-, 3.5-, and 3.4-fold, $\text{SP1}$ by 4.5-, 3.9-, and 3.3-fold, and $\text{CDK6}$ by 3.9-, 3.5-, and 9.6-fold respectively in Kasumi-1, OCI-AML3, and MV4-11 cells following the treatment with our $\text{miR-29b}$-loaded Tf-NP compared with scramble-loaded Tf-NP (Fig. 3C). Thus, the delivered $\text{miR-29b}$ fulfilled the expected function of the endogenous miR in AML cells.

We recently showed that $\text{miR-29b}$ also indirectly targets the expression of the RTKs FLT3 and KIT in AML (7, 10). Aberrant activation by activating mutations and/or overexpression of these 2 RTKs is frequently found in AML (11–13). The downregulation of these RTKs following $\text{miR-29b}$ increase is likely mediated by the disruption of a transactivation complex composed of SP1 and NF-kB, by targeting $\text{SP1}$ (7, 10). Because we observed a significant downregulation of $\text{SP1}$ on Tf-NP-$\text{miR-29b}$ treatment (Fig. 3C), we analyzed the FLT3 and KIT expression in Tf-NP-$\text{miR-29b}$-treated cells. We observed a downregulation of FLT3 by 3.3-, 2.8-, and 1.9-fold, respectively, as well as a downregulation of KIT by 7.8- 2.5-, and 1.4-fold, respectively, in Kasumi-1, OCI-AML3, and MV4-11 cells (Fig. 3D).
Figure 3. Treatment with Tf-NP-miR-29b increased mature miR-29b level, downregulated DNMT1, DNMT3A, DNMT3B, SP1, CDK6, FLT3, and KIT, and had antileukemic activity in AML cells. A, mature miR-29b expression levels; and B, miR-140 expression in Kasumi-1, OCI-AML3, and MV4-11 cells. C, DNMT1, DNMT3A, DNMT3B, SP1, and CDK6 expression; and D, FLT3 and KIT expression in Kasumi-1, OCI-AML3, and MV4-11 after treatment with Tf-NP-miR-29b compared with Tf-NP-sc treatment. The number below each band represents the ratio of the band’s intensity to actin used as a loading control. E, cell growth curve of Kasumi-1, OCI-AML3, and MV4-11 cells treated with Tf-NP-miR-29b, Tf-NP-sc, or mock. F, colony formation assays in Kasumi-1, OCI-AML3, and MV4-11 cells treated with Tf-NP-miR-29b, Tf-NP-sc, or mock.
Intracellular increase of the endogenous miR-29b primary transcripts following Tf-NP-miR-29b treatment

The endogenous mature miR-29b stems from 2 precursors (pri-miR-29b-1 and pri-miR-29b-2) encoded by 2 genes located on human chromosomes 7q32 and 1q23, respectively. We previously reported that an SP1-containing transcription repressor complex downregulated miR-29b in AML cells (7). Here, we showed that Tf-NP-miR-29b reduced SP1 expression; therefore, we hypothesized that Tf-NP-miR-29b may increase the endogenous miR-29b expression. We found 1.7-, 2.0-, and 2.3-fold increase in endogenous pri-miR—29b-1 and 2.1-, 1.8-, and 2.5-fold increase of pri-miR-29b-2 levels in Kasumi-1, OCI-AML3, and MV4-11 cells, respectively, following treatment with the miR-29b-loaded Tf-NP (Supplementary Fig. S4A).

miR-29b inhibits cell proliferation and colony formation in AML cells

We evaluated the antileukemic effects of Tf-NP-miR-29b treatment by carrying out growth curves and analyzing the colony forming ability. The Tf-NP-miR-29b treatment reduced the growth rate from 32.2% (Tf-NP-scramble) to 25.3% (Tf-NP-miR-29b) in Kasumi-1 cells, from 70.9% (Tf-NP-scramble) to 57.3% (Tf-NP-miR-29b) in OCI-AML3, and from 53.0% (Tf-NP-scramble) to 43.9% (Tf-NP-miR-29b) in MV4-11 cells (Fig. 3E) compared with the Tf-NP scramble treatment. On the last day, the Tf-NP-miR-29b treatment was associated with significantly lower cell counts than in the Tf-NP-scramble or mock (buffer only) treatment for Kasumi-1 (P = 0.01 for both), OCI-AML3 (P = 0.026 and P = 0.01, respectively), and MV4-11 cells (P = 0.007 and P = 0.002, respectively; Fig. 3E). In addition, we observed an approximately 50% reduction in colonies following the Tf-NP-miR-29b treatment (Fig. 3F). The average number of colonies (±SD) formed by mock (buffer only), Tf-NP-scramble-, and Tf-NP-miR-29b-treated cells were, respectively, 161 ± 9, 143 ± 9, and 65 ± 6 (P < 0.001 for each comparison) for Kasumi-1 cells, 289 ± 11, 269 ± 13, and 156 ± 10 (P < 0.001 for each comparison) for OCI-AML3 cells, and 234 ± 11, 213 ± 7, and 80 ± 5 (P < 0.001 for each comparison) for MV4-11 cells, respectively.

