miR-6883 Family miRNAs Target CDK4/6 to Induce G1 Phase Cell-Cycle Arrest in Colon Cancer Cells

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Abstract

CDK4/6 targeting is a promising therapeutic strategy under development for various tumor types. In this study, we used computational methods and The Cancer Genome Atlas dataset analysis to identify novel miRNAs that target CDK4/6 and exhibit potential for therapeutic development in colorectal cancer. The 3’UTR of CDK4/6 mRNAs are targeted by a family of miRNAs, which includes miR-6883-5p, miR-149*, miR-6785-5p, and miR-4728-5p. Ectopic expression of miR-6883-5p or miR-149* downregulated CDK4 and CDK6 levels in human colorectal cancer cells. RNA-seq analysis revealed an inverse relationship between the expression of CDK4/6 and miR-149* and intronic miRNA-6883-5p encoding the clock gene PER1 in colorectal cancer patient samples. Restoring expression of miR-6883-5p and miR-149* blocked cell growth leading to G0–G1 phase cell-cycle arrest and apoptosis in colorectal cancer cells. CDK4/6 targeting by miR-6883-5p and miR-149* could only partially explain the observed antiproliferative effects. Notably, both miRNAs synergized with the frontline colorectal cancer chemotherapy drug irinotecan. Further, they resensitized mutant p53-expressing cell lines resistant to 5-fluorouracil. Taken together, our results established the foundations of a candidate miRNA-based theranostic strategy to improve colorectal cancer management. Cancer Res; 77(24); 6902–13. ©2017 AACR.

Introduction

There were an estimated 134,490 cases of colorectal cancer and about 49,190 deaths from the disease in the United States in 2016 according to the National Cancer Institute Surveillance, Epidemiology, and End Results program (1). The 5-year survival rate for advanced (stage IV) disease still remains low, at 13.5% (1). Thus, there is ongoing need to develop treatments and biomarkers needed to address the mortality and morbidity from advanced colorectal cancer.

Dysregulation of the p16–CDK4/6–Rb–E2F pathway is a hallmark of cancer (2–4). CDK4 and 6, oncogenic kinases within this pathway, are validated anticancer targets. Different small molecule inhibitors of CDK4/6 are in development (abemaciclib) and some have been FDA approved (palbociclib and ribociclib) as cancer therapeutics (5–7). Emerging data suggest that the single-agent efficacy of these CDK4/6 inhibitors is limited and that they are best used in combination with drugs like aromatase inhibitors (8). Chemotherapeutic agents like FOLFOX, FOLFIRI, or FOLFIRI combinations, etoposide, taxol, or platinum are still cornerstone first-line therapy options for some solid tumors. However, these cytotoxic agents work best in actively dividing cells, and combinations of CDK4/6 inhibitors with cytotoxic agents has shown little success clinically. Hence, there is need to develop alternate therapeutics that can target the p16–CDK4–Rb–E2F pathway and synergize with frontline therapies.

miRNAs are 18 to 22 nucleotide small noncoding RNAs that can inhibit translation and/or affect mRNA stability by binding to the 3’ untranslated region (UTR) of target genes (9, 10). Studies in different tumor models have shown that miRNAs can either be oncogenic, as in the case of miR-155, miR-21, and the miR17–92 cluster, or tumor suppressive (miR-34a, let-7 family, and miR-143; refs. 11, 12). Tumor suppressive miRNAs are generally dysregulated by multiple mechanisms including methylation of promoter sites, (13) deletion or loss of expression (12, 14). Reinroduction of tumor suppressive miRNAs known as “miRNA mimics” has become an attractive therapeutic strategy that is still being translated to the clinic. Many such miRNA mimics are currently in clinical or preclinical development for cancer and other nononcological applications (15). A disadvantage of miRNA mimics is the lack of specificity as compared with targeted therapies such as the CDK inhibitor palbociclib. However, the ability of miRNA mimics to modulate different tiers of genes within a signaling network is an advantage, especially in scenarios where the targeted therapy has shown limited success.

