MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2)

Nicola Valeri a,b,c,1, Pierluigi Gasparini a,b,1, Chiara Braconi b,d, Alessio Paone a,b, Francesco Lovat a,b, Muller Fabbrini a,b,e, Khlea M. Sumani a,b, Hansjuerg Alder a,b, Dino Amadori f, Tushar Patel b,d, Gerard J. Nuovo f, Richard Fishe a,b,g,2, and Carlo M. Croce a,b,h,2

Departments of aMolecular Virology, Immunology, and Medical Genetics, bInternal Medicine, cPathology, and dPhysics, and eOhio State University Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210; Departments of Embryology and Morphology and fExperimental and Diagnostic Medicine, University of Ferrara, 44011 Ferrara, Italy; and gIstituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori, 47014 Meldola, Italy

Contributed by Carlo M. Croce, October 19, 2010 (sent for review September 22, 2010)

The overexpression of microRNA-21 (miR-21) is linked to a number of human tumors including colorectal cancer, where it appears to regulate the expression of tumor suppressor genes including p21, phosphatase and tensin homolog, TGFβ receptor II, and B-cell leukemia/lymphoma 2-α-associated X protein. Here we demonstrate that miR-21 targets and down-regulates the core mismatch repair (MMR) recognition protein complex, human mutS homolog 2 (hMSH2) and 6 (hMSH6). Colorectal tumors that express a high level of miR-21 display reduced hMSH2 protein expression. Cells that overproduce miR-21 exhibit significantly reduced 5-fluorouracil (5-FU)-induced G2/M damage arrest and apoptosis that is characteristic of defects in the core MMR component. Moreover, xenograft studies demonstrate that miR-21 overexpression dramatically reduces the therapeutic efficacy of 5-FU. These studies suggest that the down-regulation of the MMR mutator gene associated with miR-21 overexpression may be an important clinical indicator of therapeutic efficacy in colorectal cancer.


The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

1N.V. and P.G. contributed equally to this work.

2To whom correspondence may be addressed. E-mail: rfishe@osumc.edu or carlo.croce@osumc.edu

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1015541107/-/DCSupplemental.


Colorectal cancer (CRC) is one of the most frequently occurring cancers in United States with more than 140,000 new cases and about 50,000 deaths expected to occur in 2010 (1). 5-Fluorouracil (5-FU)-based chemotherapy represents the gold standard for CRC treatment both in the adjuvant and metastatic setting (2). However, primary or acquired resistance to pyrimidine analog treatments is a common problem in the management of CRC patients (2). These observations highlight the need for a better understanding of resistance mechanisms and more effective therapies.

MicroRNAs are a class of small, noncoding RNAs that act as posttranscriptional regulators of gene expression and cell homeostasis (3). Overexpression of miR-21 is a common trait of many solid and hematological malignancies (4). miR-21 overexpression has been found in blood and stool samples from patients affected by CRC (5, 6). Moreover, miR-21 overexpression is associated with poor benefit from 5-FU adjuvant chemotherapy in stage-II and -III CRC (7).

The mismatch repair (MMR) system is involved in DNA damage recognition and repair. Human mutS homolog 2 (hMSH2) and human mutL homolog 1 (hMLH1) function as core MMR proteins and form heterodimers with protein homologs hMSH3 or hMSH6 and hMLH3 or hPM2, respectively (8). Heterodimer formation is fundamental for the DNA damage recognition and represents a crucial step for the stability of the MMR protein homologs (9). Defects in MMR proteins have been associated with reduced or absent benefit from 5-FU adjuvant chemotherapy in clinical trials (10). MMR impairment appears to cause reduced incorporation of 5-FU metabolites into DNA, leading to reduced G2/M arrest and apoptosis after 5-FU treatment (11, 12).

