Targeting miR-21 Inhibits In Vitro and In Vivo Multiple Myeloma Cell Growth

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Abstract

**Purpose:** Deregulated expression of miRNAs plays a role in the pathogenesis and progression of multiple myeloma. Among upregulated miRNAs, miR-21 has oncogenic potential and therefore represents an attractive target for the treatment of multiple myeloma.

**Experimental Design:** Here, we investigated the in vitro and in vivo anti-multiple myeloma activity of miR-21 inhibitors.

**Results:** Either transient-enforced expression or lentivirus-based constitutive expression of miR-21 inhibitors triggered significant growth inhibition of primary patient multiple myeloma cells or interleukin-6–dependent/independent multiple myeloma cell lines and overcame the protective activity of human bone marrow stromal cells. Conversely, transfection of miR-21 mimics significantly increased proliferation of multiple myeloma cells, showing its tumor-promoting potential in multiple myeloma. Importantly, upregulation of miR-21 canonical validated targets (PTEN, Rho-B, and BTG2), together with functional impairment of both AKT and extracellular signal–regulated kinase signaling, were achieved by transfection of miR-21 inhibitors into multiple myeloma cells. In vivo delivery of miR-21 inhibitors in severe combined immunodeficient mice bearing human multiple myeloma xenografts expressing miR-21 induced significant antitumor activity. Upregulation of PTEN and downregulation of p-AKT were observed in retrieved xenografts following treatment with miR-21 inhibitors.

**Conclusion:** Our findings show the first evidence that in vivo antagonism of miR-21 exerts anti-multiple myeloma activity, providing the rationale for clinical development of miR-21 inhibitors in this still incurable disease. Clin Cancer Res; 19(8); 2096–106. ©2013 AACR.

Introduction

Multiple myeloma is a genetically complex hematologic malignancy characterized by abnormal infiltration of clonal plasma cells in the bone marrow (1, 2). Despite novel insights into the pathobiology of the disease and the availability of new research platforms and therapeutics (3–6), innovative treatment strategies are urgently needed. A major area of investigation is the human bone marrow microenvironment (hBMM), which plays an essential role in relapsed and refractory disease (8). Currently, there is clear evidence that changes in gene copy number, chromosomal translocations, mutations, as well as transcriptional and epigenetic events during the evolutionary process of multiple myeloma (9, 10) result in profound deregulation of the miRNA network (11, 12), which in turn leads to aberrant translation of miRNAs and cell signaling.

miRNAs are small, noncoding RNAs of 19 to 25 nucleotides, which regulate gene expression by inducing degradation or translation inhibition of target mRNAs primarily through base pairing to partially or fully complementary sites in the 3′-untranslated region (3′-UTR; ref. 13). Aberrant expression of miRNAs occurs widely in human cancers, including both solid tumors and hematologic malignancies (11, 12, 14). Deregulated miRNAs may act as oncogenes (Onco-miRNA) or tumor-suppressors.
Antitumor Activity of mir-21 Inhibitors in Multiple Myeloma

Translational Relevance

Oncogenic miRNAs are an emerging target for the treatment of human cancer. We investigated the therapeutic potential of miR-21 inhibitors in human multiple myeloma. The translational relevance of our study relies in the strong anti-multiple myeloma activity of miR-21 inhibitors, which is not abrogated by exogenous interleukin-6 or cell adherence to human bone marrow stroma, taking into account the dependency of multiple myeloma from the bone marrow milieu. Moreover, we provide evidence of a successful in vitro treatment with miR-21 inhibitors in a murine xenograft model of human multiple myeloma, offering a framework for clinical development of miR-21 inhibitors in multiple myeloma. Notably, this therapeutic activity is dependent upon miR-21 expression, which may represent a predictive biomarker to miR-21 inhibitors in this still incurable disease.

In this report, we characterized the anti-multiple myeloma activity and the molecular events triggered by miR-21 inhibition in patient multiple myeloma cells and multiple myeloma cell lines, which were maintained even when tumor cells were adherent to patient BMSCs in vitro. We then showed the in vivo cytotoxicity and mechanisms of action of miR-21 inhibitors in a murine xenograft model of human multiple myeloma, providing the framework for its clinical development.