Contemporary CDK4/6 inhibitors work best in a subset of breast cancers (8), which must express proficient retinoblastoma (Rb) protein for therapeutic benefit. Although clinical trials are under way, CDK4/6 inhibitors have not yet shown substantial...
success in colorectal cancer. Because of this, there is an urgent need to identify alternative or complementary strategies to current CDK4/6 inhibitors. To date, no miRNA mimics have been identified, specifically targeting the CDK4/6 oncoprotein network in colorectal cancer. Identification of miRNAs targeting this network independent of Rb status and understanding their regulation would provide an important complementary avenue for targeted therapy. Further, these miRNAs may serve as biomarkers of disease progression and help predict response.

In this study, we identified novel miRNAs that target CDK4/6 with potential for further therapeutic development. We discovered a novel, uncharacterized family of four miRNAs whose expression is lost in colorectal cancer. We characterized the key targets of these miRNAs as CDK4, CDK6, FOXM1, XIAP, and BCLXL, proteins with associated antiproliferative and proapoptotic effects in colorectal cancer cell lines. We also performed combination studies of both miR-6883-5p and miR-149* with irinotecan and 5-fluorouracil, used in the first-line setting to treat colorectal cancer patients in the clinic. Both miRNAs show promising synergy with either irinotecan and 5-fluorouracil. We also provide mechanistic insight and show that downregulation of both the CDK4/6-FOXM1 axis and antiapoptotic proteins BCLXL and XIAP is involved in the miRNA–drug combinations. FOXM1 is a member of the fororkhead box (FOX) transcription factors, expressed exclusively in proliferating cells (16, 17). Several reports have shown that FOXM1 expression is upregulated in different tumor types, including colorectal cancer (18–22). Overexpression of FOXM1 is associated with increased cell proliferation (18), EMT (23–26), and chemoresistance (27), suggesting a protumorigenic role of the transcription factor. FOXM1 is considered a master regulator of the cell cycle, crucial for entry of cells into both the S and G2–M cell-cycle phases (28, 29). Recently, it has been shown that CDK4/6 initiate the phosphorylation and activation of FOXM1 in G1 phase of the cell cycle (30, 31). BCLXL and XIAP have been associated with poor prognosis in colorectal cancer and are important oncogenic targets in different solid tumors as well (32–35). BCLXL in particular has been associated with colorectal cancer tumorigenesis and progression (34).

Thus, miR-6883-5p and miR-149* mediated targeting of CDK4, CDK6, FOXM1, XIAP, and BCLXL proteins identified in our study has therapeutic implications for colorectal cancer tumors.

Materials and Methods

Cell culture and reagents

All colorectal, pancreatic, and melanoma cell lines were obtained from ATCC and maintained in the recommended media. Cells were routinely verified as free of mycoplasma contamination. miRNA mimics for hsa-miR-6883-5p, hsa-miR-149*, and hsa-miR-206 (HMI2616, HMI0241, and HMI0364) were purchased from Sigma-Aldrich. siRNA for CDK4 and CDK6 (sc-29261 and sc-29264) were purchased from Santa Cruz Biotechnology. The CDK4-Luciferase construct was obtained from Origene Technologies.

Transfection of miRNA mimics, siRNA, and plasmid constructs

All miRNA mimic and siRNA transfections were performed by reverse transfection using Lipofectamine RNAiMAX (Life Technologies). siRNA (80 nmol/L) was used in all experiments for HT-29, RKO, and SW-480 cell lines. siRNA (40 nmol/L) was used for HCT-116 cells. miRNA mimics were transfected at concentrations of either 25 nmol/L or 50 or 100 nmol/L, as indicated in respective assays. CDK4-Luciferase vector was transfected in HCT-116 cells using Lipofectamine 3000 (Life Technologies) and stable cells were selected using G418 antibiotic (500 μg/mL).

