microRNA-451 Regulates Macrophage Migration Inhibitory Factor Production and Proliferation of Gastrointestinal Cancer Cells

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Abstract

Purpose: microRNAs (miRNA) are small RNAs that function as post-transcriptional regulators of gene expression. Recent evidence has shown that some miRNAs can act as oncogenes or tumor suppressors. This study was conducted to evaluate the potential association of miRNA expression with clinical outcome in patients with gastric cancer.

Experimental Design: Expression of 250 human mature miRNAs was measured by real-time PCR on paraffin-embedded tumor samples of 21 patients with gastric cancer stage III uniformly treated with surgical resection followed by chemoradiation. We identified the miRNAs correlated with disease-free and overall survival times, and the results were evaluated including 24 other patients. *In vitro* cell proliferation and radiosensitivity studies were done to support clinical data.

Results: The results revealed that down-regulation of miR-451 was associated with worse prognosis. miR-451 was detected by *in situ* hybridization in epithelial cells and showed decreased expression in gastric and colorectal cancer versus nontumoral tissues. Overexpression of miR-451 in gastric and colorectal cancer cells reduced cell proliferation and increased sensitivity to radiotherapy. Microarray and bioinformatic analysis identified the novel oncogene macrophage migration inhibitory factor (*MIF*) as a potential target of miR-451. In fact, overexpression of miR-451 down-regulated mRNA and protein levels of MIF and decreased expression of reporter genes with *MIF* target sequences. Moreover, we found a significant inverse correlation between miR-451 and *MIF* expression in tumoral gastric biopsies.

Conclusions: These findings support the role of miR-451 as a regulator of cancer proliferation and open new perspectives for the development of effective therapies for chemoradioresistant cancers.

MicroRNAs (miRNA) are a recently discovered class of short, noncoding RNAs that function as negative regulators of gene expression (1). miRNAs can regulate target genes by increasing mRNA decay or by repressing translation (2, 3). Recent evidence has shown that miRNAs control many cellular processes, such as cell growth, apoptosis, and differentiation (1, 4). Moreover, different cancer types have shown distinct miRNA expression profiles, and several miRNAs have been described to play a role in tumor progression or in tumor suppression (5–7).

Although miRNAs are becoming increasingly recognized as regulatory molecules in human cancers (8–12), the role of miRNA expression as a potential marker for diagnosis, prognosis, and pharmacogenomics is currently an emerging avenue of investigation. miRNA profiles were reported to be associated with the clinical outcome of several leukemias (6, 13) as well as lung (7, 14), breast (5), and pancreatic cancers (12). These data suggest that miRNAs are involved in carcinogenesis and could be associated with prognosis.

In western countries, patients with gastric cancer are diagnosed mostly at advanced clinical stages, typically showing lymphatic tumor dissemination, with poor prognosis and a 5-year survival rate of <30% (15, 16). Postoperative chemoradiation after radical surgery is the standard of care in locally advanced gastric cancer. Although significant advances have been achieved in the treatment of this neoplasia, the relapse rate remains high. Identification of differential risk groups based on both clinical and molecular prognostic factors is a topic of growing interest. One promising strategy for identifying molecular markers involved in gastric cancer progression is to focus on miRNA expression associated with patient survival.

Formalin-fixed paraffin-embedded tissue samples are the most readily available archival material in clinical

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

To our knowledge, this is the first report showing that microRNA expression may serve as a molecular genetic variable to predict the clinical outcome of gastric cancer patients treated by a uniform adjuvant chemoradiotherapy protocol. Patients with a low expression of miR-451 had significantly shorter disease-free survival and significantly worse overall survival. Our results suggest that miR-451 may regulate a significant number of processes whose end results promote radiosensitivity in gastric cancer cell lines, including cell proliferation rate. Because multiple interactions might need to be perturbed to observe a phenotypic consequence, the identification of macrophage migration inhibitory factor as a specific miR-451 gene target will provide a potential target to reduce tumor survival and resistance. Our results suggest that evaluation of miR-451 expression in gastric cancer may identify a subset of patients who might require more aggressive adjuvant treatment and open new perspectives for the development of effective therapies for chemoradioresistant cancers.

practice. They are generally accompanied by well-documented clinicopathologic records and represent an invaluable source for the identification of molecular prognosis markers. However, these tissues have not been widely used in molecular biology due to the poor quality of mRNA extracted from formalin-fixed paraffin-embedded blocks. It has been shown that miRNA species are less susceptible than mRNA to degradation associated with tissue processing and storage and, therefore, formalin-fixed paraffin-embedded samples are good sources to study miRNA profiling (17–19).