Materials and Methods

Reagents and cell culture

Multiple myeloma cell lines were cultured in RPMI-1640 (Gibco, Life Technologies) supplemented with 10% FBS (Lonza Group Ltd.) and 1% penicillin/streptomycin (Gibco, Life Technologies). The IL-6–dependent multiple myeloma cell line INA-6 (kindly provided from Dr. Renate Burger, University of Erlangen-Nuernberg, Erlangen, Germany) was cultured in the presence of rhIL-6 (R&D Systems), as previously reported (36). Following informed consent approved by our University Hospital Ethical Committee, primary patient multiple myeloma (ppMM) cells were isolated from bone marrow aspirates by Ficoll-Hypaque density gradient sedimentation followed by antibody-mediated positive selection using anti-CD138 magnetic-activated cell separation microbeads (Miltenyi Biotech). Purity of immunoselected cells were assessed by flow-cytometric analysis using a phycoerythrin-conjugated CD38 monoclonal antibody (mAb; CD38-PE; Imgenex) by standard procedures (37, 38). hBMSCs were obtained by long-term culture of bone marrow mononuclear cells (39). For coculture, 1 × 10^5 ppMM cells were seeded on 2 × 10^4 hBMSCs for 24 to 48 hours in 96-well plates. RNA samples of normal healthy bone marrow-derived plasma cells (nPC) were purchased (AllCells).

Overexpression and inhibition of miR-21 in multiple myeloma cells

Pre-miR-21 miRNA precursor molecules and miR-21 inhibitors were purchased from Ambion (Applied Biosystems) and were used to enforce or to antagonize mir-21 expression, respectively, at a final concentration of 100 nmol/L. Pre-miR precursor-negative control and anti-miR miRNA inhibitor-negative control were obtained from Ambion (Applied Biosystems). A total of 1 × 10^6 cells were transfected using Neon Transfection System (Invitrogen), (1 pulse at 1,050 V, 30 milliseconds), and the transfection efficiency evaluated by flow-cytometric analysis relative to a FAM dye–labeled anti-miR–negative control reached 85% to 90%. The same conditions were applied for transfection of multiple myeloma cells with 10 μg of the p3x FLAG-PTEN (kindly provided by Prof. Giuseppe Vighetto, Magna Graecia University, Catanzaro, Italy) or with the same amount of the empty p3x FLAG-CMV-7.1 vector. When cotransfected, we used 100 nmol/L of synthetic miR-21 or miR-NC together with 10 μg of p3x FLAG-PTEN or the same amount of empty p3x FLAG-CMV-7.1 vector.
Cell proliferation assays
Cell growth was evaluated by Trypan blue exclusion cell count and bromodeoxyuridine (BrdUrd) proliferation assay. Electroporated cells were incubated for 4 hours in 6-well plates; after harvesting, they were plated in 24-well plates for Trypan blue exclusion cell count and in 96-well plates for BrdUrd proliferation assay. Cells were counted at 24-hour intervals. BrdUrd uptake was measured every 24 hours by the Delfia cell proliferation assay, and luminescence was detected using a Victor 4 plate reader (PerkinElmer). Each sample was run at least in triplicate.

Survival assay
Cell survival was evaluated by MTT assay in 96-well plates. In brief, transfected cells were seeded at a density of 1 × 10^4 cells well per 100 μL of culture medium. Every 24 hours, 10 μL of 5 mg/mL MTT (Sigma) reagent were added to each well, and cells were further incubated for 4 hours at 37°C. Then medium was removed and 100 μL of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan. The optical density (OD) was evaluated at wavelength of 560 nm. Wells without cells (DMSO alone) were used as blank, and experiments were repeated at least 3 times. Data represent the mean ± SD of 3 independent experiments.

Colony formation assay
Clonogenicity was evaluated by a colony formation assay in methylcellulose-based medium (Methocult H4100, StemCell Technologies), following manufacturer’s instructions. A total of 2 × 10^4 cells/mL electroporated cells were seeded in 24-well plates and incubated for 3 weeks; colony formation was then evaluated by counting colonies of more than 100 cells. The experiments were repeated at least 3 times. Data represent the mean ± SD of 3 independent experiments.

Quantification of IL-6 production
The concentration of IL-6 in culture medium was measured by ELISA (QuantiKine Rat IL-6; R&D Systems). hBMSCs were electroporated; following incubation periods of 24 to 48 hours, aliquots of culture medium were collected and subjected to a specific ELISA for IL-6.