Luciferase assays

CDK4-Luciferase containing stable HCT-116 cells was transfected with either scramble duplex or 50 nmol/L of miRNA mimics. Luciferase signal was measured at 48 hours after transfection and relative luciferase units (RLU) were calculated by normalizing luciferase signal per μg of protein per assay well. All transfections were performed in triplicate and reported as RLU units ± SEM.

Cell proliferation assays

A total of 5K–10K cells were transfected with either scramble duplex or miRNA to a net concentration of 50 nmol/L and plated in a 96-well plate. Cell viability was measured 72 hours after transfection using CellTiter-Glo Luminescent Cell Viability Assay (Promega). The percent-cell viability was calculated by normalizing the luminescence signal to scramble duplex wells. All transfections were performed in triplicates and reported as % viability ± SEM, compared with scramble.

Cell-cycle analysis

All cell lines were transfected with either scramble duplex or miRNA mimic. At 72 hours after transfection, both floating and adherent cells were collected and fixed in 70% ethanol, followed by RNase A treatment and PI staining. Cell death (sub-G1) was quantified by propidium iodide (PI) staining and flow cytometry. FloJo analysis was performed to quantify the distribution of cells in G1, S, and G2–M phases of the cell cycle under different transfection conditions.

Colony formation assays

A total of 0.1 × 10⁴ were transfected with either scramble duplex or miRNA mimics to net concentration of 50 nmol/L (HT-29 and HCT-116) or 100 nmol/L (RKO and SW-480) for 72 hours. At 72 hours, transfected cells were harvested, and 500 cells per treatment group were plated in triplicate in 6-well plates for colony formation. Colonies were stained with 0.25% crystal violet on day 14, imaged, counted and reported as number of colonies ± SEM.

Quantitative RT-PCR

Total RNA, which includes miRNA, was isolated using the Quick-RNA MiniPrep Kit (Zymo Research). Total RNA (1 μg) from each sample was subjected to cDNA synthesis using SuperScript III Reverse Transcriptase Kit (Life Technologies), for detection of CDK4, CDK6, FOXM1, FOXM1 target genes, and housekeeping genes. For detection of miRNAs, 0.5 μg of total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). The relative expression of the reported genes and miRNAs was determined using real-time PCR performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. GAPDH and RNU6B were used as the endogenous controls for mRNA and miRNA samples, respectively. Each cDNA sample was amplified using Power SYBR Green (Applied Biosystems) and miRNA components were quantified using TaqMan Real-Time PCR Master Mix and TaqMan Human miRNA Array 2.0.
We evaluated the expression levels of CDK4 and CDK6 in colorectal cancer, focusing on the role of micro-RNAs (miRNAs). Previous reports have suggested that conserved miRNAs such as miR-206, miR-124-3p, and miR-15-5p are capable of targeting CDK4 and are dysregulated in melanoma and other tumor types. Given their ability to target CDK4/6, we investigated the functional consequences of expressing miRs-6883-5p or miR-149* and then examined the expression of CDK4 and CDK6 protein and RNA (Fig. 2A–D). Expression of miR-6883-5p repressed CDK4 and CDK6 at both the protein and RNA levels (Fig. 2A–D). By contrast, miR-149* did not alter CDK4 expression but potently repressed CDK6 (Fig. 2B–D). miR-6883-5p, but not miR-149*, was able to bind to the CDK4 3’UTR and repress the expression of the luciferase reporter gene in the pMirtarget-CDK4-luciferase vector (Fig. 2E). Of note, miR-6883-5p was able to repress luciferase activity more profoundly than miR-206, which has previously been reported to target CDK4 (40). Together, these data indicate that miR-6883-5p and miR-149* regulate the expression of CDK4 and CDK6 in colorectal cancer and may play an important role in disease pathogenesis.