The aim of this study was to identify the miRNA expression profile in an archive of paraffin-embedded human gastric carcinoma tissues from patients uniformly treated with surgery followed by radiation therapy plus 5-fluoruracil–based chemotherapy. Then, we correlated miRNA expression with clinical outcome and identified that down-regulation of miR-451 was associated with worse prognosis. Functional studies showed that miR-451 could be considered as a new suppressor gene in human gastric and colon cancer cells, as miR-451 overexpression decreased proliferation and increased response to ionizing radiation in these cells. As a potential explanation for this observation, we show that miR-451 down-regulates macrophage migration inhibitory factor (MIF), a proinflammatory cytokine involved in oncogenic transformation and tumor progression.

Materials and Patients

Patients. Patient characteristics are shown in Supplementary Table 1. This study comprised 45 patients, including 29 men (64%) and 16 women (36%), with a median age of 58 y (range, 33-74 y). All patients had been diagnosed with gastric cancer. After a gastrectomy, patients received adjuvant postoperative chemoradiotherapy according to the regimen developed by Macdonald et al. (20). The median follow-up time was 65 mo (range, 5.7-172 mo).

This retrospective study was approved and conducted in accordance with the policies of the Institutional Review Board at Navarra Hospital.

Real-time PCR profiling of mature miRNA. Total RNA was extracted from paraffin-embedded tissues with the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer's protocol. The concentration was quantified with a NanoDrop Spectrophotometer (NanoDrop Technologies). The expression of 250 mature miRNAs was profiled with the use of a real-time quantitative PCR assay as previously described (21).

miR-451 detection by chromogenic in situ hybridization. FITC 5′–labeled locked nucleic acid (LNA)-incorporated miRNA riboprobes were used for quantifying the expression of *miR-451*, *RNAU6B*, and scrambled control RNA (miRCURY LNA Detection; Exiqon). Hybridization of FITC-labeled LNA probes (50 nmol/L) was done overnight at 45°C in the StatSpin Thermobrite S500 (Iris Sample Processing). After stringency washes, the slides were incubated for 1 h with anti-FITC antibody (Dako Ltd.). The sections were revealed with the Advance System (Dako Ltd.), counterstained with hematoxylin, dehydrated, and mounted in DPX.

miRNA and small interfering RNA transfections. Pre–miR-451 (50 pmol; Ambion) and a scrambled control (50 pmol; Ambion) were transfected into AGS gastric epithelial cells and DLD1 colorectal cancer cells grown in six-well dishes (plated at 2×10^5 cells per well 24 h before transfection). Small interfering RNAs (siRNA) for human MIF (sequence: 5′-GGGTCTACATCAACTATTA-3′) and scrambled siRNA were designed and synthesized by Dharmacon. Transfection was done with Lipofectamine 2000 (Invitrogen). Transfection efficiency (>95%) was confirmed with the use of the Silencer FAM-labeled Negative Control (Ambion). Total RNA and protein were prepared 1, 3, and 6 d after transfection and were used for quantitative reverse transcription-PCR (RT-PCR) or western blot analysis.

Cell irradiation and measurement of cell viability. Transfected cells (2×10^5 cells per well) were irradiated at room temperature with 15 MeV (Primus Linear Accelerator; Siemens) at doses of 2 and 4 Gy. Nonirradiated controls were handled identically to the irradiated cells with the exception of the radiation treatment.

After irradiation, the cells were counted and plated at 3×10^3 cells per well in 96-well plates. Cell viability was measured with a CellTiter 96 Aqueous Assay Kit (Promega) at 7 d after irradiation according to the manufacturer's instructions.

Assessment of clonogenicity. AGS or DLD1 cells, transfected with scrambled oligonucleotide or *pre–miR-451*, were plated at low density (5×10^3 cells per 10-cm plate) and incubated for 6 or 10 d at 37°C in a humidified atmosphere containing 5% CO_2. Colony formation and growth were visualized with crystal violet staining. After the wells were photographed, the dye was solubilized with methanol and the optical density was measured at 570 nm by an ELISA reader (Opsys MR). The surviving fraction was calculated relative to the control nonirradiated cells.