Results

miR-21 Directly Targets hMSH2 and hMSH6 Protein Expression. In silico analysis suggested that miR-21 might target hMSH2 [National Center for Biotechnology Information (NCBI) NM_0000249.2] and hMSH6 (NCBI NM_001792.2) mRNA (TargetScan, Whitehead Institute, MIT; Fig. 1A) (13). We identified putative binding sites for miR-21 in both the hMSH2 and the hMSH6 3′ UTR. We examined the effect of miR-21 expression on endogenous hMSH2 and hMSH6 mRNA expression in CRC Colo-320DM and SW620 cells. Both cell lines display low basal miR-21 expression. We transfected these cell lines with miR-21 precursor or a scrambled miR precursor control (Fig. S1). Overexpression of an siRNA specific to hMSH2 (anti-MSH2) or hMSH6 (anti-MSH6) did not affect the levels of miR-21 (Fig. S1A). The mRNA levels of hMSH2 and hMSH6 were unaffected by overexpression of miR-21 (Fig. 1B). In contrast, anti-MSH2 and anti-MSH6 siRNA specifically reduced the expression of hMSH2 and hMSH6 mRNA, respectively (Fig. 1B). We note a consistent reduction in the expression of hMSH6 mRNA with the anti-MSH2 siRNA. This reduction could be a result of degenerate hybridization of the anti-MSH2 siRNA with the hMSH6 mRNA or of reduced hMSH6 mRNA stability resulting from the diminished heterodimeric protein partner hMSH2. These results suggest that miR-21 overexpression does not affect the mRNA levels of hMSH2 or hMSH6.

We examined the protein levels of hMSH2 and hMSH6 following transfection of miR-21 in Colo-320DM and SW620 cells by Western blot analysis (Fig. 1C and Fig. S1B). hMSH2 and hMSH6 proteins were significantly reduced in cells overexpressing miR-21 compared with cells overexpressing the scrambled miR. The anti-MSH2 and anti-MSH6 siRNAs were transfected in these cell lines in parallel. We observed that cells transfected with miR-21 displayed a down-regulation of hMSH2 and hMSH6 that appeared comparable to levels in cells transfected with siRNAs. Conversely, we transfected CRC SW480, HCT-116, and RKO cells that contain high levels of endogenous miR-21 with a locked nucleic acid (LNA) against miR-21 (anti-miR-21) or a scrambled LNA (anti-miR control). We found that cells transfected with anti-miR-21 showed an increase in both hMSH2 and hMSH6 protein expres-
sion (Fig. 1D and Fig. S2 A and B), but no changes in mRNA levels were observed (Fig. S2C).

The entire 3′ UTR of hMSH2 or hMSH6 was subcloned downstream of the luciferase gene. The luciferase reporter construct along with a precursor miR-21 or scrambled miR then was transfected into the Colo-320DM cells. We observed a 50% and 37% reduction in the luciferase activity with constructs containing the miR-21 seed regions for hMSH2 or hMSH6, respectively (P < 0.001; Fig. 1E). Deletion of the miR-21 seed regions resulted in the restoration of luciferase activity for both vectors containing hMSH2 or hMSH6 (Fig. 1E). We transfected SW480 cells that displayed high levels of miR-21 expression with a luciferase reporter vector containing the wild-type or mutated hMSH2 and hMSH6 3′ UTR seed region (Fig. 1F). As expected, we found that ablation of the miR-21 binding site resulted in increased luciferase activity for both the hMSH2 and hMSH6 vector-transfected cells. To confirm these observations, SW480 cells were cotransfected with the hMSH2 and hMSH6 3′ UTR luciferase reporter plus the LNA anti-miR-21 or anti-miR control. LNA silencing of miR-21 induced an increase in luciferase activity (Fig. 1F). Taken as a whole, these results suggest that miR-21 exerts a direct effect on the hMSH2 and hMSH6 3′ UTR that ultimately regulates hMSH2 and hMSH6 protein expression. Because hMSH2 protein status can affect hMSH6 protein stability and expression (9), we cannot exclude the possibility that...
miR-21 regulation and hMSH2 protein loss can contribute to hMSH6 down-regulation.