Reverse transcription and quantitative real-time PCR
Total RNA-containing miRNAs and mRNAs were extracted from cells with Trizol Reagent (Invitrogen), according to the manufacturer’s instructions. All RNA extractions were carried out in a sterile laminar flow hood using RNase/DNase–free laboratory ware. The integrity of total RNA was verified by nanodrop (Cebio Nanodrop Spectrophotometer nd-1000). The single-tube TaqMan miRNA assay (Applied Biosystems) was used to detect and quantify mature miR-21, using VIas7 RT reader (Applied Biosystems); the protocol was conducted for 40 cycles at 95°C for 3 minutes, 95°C for 15 seconds, and 60°C for 30 seconds. miR-21 expression was normalized on RNU44 and then expressed as fold change (2^{ΔΔCt}). For mRNA dosage studies, oligo-dT–primed cDNA was obtained through the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and then used as a template to quantify PTEN (Hs01026371_m1), Rho-B (Hs1085292_m1), and Btg2 (Hs1105077_m1) levels by TaqMan assay (Applied Biosystems); normalization was conducted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs03929071_g1). Comparative real-time PCR (RT-PCR) was conducted in triplicate, including no-template controls. Relative expression was calculated using the comparative cross-threshold (Ct) method.

Protein extraction and Western blot analysis
Total proteins were extracted with a lysis buffer (Tris–HCl 15 mmol/L pH 7.5, NaCl 120 mmol/L, KCl 25 mmol/L, Tryton X-100 0.5%) and addition of Halt Protease Inhibitor Single-Use Cocktail 1× (Thermo Scientific). After lysis in ice for 30 minutes, the solution was disrupted by gentle pipetting followed by 3 × 10 sonication cycles; the lysate was centrifuged at 12,000 rpm for 20 minutes and the supernatant was collected. For Western blot analysis, 60 μg of lysate were separated by electrophoresis on Mini Protein TGX precast gels (4%-20%) and electro-transferred onto a nitrocellulose membrane (Invitrogen). The membranes were blocked for 1 hour in 5% milk at room temperature and then incubated overnight at 4°C in milk 5% with the following antibodies: PTEN (A2B1; Santa Cruz Biotechnol-ogy); phospho-Akt (Ser473) rabbit mAb (Cell Signaling); AKT (pan; 11E7) rabbit mAb (Cell Signaling); phospho-44/42 mitogen-activated protein kinase (MAPK; Erk1/2; Thr202/Tyr204) rabbit mAb (Cell Signaling); p44/42 MAPK (Erk1/2) rabbit mAb (Cell Signaling); γ-tubulin Antibody (C-20) goat polyclonal; GAPDH–horseradish peroxidase rabbit polyclonal immunoglobulin G (IgG; Santa Cruz Biotechnology). Membranes were washed 3 times in PBST and then incubated with a secondary antibody conjugated with horseradish peroxidase in 0.5% milk for 2 hours at room temperature. After 3 washes with PBSTween, chemiluminescence was detected using Pierce ECL Western Blotting Substrate (cat. 32109; Pierce). Intensity of the bands was analyzed using Quantity One analyzing system (Bio-Rad).

Virus generation and infection of multiple myeloma cells
Multiple myeloma cells stably expressing miR-21 inhibitor were transduced by the lentiviral vector miR-Zip-21 anti-miR-21 construct (System Biosciences). The supernatant was collected 48 hours posttransfection and was centrifuged (3,000 × g for 15 minutes at 4°C) to remove cell debris; it was then passed through a 0.25-μm filter and used for 2 rounds of transduction of U-266 and MM.1S cells (1 × 10^5) in the presence of 8 μg/mL polybrene (Sigma-Aldrich). Multiple myeloma cells underwent 3 rounds of infection (8 hours each round), and transduced cells were selected in medium containing 1 μg/mL puromycin.
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Animals and in vivo model of human multiple myeloma

Male CB-17 severe combined immunodeficient (SCID) mice (6- to 8-weeks old; Harlan Laboratories, Inc.) were housed and monitored in our Animal Research Facility. All experimental procedures and protocols had been approved by the Institutional Ethical Committee (Magna Graecia University) and conducted according to protocols approved by the National Directorate of Veterinary Services (Italy). In accordance with institutional guidelines, mice were sacrificed when tumors reached 2 cm in diameter or in the event of paralysis or major compromise, to prevent unnecessary suffering. Animal experimental procedures have been carried out as in previous reports (40–42). Briefly, mice were subcutaneously inoculated with $1 \times 10^6$ OPM-2 cells, and treatment started when palpable tumors became detectable, approximately 3 weeks following injection of multiple myeloma cells. Each dose contained 20 µg of NLE-formulated (MaxSuppressor In Vivo RNA-LANCer II) synthetic oligo, which equals 1 mg/kg per mouse, as previously described (21). Treatments were conducted intratumorally every 2 days for a total of 8 injections.