RNA microarray hybridization. Transcriptional profiling was done with the Affymetrix HG-U133 Plus 2.0 Array, as previously described, 48 h after transfection of AGS gastric cancer cells transfected with the scrambled control or *pre–miR-451*. Raw signal intensities for each probe were recorded in CEL files and were analyzed with the robust multi-array averaging normalization method. The fold changes were calculated between the treated and control cells of both cell lines.

Real-time PCR assays for MIF gene. RNA from each cell line (1 μg) was used to generate complementary DNA with a High-Capacity cDNA Archive Kit (Applied Biosystems). Each complementary DNA sample was analyzed in triplicate with the ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Primers and probes were purchased from Applied Biosystems as assay-on-demand: *MIF* (Hs00236988_g1), *RNA18s* (Hs99999901_s1), or β2-microglobulin (Hs99999907_m1). The data were represented as $2^{-\Delta\Delta CT}$.

Western blotting for MIF protein. Proteins were analyzed by SDS PAGE, and western blotting was done with the use of nitrocellulose membranes (Bio-Rad). The mouse monoclonal anti-human MIF antibody (R&D Systems Europe Ltd.) was diluted 1:500, and the mouse monoclonal anti-human β-tubulin antibody (Sigma) was diluted 1:1,000. The secondary antibody was goat anti-mouse horseradish peroxidase (Sigma). Proteins were detected with Western Lightning Chemiluminescence (PerkinElmer LAS) and ImageQuant