Expression of miR-6883-5p and miR-149* results in G0–G1 arrest and cell death

Given their ability to target CDK4/6, we investigated the functional consequences of expressing miRs-6883-5p and 149* compared with miR-206 (a known CDK4 targeting miR) in the panel of colorectal cancer cell lines. We rationalized that because the miRNAs target proteins in the cell cycle, they could affect cell proliferation, both short term and long term. All three miRNAs had comparable short-term antiproliferative effects, reducing cell viability by 30% to 75% in the panel of colorectal cancer cell lines 72 hours after transfection (Fig. 3A). There was a greater than 50% to 70% inhibition of the long-term proliferation of these cells as observed in a colony formation assay in all four cell lines (Fig. 3B and C). We also checked for antiproliferative effects of miR-6883-5p and miR-149* in three melanoma cell lines. Although both miRs reduced protein levels of CDK4 and CDK6 (Supplementary Fig. S3A), there was no substantial effect on short-term proliferation of the...
melanoma lines tested (Supplementary Fig. S3B). We next investigated the effects of the miRNAs on cell-cycle markers. We expected the inhibition of CDK4/6 would lead to G0–G1 arrest in all the cell lines. As seen in Fig. 3D, in all four cell lines, miR-6883-5p and miR-149/C3 did indeed reduce levels of phosphorylated Rb (S795), indicating G0–G1 arrest and inhibition of CDK4/6 activity. We further confirmed our findings through propidium iodide staining and cell-cycle profile analysis. As seen in Fig. 3E(left), both RKO and HT-29 cells showed G0–G1 arrest following transfection with each of the miRNAs and a fraction of cells underwent cell death. However, in HCT116, expression of all miRNAs led to apoptosis, indicated by 30% to 50% cells in sub-G₁ phase (Fig. 3E, right). This result was further supported with the observation of PARP cleavage by Western blot analysis indicating apoptosis (Fig. 3F). In HCT116 cells, all three miRNAs promoted increased levels of apoptosis; but in RKO and SW480 cells, miR-6883-5p was the most potent at inducing apoptosis, measured by PARP cleavage (Fig. 3F). No change in the p53 levels was noted, indicating that apoptosis was likely not p53-dependent in the p53⁺/⁺ cells. Interestingly, miR-6883-5p downregulated XIAP and BCLXL which could in part explain the induction of apoptosis by miR-6883-5p. While XIAP is a predicted target of the family of miRNAs, BCLXL is not.
miR-6883-5p and miR-149* synergize with FDA-approved therapeutics for colorectal cancer

We evaluated the combinatorial potential of miR-6883-5p and miR-149* with colorectal cancer first-line chemotherapeutics irinotecan and 5-fluorouracil. As seen in Fig. 5A and B, in all four cell lines both miR-6883-5p and miR-149* synergized with irinotecan to reduce cell viability. Furthermore, the irinotecan–miRNA combination engaged the intrinsic pathway of cell death as measured by increased levels of cleaved caspase-9 and decreased levels of BCLXL and XIAP (Fig. 5C and D). Both miR-6883-5p and miR-149* increased the sensitivity of p53-mutant cell lines HT-29 and SW480 to 5-fluorouracil (Fig. 6A and B). Cells treated with miR-149* alone showed p53-independent cell-cycle arrest, as seen by induction of p21 levels (Fig. 6C and D). The combination with 5-fluorouracil led to apoptosis indicated by increased levels of cleaved PARP (Fig. 6C and D). This suggests that combinations of both miR-6883-5p and miR-149* with irinotecan/5-fluorouracil have translational potential in colorectal cancer.

miR-6883-5p and miR-149* combinations inhibit CDK4/6-FOXM1 signaling in colorectal cancer cell lines

Recently, it has been shown that CDK4/6 initiate the phosphorylation and activation of FOXM1 in G1 phase of the cell cycle (30, 31) and thereby promote downstream signaling. We
hypothesized that targeting of CDK4/6 by miR-6883-5p and miR-149 could downregulate the CDK4/6-FOXM1 signaling pathway, particularly in combination with irinotecan and 5-fluorouracil. miR-6883-5p alone reduced levels of both p-FOXM1 and FOXM1 and its transcriptional target cyclin B1 in HT-29 cells (Fig. 7A). Combination with irinotecan and 5-fluorouracil further exacerbated this signaling in HT-29 cells (Fig. 7A). Although miR-6883-5p had no effect on FOXM1 protein levels on its own in HCT116 cells, the combinations downregulated the CDK4/6-FOXM1 pathway (Fig. 7B). For HT-29 cells, we also investigated a subset of FOXM1 transcriptional targets required for progression to S or G2-M phases of the cell cycle.
cycle. qRT-PCR results indicate that miR-6883-5p downregulated expression of FOXM1 transcript by 40% and the corresponding target genes by 50% to 85% (Fig. 7C and D). Combination with both irinotecan and 5-fluorouracil further synergized with miR-6883-5p in downregulating the indicated genes (Fig. 7C and D).