miR-21 is inversely correlated with the MMR core protein hMSH2 in CRC Tissues. We examined miR-21 and hMSH2 expression in two different CRC cohorts (Fig. 2). A tissue microarray containing 50 unselected cases of CRC and paired normal adjacent tissue was hybridized with an LNA anti-miR-21 or nonspecific LNA anti-miR control combined with immunohistochemical staining for hMSH2 protein (Fig. 2A) (14). A score for both miR-21 and hMSH2 protein expression was given according to the percentage of positive cells in the core. Forty-two of 50 cores were available for matched analysis of tumor and paired normal tissue. We found that miR-21 was up-regulated in 28 (66%) of these cases when tumor was compared with normal paired tissue. In 14 of 42 cases (33%), there was a strong down-regulation of hMSH2 in tumor compared with normal tissue. In all these cases miR-21 was found to be up-regulated. Pearson’s correlation analysis in this subgroup of patients showed an r value of −0.82 (P < 0.001). Correlation analysis on the entire cohort of cases showed an r value of −0.63. CRC tissues that scored positive for both miR-21 and hMSH2 showed no coexpression in the same cancer nest (Fig. 2A, colabeling).

We examined fresh-frozen tumors from a second cohort of CRC samples for which cancer and normal adjacent tissues were available (Fig. 2B). miR-21 expression was determined by Northern analysis and RT-PCR, and hMSH2 protein expression was determined by Western analysis. In 26 cases, hMSH2 was down-regulated in tumors compared with normal adjacent tissue. In 24 of these cases (90%), miR-21 expression was increased in tumor as compared with adjacent normal tissues. Because we previously have reported that miR-155 can affect the expression of hMSH2 and other MMR proteins (15), we excluded from analysis 16 cases showing simultaneous overexpression of miR-155 and miR-21. An inverse correlation (r = −0.81; P < 0.02) still was evident in the remaining eight cases, highlighting the inverse correlation between miR-21 overexpression and hMSH2 down-regulation in CRC tumors (Fig. 2B and Fig. S3).

miR-21 reduces G2/M arrest and apoptosis following exposure to 5-FU. MMR-defective cell lines display resistance to a variety of therapeutic drugs, including 5-FU (16). These studies have demonstrated that resistance is the result of defective in-
corporation of 5-FU metabolites into DNA, leading to reduced damage-dependent G2/M arrest and subsequent apoptosis (11). We examined 5-FU–induced cell-cycle arrest and apoptosis in Colo-320DM and SW620 cells following transfection of miR-21. We used a scrambled miR as a control and compared our results with a similar transfection with an siRNA anti-MSH2 (Fig. 3). We found that miR-21 overexpression decreased the percentage of sub-G1 (apoptosis) and G2/M cells following treatment with 5-FU. Cells transfected with miR-21 displayed reduced G2/M arrest and apoptosis, similar to cells transfected with siRNA to hMSH2 (Fig. 3). The effect of miR-21 expression on 5-FU–mediated apoptosis was confirmed further in Colo-320DM and SW620 cells by Annexin V staining (Fig. S4). A similar response was observed in isogenic Lovo cells where the hMSH2 mutation [Lovo(MSH2−)] has been complemented with the introduction of chromosome 2 [Lovo(MSH2+)] (17). miR-21 overexpression, as well as siRNA to hMSH2, reduced sub-G1 and G2/M accumulation in Lovo(MSH2−) cells, whereas no effects were observed in Lovo(MSH2+) cells (Fig. 4). Cotransfection of Lovo (MSH2−) and Lovo(MSH2+) cells with a plasmid encoding the full-length hMSH2 cDNA rendered the message insensitive to miR-21 regulation, and cells retained a normal damage-induced G2/M arrest and apoptosis. Taken as a whole, these results are consistent with the conclusion that down-regulation of hMSH2 expression by miR-21 results in cellular resistance to 5-FU.