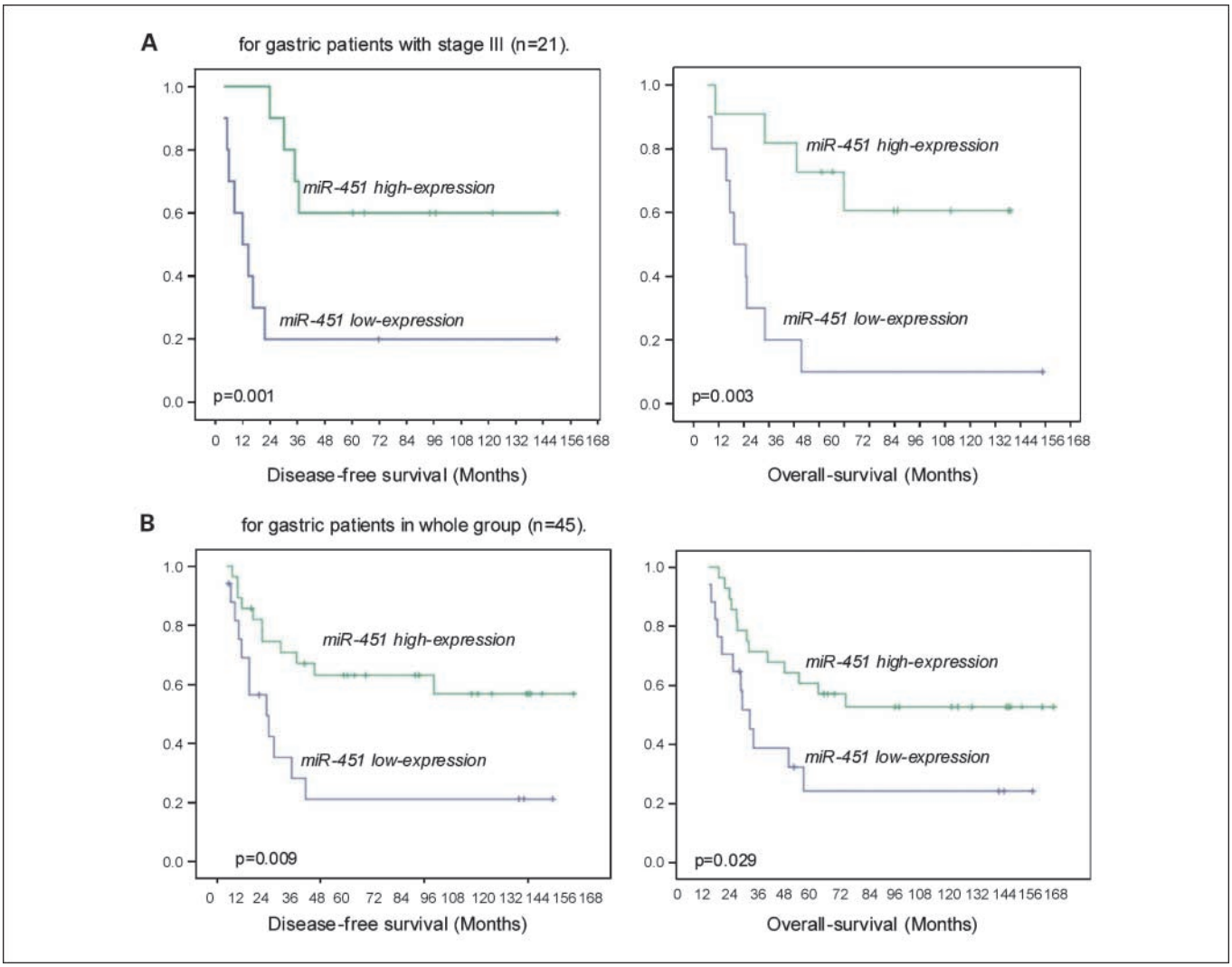

Fig. 1. Kaplan-Meier DFS and overall survival analysis. *A,* Kaplan-Meier DFS (*left*) and overall survival (*right*) analysis for stage III gastric patients based on the expression of miR-451 (n = 21). The rate of DFS or overall survival time in the miR-451 low-expression group was significantly lower than that of patients with high expression of miR-451. *B,* Kaplan-Meier DFS (*left*) and overall survival (*right*) analysis for gastric patients in the whole testing group (n = 45). The rate of DFS or overall survival time in the miR-451 low-expression group was significantly lower than that of patients with high expression of miR-451.

ECL imager, and signals were quantified with ImageQuant TL software (GE Healthcare).

Renilla luciferase assay. Renilla luciferase constructs, Renilla-*MIF*-3′untranslated region (UTR), and Renilla-*MIF*-3′UTR-*Mut* were made with the following oligonucleotides:

(*MIF*-3′UTR-F: 5′-CTAGAGCCCACCCCAACCTTCTGGTG-GGGAGAAATAAACGGTTTAGAGACTGC-3′,
MIF-3′UTR-R: 5′-GGCCGCAGTCTCTAAACCGTTTATTTC-TCCCCACCAGAAGGTTGGGGTGGGCT-3′,
MIF-3′UTR-Mut-F: 5′-CTAGAGCCCACCCCAACCTT-CTGGTGGGGAGAAATAGGTACTGAAGAGACTGC-3′, and
MIF-3′UTR-Mut-R: 5′-GGCCGCAGTCTCTTCAGTACC-TATTTCTCCCCACCAGAAGGTTGGGGTGGGCT-3′).

These oligonucleotides contain the complete putative target site of *MIF* 3′UTR or a mutated version. Hybridized oligonucleotides were ligated into the Xba I–Not I site of the Renilla reporter vector (pRL-SV40; Promega). With the use of Lipofectamine 2000 (Invitrogen), 75,000 DLD1 cells were cotransfected with the Renilla-*MIF* 3′UTR vector or the Renilla-*MIF*-3′UTR-mut, a control vector containing firefly luciferase (pGL3-Promoter; Promega), and either pre–miR-451 or scrambled precursor-miR molecules. Renilla luciferase activity was measured 48 h after transfection with the use of a Dual-Luciferase System (Promega) in a Berthold Luminometer (Lumat LB 9507) as previously described (22).