miR-149 did not have significant single agent impact on any of the tested genes except CDC25B, which was reduced to 50% expression (Fig. 7C and D). However, the combination of miR-149 and irinotecan/5-fluorouracil still reduced the CDK4/6-FOXM1 signature in HT-29 cells (Fig. 7C and D).
In summary, our data demonstrate that miR (6883-5p and 149*) mediated targeting of cell-cycle genes CDK4/6 and anti-apoptotic genes XIAP and BCLXL has antiproliferative and proapoptotic effects in colorectal cancer cell lines (Fig. 7E). miR-6883-5p in particular is a strong candidate for combination with current colorectal cancer chemotherapy. Combinations with miR-6883-5p and miR-149* have an advantage of promoting cancer cells to apoptosis compared to growth arrest alone as observed with CDK4/6 inhibitors (Supplementary Fig. S4). We have independently reproduced the combination results observed with CDK4/6 inhibitor palbociclib and irinotecan (Supplementary Fig. S4A and S4B) or 5-fluorouracil (Supplementary Fig. S4C) in the recent by paper by Zhang and colleagues (41). As proof of concept, we also transiently knocked down CDK4 using siRNA in HT-29 and HCT-116 cell lines, combined treatment with irinotecan and observed reduction in short-term proliferation with the combination compared siRNA or irinotecan alone (Supplementary Fig. S5A). As expected, CDK4 siRNA knockdown alone reduced levels of p-FOXM1 and its downstream target cyclin B1 (Supplementary Fig. S5B and S5C). Similar to the miR-6883-5p and miR-149* combination results, CDK4 siRNA + irinotecan induced apoptosis as assessed by PARP and cleaved caspase-3 (CC3) data (Supplementary Fig. S5C).

**Discussion**

Dysregulation of genes in the CDK4/6-Rb cell-cycle pathway is a hallmark of cancer (42). Cancer cells subvert cell-cycle checkpoints for continued growth and proliferation. Overcoming
cell-cycle checkpoints involve either loss of tumor-suppressor proteins such as p16
INK4A, Rb or overexpression of oncogenes like CDK4, CDK6, and cyclin D. p16
INK4A inactivation caused by homozygous deletion, frameshift mutation or methylation is observed in tumors such as pancreatic cancer and a subset of melanoma (3, 43). Loss of p16
INK4A permits escape from senescence and provides an advantage for tumor progression. As a result, cancer cells express increased CDK4, CDK6, and cyclin D–dependent kinase activities. Tumors like melanoma and colorectal cancer, however, show amplification of CDK4 and/or CDK4R24C mutation (loss of INK4 binding site). Hence, targeting these tumors with CDK4/6 inhibitors, such as palbociclib and ribociclib, is an ongoing therapeutic strategy in clinical trials for pancreatic, melanoma, and colorectal cancer patients. Emergent knowledge with CDK4/6 inhibitors in clinical trials indicates that these therapies have limitations as monotherapy (44). Thus, there is an imminent need to develop alternate strategies to target CDK4/6.

In the present study, we used a combination of in silico prediction algorithms and TCGA dataset analysis to identify tumor suppressor miRNAs that can translate to single agent/combinatorial therapies for colorectal cancer. Previous studies described miRNAs regulating CDK4 expression in melanoma and nonsmall cell lung carcinoma (40, 45–47). However, there has been no report of tumor suppressor miRs identified in colorectal cancer and/or studies evaluating their use as therapeutics.