Statistical analysis

Each experiment was carried out at least 3 times and all values are reported as means ± SD. Comparisons between groups were made with Student t test, whereas statistical significance of differences among multiple groups was determined by GraphPad software (www.graphpad.com). Graphs were obtained using SigmaPlot version 12.0. P value of less than 0.05 was accepted as statistically significant.

Results

miR-21 expression in multiple myeloma cell lines and primary patient multiple myeloma cells

By RT-PCR, we evaluated the miR-21 expression in INA-6, MM.1S, NCI-H929, RPMI-8226, OPM-2, U-266, and KMS-26 multiple myeloma cell lines as compared with nPCs. Among these cell lines, we found variable miR-21 expression: KMS-26, U-266, and OPM-2 showed the highest, whereas other cells expressed very low levels of miR-21 (Fig. 1A). Notably, we found more than 3-fold increase in miR-21 expression when INA-6 cells, which are IL-6-dependent, were cultured adherent to hBMSCs (Fig. 1B). Consistent with data achieved in cell lines, miR-21 levels were upregulated in ppMM cells as compared with nPCs; moreover, ppMM cells further increased miR-21 expression (>4.18-fold) when cells were cultured adherent to hBMSCs (Fig. 1C), showing that miR-21 in multiple myeloma is indeed upregulated by the bone marrow milieu.

Transfected miR-21 inhibitors exert anti-multiple myeloma effects in vitro

To study the anti-multiple myeloma activity of miR-21 inhibition, we transfected tumor cells with miR-21 inhibitors. By Trypan blue exclusion cell count and BrdUrd proliferation assay, we found significantly decreased cell growth of multiple myeloma cell lines highly expressing miR-21 levels (KMS-26, U-266, and OPM-2; Fig. 2A and B). In these cells, we also observed reduction of cell survival by MTT assay (Fig. 2C). Conversely, miR-21 inhibitors did not affect cell proliferation or survival of multiple myeloma cell

![Figure 1](https://www.aacrjournals.org/clinicanalysis/19(8)/April15,2013/2099/antitumoractivityofmir21 inhibitorsinmultiplemyeloma.png)

Figure 1. miR-21 expression in patient with multiple myeloma cells and cell lines. A, qRT-PCR analysis of miR-21 expression using total RNA from 7 multiple myeloma cell lines and bone marrow–derived plasma cells (nPCs) from healthy donors. Raw C, values were normalized to RNU44 housekeeping snoRNA and expressed as $\Delta$Ct values relative to miR-21 levels in nPCs. Values represent mean ± SE of 3 different experiments. B, qRT-PCR of miR-21 expression in INA-6 cells cultured in the presence or absence of IL-6 (2.5 ng/mL) or cocultured with hBMSCs and then immunopurified by immunomagnetic sorting with anti-CD138 beads. Raw C, values were normalized to RNU44 housekeeping snoRNA and expressed as $\Delta$Ct values calculated using the comparative cross-threshold method. miR-21 expression levels in INA-6 cells cultured in the absence of IL-6 were set as an internal reference. Values represent mean ± SE of 3 different experiments. C, qRT-PCR of miR-21 expression in nPCs ($n = 3$) and ppMM ($n = 3$) cells before and 6 hours after seeding on hBMSCs, and then immunopurified by immunomagnetic sorting with anti-CD138 beads. Raw C, values were normalized to RNU44 housekeeping snoRNA and expressed as $\Delta$Ct values calculated using the comparative cross-threshold method. miR-21 expression levels in nPCs were used as an internal reference. Data are the average of 3 independent experiments carried out in triplicate. P values were obtained using two-tailed t test.
lines with low miR-21 expression (NCI-H929, MM.1S, and RPMI-8226; Supplementary Fig. S2).