Validation in AML patient blasts

The antileukemic activity of Tf-NP-miR-29b was further validated in primary blasts from 3 patients with newly diagnosed AML. Patient 1 had a secondary AML with validated in primary blasts from 3 patients with newly diagnosed AML. Patient 1 had a secondary AML with unknown karyotype (standard cytogenetic analysis failed in this patient). Patients 2 and 3 had a de novo cytogenetically normal AML. After Tf-NP-miR-29b treatment, we observed an approximate 860-, 400-, and 750-fold increase in miR-29b levels, compared with Tf-NP-scramble after 24 hours in blasts sample from all 3 patients (Fig. 4A). No significant change of the expression of an unrelated miR (i.e., miR-140) was observed (Fig. 4B). In addition, we observed 2.2-, 2.1-, and 1.9-fold increase in endogenous pri-miR-29b-1, and 1.6-, 1.4-, and 2-fold increase in endogenous pri-miR-29b-2 compared with controls after Tf-NP-miR-29b treatment in the blasts from all 3 patients (Supplementary Fig. S4B).

After 48-hour Tf-NP-miR-29b treatment, 1.3-, 2.9-, and 6.6-fold DNMT1 downregulation was observed in all 3 patients’ blast samples, as well as 3.5-, 4.4-, and 6.4-fold DNMT3A, 6.9-, 9.7-, and 6.7-fold DNMT3B, 7.6-, 5.3-, and 6-fold SP1, and 8.1-, 4.9-, and 2.8-fold CDK6 downregulation compared with Tf-NP-scramble treatment (Fig. 4C). In addition, 1.9- and 2.1-fold FLT3 decrease in patient 1 and patient 2, and 1.9- and 3.3-fold decrease Kit in patient 1 and patient 3 were observed (Fig. 4D). Tf-NP-miR-29b decreased cell viability, respectively, by approximately 19% (P = 0.03), 15% (P = 0.017), and 21% (P = 0.001) respectively, compared with Tf-NP-scramble in all three AML patients’ blasts (Fig. 4E).

In vivo evaluation of Tf-NP-miR-29b in preclinical models

To assess the safety profile of systemic nanoparticle treatment, we evaluated basic hepatic and renal functions in immunocompetent mice after saline, empty nanoparticles, or Tf-NP-miR-29b treatment. No significant organ impairment was observed (Supplementary Fig. S5A). Moreover, TF-NP treatment did not result in body weight changes (Supplementary Fig. S5B) or significant changes in hemoglobin (Hb) level, white blood count (WBC), or platelet (PLT) count (Supplementary Fig. S5C). Next, we evaluated the in vivo therapeutic efficacy of TF-NP-miR-29b. In the first trial, the MV4-11-engrafted mice were treated with free miR-29b (n = 3; 1 mg/kg/d miR intravenously), Tf-NP-scramble (n = 6), or TF-NP-miR-29b (n = 6) starting on day 10 after cell injection. The median survival time was 27, 28, and 32.5 days for free miR-29b, Tf-NP-scramble, and TF-NP-miR-29b–treated mice, respectively. The survival in the TF-NP-miR-29b–treated group was significantly longer compared with free miR-29b–treated group (P = 0.003, log-rank test) as well as when compared with the TF-NP-scramble–treated group (P = 0.015, Fig. 5A). Consistent with the longer survival, the spleen sizes in the TF-NP-miR-29b–treated group were significantly smaller than in the free miR-29b–treated mice (P = 0.033) or in the TF-NP-scramble–treated group (P = 0.049). The mean spleen weight was 29.3 ± 4.1, 26.6 ± 1.6, and 19.3 ± 3.4 mg for the free miR-29b, TF-NP-scramble, and TF-NP-miR-29b–treated mice, respectively (Fig. 5A). To validate these results, we conducted a second independent trial, testing a slightly different schedule and dosing (see Materials and Methods). The engrafted mice were treated with saline (n = 5), TF-NP-scramble (n = 7; 1.5 mg miR/kg/d intravenously), or TF-NP-miR-29b (n = 7) starting at day 10 after cell injection. The median survival time in this trial was 26, 27, and 34 days for saline, TF-NP-scramble, and TF-NP-miR-29b–treated mice, respectively. Similar to the first trial, the TF-NP-miR-29b treatment prolonged the survival of the leukemic mice compared with the TF-NP-scramble–treated group (P = 0.01, Fig. S5B).
Blood samples at day 24 (after 6 doses; second trial) showed a 20-fold increase in intracellular miR-29b levels in the Tf-NP-miR-29b-treated mice compared with the Tf-NP-scramble–treated group (\(P = 0.003\), Fig. 5B). Furthermore, we observed a decreased expression of the miR-29b targets, DNMT3A by 2.9-fold (\(P = 0.02\)), DNMT3B by 4-fold (\(P = 0.002\)), SP1 by 2.9-fold (\(P = 0.039\)), CDK6 by 1.6-fold (\(P = 0.015\)), KIT by 3.6-fold (\(P = 0.018\)), and FLT3 by 1.5-fold (\(P = 0.029\)) compared with the Tf-NP-scramble–treated group in vivo.