Statistics. First, the 250 miRNA expression profile was calculated as 2^{-Ct}, and data were multiplied by 10^8 and $\log_{10}$. The relative miRNA expression was median centered, and clustering of k-means was done to identify miRNA with high, moderate, low, and no expression (Supplementary Fig. S1). The reproducibility of real-time PCR tends to become worse when very low copies of templates are amplified. For this reason, the miRNAs included in cluster IV (no expression) were eliminated from further analysis. To identify miRNAs whose expression was significantly related to time to progression, we computed a statistical significance level for each miRNA based on a Cox proportional hazards regression model with a global test permutation with the use of BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam.[8]

[8] http://linus.nci.nih.gov/BRB-ArrayTools.html

Table 1. Multivariate Cox regression analysis of *miR-451* expression and DFS in gastric cancer patients

A. Multivariate analysis of postoperative DFS of 21 patients in stage III gastric carcinoma treated with adjuvant chemoradiotherapy in relation to stage, *miR-451,* and *hsa-miR-126* expression normalized by the median or *RNAU6B* (control). miRNA expression was categorized as low or high in relation to median values

Variable	Subset	HR (95% CI)	*P*
Data of miRNA expression normalized by median (stage III)			
Stage	IIIA/IIIB	1/2.5 (0.72-9.14)	0.148
miR-451	Low/High	4.78 (1.2-18.5)/1	0.025
miR-126	Low/High	2.56 (0.76-9.6)/1	0.122
Data of miRNA expression normalized by U6B snRNA (stage III)			
Stage	IIIA/IIIB	1/2.7 (0.8-9.3)	0.103
miR-451	Low/High	4.74 (1.3-17.3)/1	0.001

B. Multivariate analysis of postoperative DFS of 45 patients with gastric carcinoma (stages I-IV) treated with adjuvant chemoradiotherapy in relation to stage and *miR-451* expression

Variable	Subset	HR (95% CI)	*P*
Stage	I+II/III+IV	1/6 (1.7-21.2)	0.005
miR-451	Low/High	3.7 (1.5-8.7)/1	0.003

Abbreviations: HR, hazards ratio; 95% CI, 95% confidence interval; snRNA, small nuclear RNA.

Validation of miRNA expression was calculated through the comparative Ct method ($2^{-\Delta CT}$) with *RNAU6B* as reference gene.

Survival rate was estimated with the use of the Kaplan-Meier method. For this analysis, the medians of normalized data for miRNAs were calculated, and the cases were classified according to the median. The difference in survival rates between groups was tested for significance with the use of the log rank test. A Cox proportional hazards regression was done to estimate prognostic factors associated with disease-free survival (DFS). DFS was calculated from the date of surgery to the date of progression (local and/or distal tumor recurrence) or to the date of death. Overall survival was defined as time from the date of diagnosis to the date of death or last follow-up.

Relationships between miR-451 and *MIF* expression were explored by Spearman's correlation.

Statistical analysis was done with the use of SPSS version 13.1.

Results

miRNA expression profile and correlation with clinical outcome in gastric cancer patients. We analyzed the expression of 250 human mature miRNAs by real-time PCR on paraffin-embedded cancer biopsies of 21 patients with gastric cancer stage III. Initially, we did k-means clustering to classify the expression levels of miRNAs (Supplementary Fig. S1). We observed a uniform expression of the miRNA in all gastric cancer samples analyzed. miRNA expression could be divided into four groups representing high, intermediate, low, or nondetectable expression. Cluster 1 included a group of 74 miRNAs with low Ct values and, therefore, high expression levels. Fourteen of 22 miRNAs that were up-regulated in neoplastic gastric tissues studied by Volinia et al. (23) are in Cluster 1, suggesting that our approach with the use of formalin-fixed paraffin-embedded samples compares well with previous studies done with frozen gastric tumors. In our cohort, miR-21 and miR-24 were the miRNAs with the highest levels of expression. Previous studies have reported the overexpression of miR-21 in several cancer types (24–26), including gastric cancer (27). Cluster 1 also contains several members of the miR-17-92 cluster (miR-17-5p, miR-20a, and miR-92), which have been implicated in potent oncogenic behavior (28, 29).

Then, we investigated the correlation between the miRNA expression of 250 miRNAs and the rate of DFS. The univariate Cox proportional hazards regression model indicated that two miRNAs (miR-451 and miR-126) were related to the DFS of patients. Patients with lower expression of these miRNAs were found to have significantly worse prognoses than those with higher expression.

Kaplan-Meier analysis also showed that patients with either lower miR-451 or lower miR-126 had shorter DFS than patients with higher miR-451 or miR-126 expression. However, the difference in DFS for these two groups was only significant for miR-451 expression ($P = 0.008$; log rank test) because differences in miR-126 expression did not reach the level of statistical significance ($P = 0.096$, log rank test).