Overexpression of miR-21 Induces 5-FU Resistance in a CRC Xenograft Model. Our cellular studies suggested that miR-21 inhibits 5-FU–induced G2/M arrest and apoptosis by reducing the expression of hMSH2. We developed a xenograft colon cancer tumor model in which we generated stable clones of Lovo(MSH2+) cells that overexpressed miR-21 [Lovo(MSH2+)-miR-21] or an siRNA to hMSH2 [Lovo(MSH2+)-anti-MSH2] using a lentiviral expression system. Lovo(MSH2−) cells and Lovo(MSH2+) cells containing the stable insertion of an empty vector served as controls. We injected 5 × 106 cells containing stable lentiviral expression into the flank of nude mice. When xenografts reached a palpable volume, 5-FU (50 mg·kg−1·d−1) was administered by i.p. injection for 5 consecutive days per week for 2 wk (19). The 5-FU treatment proved more efficacious with Lovo (MSH2+) tumor xenografts than with Lovo(MSH2−) tumor xenografts (Fig. 5A and Table S1). These results are consistent with previous studies and suggest that MMR-proficient cells respond better to 5-FU therapy (11, 12). Importantly, stable
overexpression of miR-21 [Lovo(MSH2⁺)-miR-21] resulted in a reduced response to 5-FU and caused a tumor growth rate comparable to that in Lovo(MSH2⁺) tumor cells infected with siRNA to hMSH2 [Lovo(MSH2⁺)-anti-MSH2] (Fig. 5A and Table S1). Furthermore, 2 wk after discontinuation of 5-FU, tumor growth appeared significantly greater in the cells infected with Lovo(MSH2⁺)-miR-21 than in controls, suggesting that miR-21 overexpression enhances cancer progression. We confirmed that the expression of hMSH2 was reduced dramatically in Lovo(MSH2⁺) tumor xenografts expressing miR-21 or the anti-hMSH2 siRNA compared with the empty vector (Fig. 5 B and C). Taken together, our results support a central role for miR-21–dependent down-regulation of the hMSH2-hMSH6 heterodimer MMR protein in 5-FU resistance.

Discussion

miR-21 is commonly overexpressed in a number of human tumors, including CRC (4, 7). In recent years several miR-21 tumor-suppressor targets have been identified that may accelerate the progression of cancer (20–24). We found an inverse relationship between colorectal tumor cells that overexpress miR-21 and those that express the hMSH2 tumor-suppressor protein. Moreover, we determined that miR-21 appears to target directly the 3′ UTR of both the hMSH2 and hMSH6 mRNAs, resulting in significant down-regulation of protein expression. Because the hMSH2-hMSH6 heterodimer is the key initiation component, we expect that down-regulation of its core components is likely to affect mutation suppression by MMR and thereby enhance tumor progression. A clear prediction is that overexpression of miR-21 should enhance mutation rates via down-regulation of hMSH2 protein. Although these studies are ongoing, there are a number of other distinctive phenotypic effects that are indicators of MMR defects.

The state of the art therapeutic treatment of CRC includes 5-FU. 5-FU exerts its cytotoxic effects by misincorporation of fluoronucleotides into RNA and DNA and by inhibiting nucleotide synthesis by targeting the thymidylate synthetase enzyme. The overexpression of thymidylate synthetase, defects in 5-FU metabolism, TP53 mutations, and impairment of the MMR system are all hallmarks of 5-FU resistance and are predictors of clinical outcome (2). More recently both microRNA and gene expression analysis have revealed a higher level of complexity in predicting 5-FU benefit in stage-II and -III CRC patients who underwent adjuvant chemotherapy (7, 25). Indeed, a retrospective analysis of stage-II and -III CRC patients treated with 5-FU analogs showed reduced survival in patients with high miR-21 expression (7). The same findings were confirmed in the subgroup of stage-III CRC patients alone, whereas stage-II CRC patients showed no statistically significant correlation (7). The low number of patients may account for this latter result. Cells with genetic or epigenetic defects of the MMR machinery appear to tolerate 5-FU metabolites as a result of defects in G2/M arrest and apoptosis (11). Our studies have demonstrated that down-regulation of hMSH2 by miR-21 induces resistance to 5-FU in both a cellular model and a xenograft tumor model. Taken together, these results suggest that miR-21 tumor status is likely to be an important indicator of 5-FU therapeutic efficacy.