We identified a novel family of miRNAs, whose expression is lost in colorectal cancer. We have characterized the primary gene network—G1–S phase of the cell cycle—and selected apoptosis markers that are regulated by two of the four miRNAs in the family. These critical gene targets mediating the antiproliferative and proapoptotic effects of miR-6883-5p and miR-149* include CDK4, CDK6, FOXM1, XIAP, and BCLXL. FOXM1 is a master regulator of the cell cycle and has recently been identified to be phosphorylated and activated by CDK4/6 (31). FOXM1 has also been linked to resistance to different chemotherapies like cisplatin, 5-fluorouracil (27) and is an important oncogenic target in preclinical development. Further, FOXM1 is a crucial effector of mutant p53 gain of function (GOF) toward cell-cycle progression (48). Downregulation of
miR-6883 Family Targets CDK4/6 to Suppress Cancer

Figure 7.
miR-6883-5p and miR-149* combinations abrogate CDK4/6-FOXM1 signaling in colorectal cancer cell lines. HT-29 and HCT116 cell lines were transfected with either 50 nM or SCR or the indicated miRNA mimics. Sixteen hours after transfection, irinotecan or 5-fluorouracil were added at the indicated doses. Expression of FOXM1 and target genes with miRNA alone or combination was assessed by Western blot and qRT-PCR. Representative Western blots are for HT-29 and HCT116 cells shown in A and B, respectively. Representative qRT-PCR results for FOXM1 and panel of target genes in HT-29 cells combined with irinotecan (C) or 5-fluorouracil (D), respectively. E, Model depicting described role of miR-6883-5p and miR-149* in colorectal cancer cell lines.

the CDK4/6-FOXM1 axis and antiapoptotic proteins BCLXL and XIAP by our miRNA combinations thus has wide clinical implications. The investigated miRNA-drug combinations are particularly relevant in mutant p53-expressing tumors, which have varied GOFs in the cell cycle. Our study also suggests the use of miRNAs as potential adjuvant therapeutics.

miR-6883-5p is an intronic miRNA transcribed as part of the PER1 gene, a tumor suppressor gene whose loss is correlated...
with liver metastasis in colorectal cancer (38). PER1 also interacts with DNA-damage response proteins ATM, CHK2, and cell-cycle regulators Wee1, cyclin B1, and Cdc2 to contribute to apoptosis or cell-cycle arrest in colorectal cancer (39). By evaluating the role of miR-6883-5p, which is transcribed as part of the PER1 gene, we add the link between the circadian clock genes and their contribution to cell division and response to DNA damage.

miR-149-5p has previously been shown to target AKT1 and E2F1 and to contribute to apoptosis in neuroblastoma cell lines (49). Loss of miR-149-5p has been attributed to methylation and further linked to increased expression of its target gene SP1 (50). Our findings of miR-149-5p targeting CDK6 and XIAP bring to light a novel role of miR-149-5p, along with finding a family of miRNAs with similar functions. Also, the tumor suppressor roles of miR-149-5p and its loss in colorectal cancer is consistent with previous findings. In melanoma, miR-149-5p has been described as an oncogenic miR (51). miR-149-5p has been better characterized than miR-149-3p for its target–gene network interactions. Overall studies suggest that miR-149-5p is a tumor suppressor and inhibits genes involved in the epithelial-to-mesenchymal (EMT) transition and chemoresistance networks (52–55). Thus, our study provides further evidence for the role of miR-149-5p as a tumour suppressive miRNA.

In summary, we have identified two novel miRNAs, miR-6883-5p, and miR-149-5p as direct negative regulators of CDK4 and CDK6. Restoring the expression of miR-6883-5p and miR-149-5p in cancer cells shows promise for antiproliferative and proapoptotic effects when used alone or in combination. Thus, this study confirms miRNA mimics as potential adjuvant therapy for colorectal cancer, providing a promising avenue of investigation to supplement the small molecule inhibitors and chemotherapeutics currently in use.

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

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