To validate our results, we did real-time RT-PCR analysis and normalized miR-451 expression to U6 small nuclear RNA levels. The analysis of the results showed a significantly shorter DFS ($P = 0.001$; Fig. 1A) and shorter overall survival ($P = 0.003$; Fig. 1A) for patients with low miR-451 expression. Moreover, the multivariate Cox proportional hazards regression analysis indicated that low miR-451 expression was an unfavorable independent factor ($P = 0.001$; Table 1A).

With the use of the previously defined cutoff point for 21 patients in stage III, a new set of 24 patients with stages I to IV was added. We used the whole group to evaluate miR-451 expression as a potential marker associated with DFS ($P = 0.009$; Fig. 1B) and overall survival ($P = 0.029$; Fig. 1B). The multivariate analysis showed that low expression of miR-451 was an unfavorable independent factor associated with lower DFS ($P = 0.003$; Table 1B).

Although further prospective controlled studies are necessary to validate the use of miR-451 as a prognostic molecular marker, our results suggest that miR-451 could play a critical role in the pathogenesis of gastric cancer.

miR-451 is down-regulated in primary gastric and colorectal tumors. To further verify the results showed so far about the biological role of miR-451 in human gastric carcinogenesis, we examined by quantitative RT-PCR the miR-451 expression in a new matched set of healthy tissues and gastric tumors. As

shown in Supplementary Fig. S2A, miR-451 was down-regulated in 78% of gastric tumors (17 of 22 samples).

Moreover, we tested whether down-regulation of miR-451 was also relevant in other gastrointestinal tumors. We found that miR-451 was also down-regulated in 75% of the colorectal tumors analyzed (9 of 12 paired samples; Supplementary Fig. S2B), suggesting that expression of this miRNA could play a role in different tumors.

miR-451 detection in epithelial cells. miR-451 is highly expressed in mature RBCs (30), but its expression has not been reported in gastric or colon cells. On gastric sections, *in situ* hybridization revealed cytoplasmic expression of miR-451 in epithelial cells of the mucous normal glands (Fig. 2A and B). Moreover, some cells of the gastric glands, with morphology consistent with oxyntic cells, were also positive for miR-451 (Supplementary Fig. S3A and B). As expected, we also found intense expression of miR-451 in mature erythrocytes (Supplementary Fig. S3C). The absence of staining with a scramble probe showed the specificity of the technique (Fig. 2C; Supplementary Fig. S3B and D). Moreover, RNAU6B nuclear expression was used as a positive control of the technique (Fig. 2D).

miR-451 regulates cell proliferation and cell survival after radiation therapy. miR-451 is expressed in epithelial cells and down-regulated in gastric and colorectal cancers, suggesting that miR-451 overexpression could be detrimental for the tumor. The effects of miR-451 on gastric and colorectal cancer cells were characterized by overexpressing miR-451 precursor molecules (pre–miR-451) that are processed to a miRNA that mimics endogenous mature miRNA. The overexpression of miR-451 was confirmed by real-time PCR (Supplementary Table S2), which showed that the levels of miR-451 expressed from transfected pre–miR-451 were similar to levels of abundant endogenous miRNAs.

Initially, we determined whether ectopic expression of miR-451 had a biological effect in AGS gastric or DLD1 colorectal cancer cell lines with the use of the MTS cell viability assay. Cell lines transfected with pre–miR-451 showed a significant reduction in metabolically active cells compared with those transfected with the scrambled negative control (Fig. 3A). Moreover, the effect of ectopic expression of this miRNA on cell proliferation was evaluated through clonogenic assays. Transfection of both cancer epithelial cell lines with pre–miR-451 decreased proliferation by 50% compared with controls 6 days after transfection (Fig. 3B).