miR-21 appears to regulate a number of cell-cycle and tumor suppressor genes. It is possible that miR-21 could regulate other genes associated with cell-cycle arrest and/or drug resistance that might contribute to 5-FU resistance (22, 23). However, our study suggests that down-regulation of the MMR machinery may contribute to 5-FU resistance (22, 23). Interestingly, miR-21 expression appears to have a central role in the development of 5-FU resistance. Indeed, inhibition of 5-FU–induced apoptosis and G2/M arrest by miR-21 was comparable to that caused by siRNA-mediated selective inhibition of hMSH2. Moreover, transfection of Lovo(MSH2⁺) cells with miR-21 did not alter cell-cycle arrest or apoptosis, demonstrating that miR-21–induced effects are dependent on hMSH2 expression. Taken together, our results clearly suggest that inhibition of miR-21 might represent a synergistic treatment to overcome 5-FU resistance. Indeed, it has shown recently that antisense-miR-21 and 5-FU by a poly(amideamine) dendrimer can sensitize cells to 5-FU by increasing cell-cycle arrest and apoptosis in glioma cell lines (26).

Our results suggest that miR-21–dependent down-regulation of hMSH2-hMSH6 might be responsible for both primary and acquired resistance to 5-FU. In clinical practice 5-FU usually is administered as a continuous infusion over a 48-h period (27). Interestingly, miR-21 expression appears to increase in cell lines continuously exposed to 5-FU (28). We speculate that this overexpression may be a secondary mechanism of resistance and that cells may acquire miR-21 overexpression to overcome 5-FU cytotoxicity. There is additional clinical relevance if one considers that frequently hMSH2 is down-regulated after primary chemotherapy including 5-FU or cisplatin in rectal and ovarian cancers (29, 30).

In summary, we have correlated 5-FU drug resistance in colorectal tumors to the overexpression of miR-21 that directly down-regulates the core MMR proteins hMSH2 and hMSH6, ultimately leading to a defect in damage-induced G2/M arrest and apoptosis. These studies suggest that miR-21 is likely to be a useful marker for therapeutic protocols in CRC.

Materials and Methods

Colo-320 DM, SW620, HCT-116, SW480, RKO, and cells were transfected using Lipofectamine 2000 (Invitrogen). Luciferase constructs were subcloned using the primers listed in Table S2. Lentiviral vectors for miR-21 overexpression and empty vectors were generated by using System Biosciences kit. Results
of statistical analyses are expressed as mean ± SD unless indicated otherwise. Comparisons between groups were performed using the two-tailed Student’s t test. A P value <0.05 was considered significant. Graphpad Prism version 5.0 was used for Pearson correlations. More detailed information is provided in SI Materials and Methods.


ACKNOWLEDGMENTS. We thank Dr. Richard Boland (Baylor University Medical Center, Dallas) and Dr. Christoph Gasche (Medical University of Vienna) for the Lovo hMSH2−/− and Lovo(DT40.2)-1 hMSH2−/− cell lines. This work was supported by National Institutes of Health Grants CA067007 and GM080176 (to R.F.) and CA152758 (to C.M.C.) and by the Associazione Italiana per la Ricerca sul Cancro (N.V.). N.V. is the recipient of a Kimmel Translational Science Award.