**Lentiviral transduced miR-21 inhibitors affect multiple myeloma cell proliferation and clonogenicity**

We next evaluated the effects of constitutive inhibition of miR-21. U-266 cells (expressing high miR-21) or MM.1S cells (expressing low miR-21) were transduced with a lentiviral vector carrying a miR-21 inhibitory sequence or with a lentiviral empty vector. By BrdUrd proliferation assay, we found significantly decreased cell growth of U-266 cells transduced with miR-21 inhibitors as compared with controls (Fig. 3B). In contrast, no significant effects on cell proliferation were observed in MM.1S cells transduced with miR-21 inhibitors (Fig. 3A). Constitutive expression of miR-21 inhibitors in U-266 cells also significantly inhibited colony formation in methylcellulose cultures (75% reduction in colonies; Fig. 3B), whereas clonogenicity of MM.1S cells was not affected (Fig. 3A). Taken together, these results show that miR-21 inhibition obtained either by transfection or transduction of miR-21 inhibitors exerts antiproliferative activity in multiple myeloma cells. The growth-inhibitory activity strongly relies on high basal miR-21 expression, as multiple myeloma cells with low miR-21 were not affected.
miR-21 inhibition modulates the expression and activity of several signaling molecules

miR-21 is known as a growth promoting and antiapoptotic factor in several human cancers through the targeting of multiple tumor suppressor genes (26). Among validated targets of miR-21, PTEN, Rho-B, and BTG2 are the most studied and involved in cell-cycle progression and/or apoptosis regulation (43–45). We therefore evaluated the effects of miR-21 inhibition on the expression of these targets and found that these genes were upregulated at mRNA level after transfection of miR-21 inhibitors, as compared with control cells transfected with scrambled sequences (miR-NC inhibitors; Fig. 3C). Moreover, by Western blot analysis, we found a marked upregulation of PTEN protein expression 24 to 48 hours after transfection with miR-21 inhibitors (Fig. 3D). Notably, we found

Figure 3. Activity of miR-21 inhibitors in multiple myeloma cell lines. BrdUrd proliferation assay and colony formation assay of MM.1S (A) and U-266 (B) cells transduced with a lentivirus carrying either the empty vector or miR-21 inhibitory sequences. Averaged values ± SD of 3 independent experiments are plotted. P values were obtained using two-tailed t test. C, qRT-PCR of PTEN, BTG2, and Rho-B expression was conducted in U-266 cells 48 hours after transfection with either miR-21 inhibitors or scrambled controls (miR-NC inhibitors). The results are shown as average mRNA expression after normalization with GAPDH and ΔΔCt calculations. Data represent the average ± SD of 3 independent experiments. D, Western blot analysis of PTEN 24 to 48 hours after transfection of U-266 cells with miR-21 inhibitors or scrambled controls. E, levels of total AKT, total ERK, p-AKT, and p-ERK 24 to 48 hours after transfection of U-266 cells with either miR-21 inhibitors or scrambled controls. Relative protein level values are derived from densitometric scan.
that both p-AKT and p-extracellular signal–regulated kinase (ERK) were reduced by miR-21 inhibitors, whereas total AKT and ERK were unaffected (Fig. 3E).

Enforced expression of miR-21 mimics enhances proliferation of multiple myeloma cells

To establish the oncogenic role of miR-21 in our model, we evaluated the effects of transiently enforced expression of synthetic miR-21 mimics in tumor cells. Specifically, we examined whether miR-21 mimics exerted a growth-promoting activity in MM.1S (low miR-21) or in U-266 (high miR-21) cells. We found that miR-21 mimics enhanced growth of MM.1S cells (Fig. 4B), whereas no effects were observed in U-266 cells (Fig. 4A). We then evaluated whether miR-21 overexpression downregulated canonical targets. As shown in Fig. 4C, quantitative RT-PCR (qRT-PCR) analyses showed a decrease in PTEN, BTG2, and Rho-B mRNA expression (78%, 62%, and 42%, respectively).
Moreover, Western blot analysis showed that levels of PTEN protein were reduced in MM.1S cells overexpressing miR-21 compared with controls (Fig. 4D). To investigate if PTEN downregulation mediates the growth promoting activity of miR-21, we transfected MM.1S cells with an expression vector encoding PTEN gene lacking regulatory 3'-UTR sequence. We showed that transfected cells indeed over-expressed PTEN gene, which was not downregulated by miR-21 (Fig. 4E). Importantly, the PTEN rescue completely abrogated the miR-21 growth-promoting activity (Fig. 4B). We conclude that the proliferative effect of miR-21 is dependent on PTEN suppression in multiple myeloma cells.