The aberrant activation of survival pathways in tumor cells can enhance resistance to treatment. To explore the role of miR-451 in cell survival and the response to radiotherapy, AGS gastric and DLD1 colorectal cancer cells were transfected with the miR-451 precursor, and their sensitivity to radiotherapy was

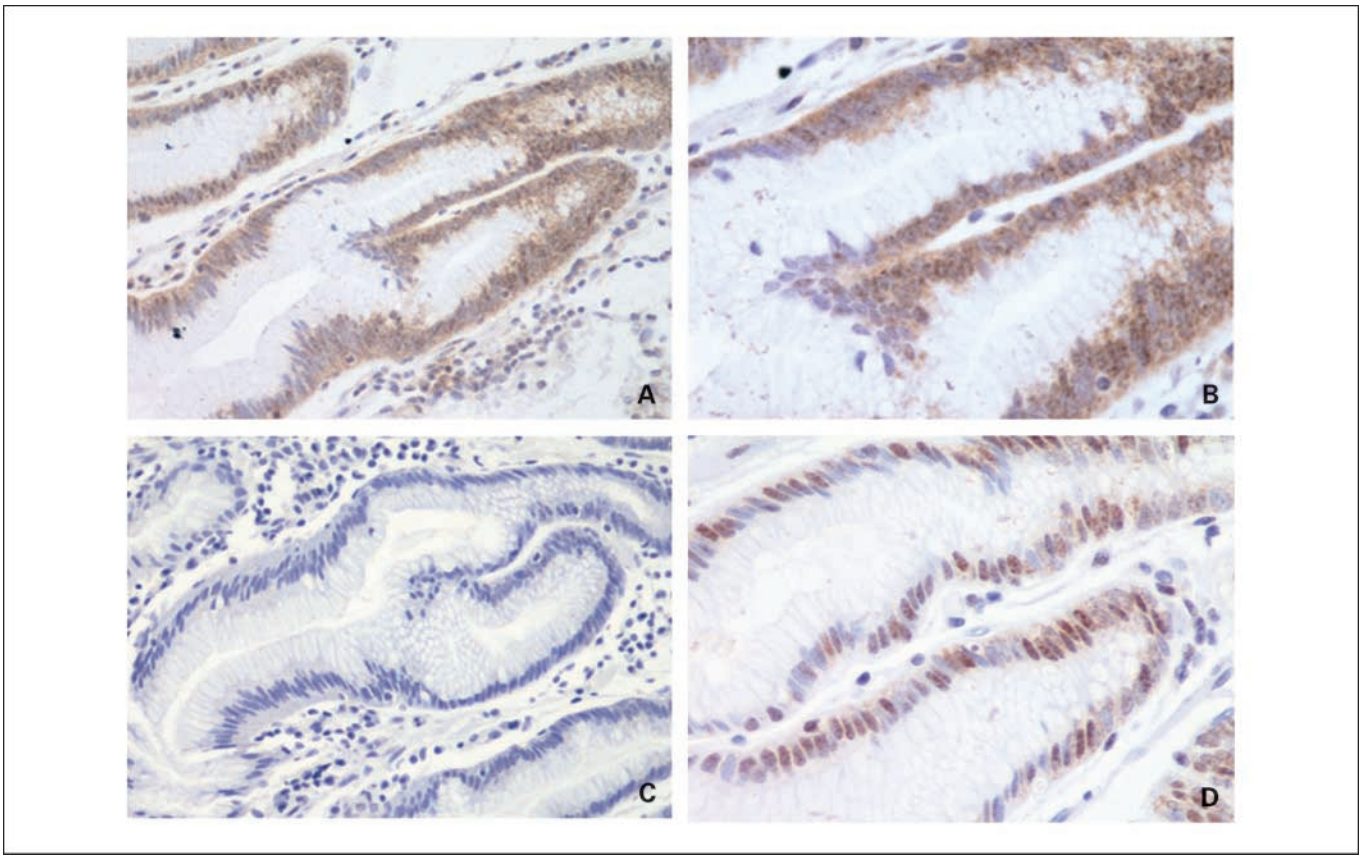

Fig. 2. *In situ* hybridization of *miR-451* in gastric mucous normal glands. ISH analyses with the use of 5′fluorescein-conjugated LNA-modified DNA probes complementary to mature miR-451 were done on 4-μm sections from archived formalin-fixed paraffin-embedded whole tissue blocks. *A* and *B,* gastric mucous normal glands showed significant amounts of miR-451 in the cytoplasm. *C,* as expected, the scramble control probe showed no significant staining in a serial section. *D,* ISH analysis with the use of 5′fluorescein-conjugated LNA-modified DNA probes complementary to RNAU6B; nuclear expression was used as a positive control of the technique. *A, C* ×20; *B, D* ×40. ISH, *in situ* hybridization; LNA, locked nucleic acid.