Figure 4. Validation of Tf-conjugated-NP-miR-29b treatment effects in AML patient blasts. A, expression levels of mature miR-29b. B, miR-140 expression levels in AML patient blasts from 3 different patients with AML treated with Tf-NP-miR-29b, Tf-NP-sc, or mock. C, DNMT1, DNMT3A, DNMT3B, SP1, and CDK6 expression; and D, FLT3 and KIT expression in AML patient blasts. The number below each band represents the ratio of the band’s intensity to the respective Actin band intensity. E, cell viability of 3 patient blast samples treated with Tf-NP-miR-29b, Tf-NP-sc, or mock.
Figure 5. *In vivo* evaluation of Tf-NP-miR-29b in preclinical model. A, first trial: leukemic mice were treated with free miR-29b mimic, Tf-NP-scramble, or Tf-NP-miR-29b. Survival curves of the mice according to distinct treatments are shown (left). Corresponding spleen weights are shown (right). B, second trial: leukemic mice were treated with saline, Tf-NP-sc, or Tf-NP-miR-29b. Survival curves of the mice according to distinct treatments are shown (top). Intracellular levels of miR-29b, DNMT1, DNMT3A, DNMT3B, SP1, CDK6, FLT3, and KIT at day 24.
These findings indicate that the miR-29b mimic molecules were successfully delivered to the leukemic cells and decreased miR-29b targets in vivo.

Antileukemic activity of Tf-NP-miR-29b priming followed by decitabine

Because we showed that higher pretreatment miR-29b levels were associated with improved clinical response to decitabine (24), we tested here whether Tf-NP-miR-29b treatment would improve the antileukemic activity of decitabine in AML cells. As we observed a miR-29b target downregulation at 48 hours, we pretreated AML cell lines and primary blasts with Tf-NP-scramble or Tf-NP-miR-29b for 48 hours before exposing them to decitabine.

Pretreatment with Tf-NP-miR-29b decreased the cell viability by approximately 40% (P = 0.001) compared with Tf-NP-scramble pretreatment after treatment with 0.5 μmol/L decitabine in Kasumi-1, approximately 20% (P<0.001) after treatment with 2.5 μmol/L decitabine in OCI-AML3 cells, and approximately 18% (P<0.001) after treatment with 2.5 μmol/L decitabine in MV4-11 cells (Fig. 6A).

Next, we evaluated the in vivo the Tf-NP-miR-29b priming activity. We engrafted NSG mice with MV4-11 cells and treated them with decitabine alone (n = 7; 0.4 mg/kg/d intraperitoneally), Tf-NP-scramble (n = 9), or Tf-NP-miR-29b—decitabine (n = 9). The median survival time was 27, 28, and 37 days for the decitabine alone, Tf-NP-scramble → decitabine, and Tf-NP-miR-29b → decitabine, respectively. The combination treatment of Tf-NP-miR-29 — decitabine significantly prolonged the survival of the leukemic mice compared with decitabine alone (P = 0.001) and compared with the combination treatment of Tf-NP-scramble → decitabine (P = 0.001) and by trend also when compared with Tf-NP-miR-29b alone (P = 0.06).

Discussion

The differential expression of some miRs has been associated with myeloid leukemogenesis and/or patient outcome (4–7). The expression level of miR-29b has been found to be downregulated in AML blasts compared with normal bone marrow cells (Supplementary Fig. S3; ref. 8). Furthermore, high expression of miR-29b has been shown to have antileukemic activity, and to be associated with longer survival in patients treated with conventional chemotherapy and higher odds for achieving a complete remission following decitabine treatment (7, 11, 24). Thus, a therapeutic increase of miR-29b in AML blasts could provide substantial clinical benefit. However, the delivery of miRs
remains a challenging goal and, to our knowledge, an efficient miR-delivery system has not been reported for AML blasts.