miR-21 inhibitors antagonize the hBMSCs protective role on multiple myeloma cells

It is well known that the human bone marrow milieu strongly support survival and proliferation of multiple myeloma cells. Because miR-21 expression in multiple myeloma cells was significantly enhanced by adherence of cells to hBMSCs (Fig. 1B and C), we next evaluated whether miR-21 inhibition could overcome the supportive effects of the human bone marrow milieu. To this aim, we evaluated the antitumor activity of miR-21 inhibition in a context closely resembling the intramedullary stage of multiple myeloma. Specifically, we cultured IL-6–dependent multiple myeloma cell line INA-6 adherent to hBMSCs and enforced the expression of miR-21 inhibitors in one or both cell types. As shown in Fig. 6A, miR-21 inhibition affected viability of multiple myeloma cells to a similar extent as hBMSCs deprivation. miR-21 inhibition was also observed in INA-6 cells cultured in an IL-6–enriched culture medium (Fig. 5A). We wondered whether this effect was due to miR-21 inhibition directly in INA-6 cells, in hBMSCs, or in both. Therefore, we investigated the effects of miR-21 inhibition in INA-6 cells cocultured with nontransfected hBMSCs and found that the antitumor effect was similar to that obtained when miR-21 inhibition was induced in both cell types (Fig. 5B). In contrast, no effects were observed when miR-21 was inhibited only in hBMSCs adherent to INA-6 cells (Fig. 5B). Furthermore, viability and IL-6 secretion in culture medium were not affected by miR-21 inhibition in hBMSCs (data not shown). Finally, ppMM cells were cultured adherent to hBMSCs and then exposed to miR-21 inhibitors, which were able to overcome the supportive effect of human bone marrow milieu (Fig. 5C). On these findings, we conclude that miR-21 inhibitors abrogate the supporting activity of hBMSC on multiple myeloma cell lines and ppMM cells.

In vivo delivery of miR-21 inhibitors exert anti-multiple myeloma activity against human multiple myeloma xenografts

Finally, we studied the in vivo antitumor potential of miR-21 inhibitor oligonucleotides in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice bearing human multiple myeloma xenografts. On the basis of in vitro findings, we induced OPM-2 xenografts into a cohort of 12 mice. When tumors became palpable, mice were randomized into 2 groups and treated intra-tumorally with miR-21 inhibitors or scrambled inhibitors (miR-NC inhibitors). As shown in Fig. 6A, repeated injection of miR-21 inhibitors (1 mg/kg; 8 injections, 2 days apart), significantly reduced growth of multiple myeloma xenografts. Importantly, we next evaluated if

![Figure 5. miR-21 inhibition antagonizes prosurvival effect of bone marrow milieu. A, MTT assay of INA-6 cells cultured adherent to hBMSCs, in IL-6–enriched culture medium, or in IL-6–free culture medium. The assay was conducted 48 hours after transfection of cocultured cells with miR-21 inhibitors or scrambled controls (miR-NC inhibitors). B, MTT assay of INA-6 cells cultured with hBMSCs was conducted 48 hours after either INA-6 cells or hBMSCs were independently transfected with miR-21 inhibitors or scrambled controls. C, MTT assay conducted in ppMM cells cultured in the presence or absence of hBMSCs. The assay was conducted 48 hours after transfection with miR-21 inhibitors or scrambled controls. Averaged values ± SD of 3 independent experiments are plotted including. P values were obtained using two-tailed t test.](http://www.aacrjournals.org/doi/fig/10.1158/1078-0432.CCR-12-3325)
the antiproliferative activity of miR-21 inhibitors was related to modulation of miR-21 canonical targets. Consistent with our in vitro experiments, we found upregulation of PTEN both at mRNA and protein levels in retrieved tumors from animals treated with miR-21 inhibitors (Fig. 6B and C). Moreover, inhibition of miR-21 significantly reduced the phosphorylation of AKT, a downstream target of PTEN and key mediator of tumor cell survival (Fig. 6C), suggesting that the in vivo antitumor activity of miR-21 inhibitors is related to PTEN upregulation and AKT activation impairment within tumors.

Discussion

In this report, we show that antagonism of miR-21 by oligonucleotide inhibitors exerts antitumor activity in vitro and in vivo against human multiple myeloma xenografts in SCID/NOD mice. To our knowledge, this is the first evidence of a successful in vivo treatment with miR-21 inhibitors in a murine xenograft model of human multiple myeloma, which has important potential clinical applications. We show that efficacy of strategies based on miR-21 inhibition is dependent upon miR-21 expression levels in multiple myeloma cells. Indeed, in multiple myeloma cells expressing high miR-21 levels (KMS-26, U-226, and OPM-2), miR-21 inhibitors reduce cell proliferation, survival, and clonogenicity. In contrast, no antitumor effects were observed in cells with low endogenous miR-21 expression. These data suggest that miR-21 expression is a potential biomarker predictive of therapeutic response to miR-21 inhibitors in future clinical trials.

The oncogenic role exerted by miR-21 in multiple myeloma pathogenesis is predicted upon its upregulated levels even at early stages of disease. This notion is further supported by the role of miR-21 in IL-6/Stat3 signaling, a central pathway for multiple myeloma cell growth and drug resistance (33, 46, 47). In this report, we show increased proliferation in multiple myeloma cells transfected with miR-21 mimics, further supporting this view. Consistent with prior reports (35), we found that miR-21 expression in multiple myeloma cells is upregulated by adherence to hBMSCs. Importantly, we show that the antitumor properties of miR-21 inhibitors were not attenuated either by exogenous IL-6 or by adherence of ppMM cells or multiple myeloma cell lines to hBMSCs. These findings strongly suggest the clinical potential of targeting miR-21. Specifically, the human bone marrow milieu induces resistance to conventional therapies including melphalan, doxorubicin, and dexamethasone (7); in contrast, upregulation of miR-21 promoted by the bone marrow suggests that targeting this miRNA may be even more active against multiple myeloma cells in this milieu.

We investigated the molecular basis of miR-21 inhibitor anti-multiple myeloma activity. It is well known that tumor suppressor genes, including PTEN, BTG2, and Rho-B, are validated targets of miR-21 (26). Here, we showed that miR-21 inhibition triggered upregulation of these genes in multiple myeloma cell lines, whereas miR-21 mimics had the opposite effect. Modulation of PTEN expression and activity may be of particular clinical relevance in multiple myeloma, as PTEN mutations are rare events in this disease (43) and PTEN downregulates AKT and ERK activity (48).
thereby decreasing multiple myeloma cell proliferation, survival, and drug resistance (49). Importantly, upregulation of miR-21, p-AKT, and p-ERK in multiple myeloma cells mediates hBM-MC-induced tumor cell growth and survival (7), and a p-AKT/miR-21 positive feedback loop has been already suggested (50). It is therefore of note that miR-21 inhibitors upregulate PTEN and decrease AKT and ERK phosphorylation in multiple myeloma cells in our study. By transfection of a PTEN expression vector not containing 3’-miR-21 target sequence, which could be not downregulated by miR-21, we showed that PTEN suppression was indeed essential for miR-21–induced proliferative activity. Moreover, while miR-21 inhibitors produced a prolonged growth inhibitory activity with pERK impairment at 48 hours, pAKT was strongly suppressed only at 24 hours and almost recovered at 48 hours. We interpretate these findings as a rebound activation of pAKT, which does not translate in growth rescue.

In conclusion, our results both further highlight the oncogenic role of miR-21 in multiple myeloma and provide the framework for clinical development of miR-21 inhibitors to improve patient outcome in multiple myeloma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Correction: Targeting miR21 Inhibits *In Vitro* and *In Vivo* Multiple Myeloma Cell Growth

In this article (Clin Cancer Res 2013;19:2096–106), which was published in the April 15, 2013, issue of *Clinical Cancer Research* (1), a section of the grant support was mistakenly omitted. It should read as follows: "This work was supported in part by NIH grant P50CA100707 SPORE in Multiple Myeloma (to K.C. Anderson), and by funding from the Italian Association for Cancer Research (AIRC), PI: PT. 'Special Program Molecular Clinical Oncology—5 per mille’ n. 9980, 2010/15. KCA is an American Cancer Society Clinical Research Professor." The authors regret this error.

Reference


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