Currently, the miR-delivery for potential cancer therapy is based on viral (25–28) and nonviral (29–43) systems. Among the reported viral-based systems, the adeno-associated virus (AAV)-based approaches seem promising, as supported by significant therapeutic effects in a murine liver cancer models (26). Nonviral cationic polymer or cationic lipid carrier systems have also been used to deliver miR-expressing plasmids to solid tumors by other researchers (29–41). However, the miR-expressing vectors and the AAV approach share some drawbacks, including limited efficiencies for hematopoietic cells, need for nuclear translocation of large DNA vectors, and limitations in expression of the mature miRs (44). With regard to hematopoietic cells, the shortcomings for both viral and nonviral approaches could be bypassed by engineering a targeting delivery system for mature miRs or miR mimic molecules (45). Most delivery systems for solid tumors use cationic or neutral lipid particles to deliver miR molecules due to their tendency of organ accumulation (34–40, 43). Thus, here we developed a novel anionic lipopolyplex nanocarrier system for miR delivery to AML cells.

The nanoparticles presented here had several remarkable differences from the conventional cationic lipid nanoparticles used in solid tumors that have the tendency to accumulate preferentially in lungs, kidney, and liver due to their charge property (46). The neutral and anionic lipid formulation of our nanoparticles was designed to avoid the nonspecific immune response caused by cationic lipids through activation of TLR4 and NF-kappaB pathways and, in turn, proinflammatory cytokine production (47, 48). Moreover, the overall neutral surface charge results in reduced plasma protein binding and low rate of nonspecific cellular uptake (23). Low-molecular weight polyethylenimine was selected as a core to condense miR molecules because it is known to be relatively biocompatible and to provide a positive charge, which allows for easily capture of the negatively charged miR molecules, and in turn high entrapment efficiency. The lipid-based carrier was made of DOPE, linoleic acid, and DMG-PEG. The low binding affinity between linoleic acid and small RNA may also enhance the dissociation of miRs from the lipopolyplex after endocytosis to facilitate target gene downregulation (49). Furthermore, the nanoparticles are protected from reticuloendothelial system clearance by 2% (molar ratio) of DMG-PEG to achieve long circulation times (22) and, thus, more efficient delivery in hematopoietic organs, including bone marrow. To increase the specific effect on tumor cells, nanoparticles may be conjugated with molecules that enhance their targeting specificity (35).

We showed that our nanoparticles were able to efficiently deliver miR-29b mimics, increase mature miR-29b levels, and effectively target a panel of AML-relevant genes and mechanisms involved in epigenetics, cell-cycle control, and kinase-signaling pathways. Unlike the delivery of siRNA or short hairpin RNAs (shRNA) that are usually designed to target single genes, miRs can concurrently target multiple genes and pathways involved in leukemia that could potentially result in a better antileukemic activity and reduced emergence of resistance mechanisms. Indeed, we showed that TF-NP-miR-29b treatment resulted in an in vitro growth inhibition, a reduction of colony formation in AML cells, and in a significant therapeutic activity and prolonged survival in 2, independent AML in vivo trials. Interestingly, approximately 80% of the mice treated with TF-NP-miR-29b were still alive at the time when the control-treated mice (i.e., saline, free miR-29b, or TF-NP-scramble) had died. Although, several studies investigating miR-anti-sense/plasmid/mimic delivery-approaches were shown to reduce tumor burden in vivo (29–43), only a few of them were able to show that miR-based therapies (i.e., miR-145, miR-34a, and miR-107) prolonged survival in mice with an aggressive cancer (31, 32, 36, 39).

Finally, in this study we also showed that priming AML cells with TF-NP-miR-29b led to an improved antileukemic activity of decitabine in vitro and in vivo, thereby also supporting our earlier finding that higher endogenous miR-29b pretreatment levels associate with improved response to decitabine (20, 24). We now showed that miR-29b expression may not only be a predictor of treatment response to decitabine, but miR-29b priming may indeed be integral to decitabine-based regimens, especially for those patients with AML with downregulated endogenous miR-29b.

In conclusion, we developed a novel transferrin-conjugated nanoparticle system to efficiently deliver synthetic miR mimics to AML blasts. TF-NP-miR-29b treatment increased mature miR-29b levels, downregulated known miR-29b targets, and showed antileukemic activity by improving survival in in vivo AML models. Our nanoparticle delivery approach is a promising new antileukemic strategy that may be rapidly translated into the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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# Targeted Delivery of microRNA-29b by Transferrin-Conjugated Anionic Lipopolypolplex Nanoparticles: A Novel Therapeutic Strategy in Acute Myeloid Leukemia

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