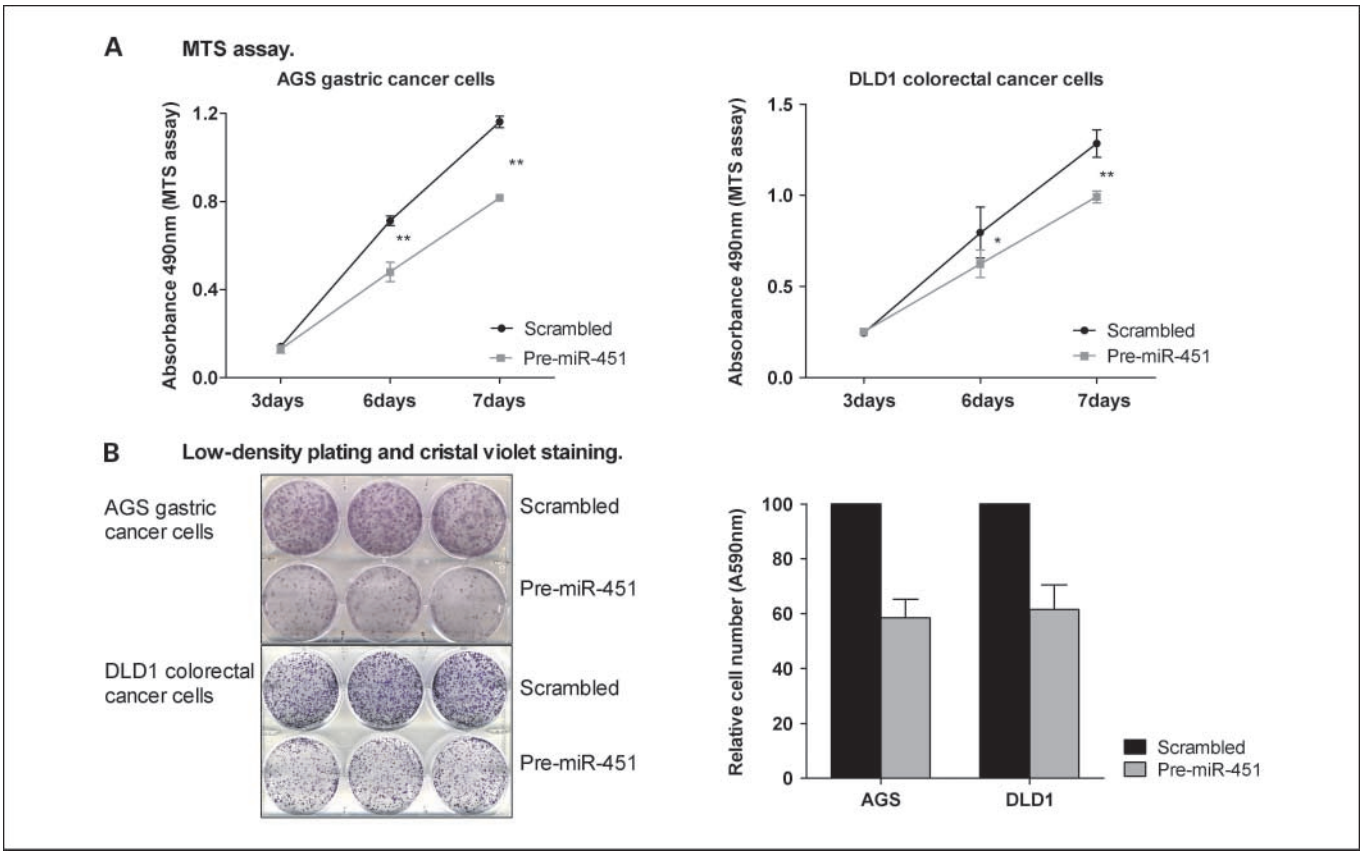

Fig. 3. Overexpression of miR-451 in gastric and colorectal cancer cells affected cell proliferation. *A,* the MTS cell viability assay was done on days 3 to 7 after Lipofectamine transfection of AGS cells and DLD cells with either a pre – miR-451 molecule (50 nmol/L) or a negative control scrambled oligonucleotide that does not encode any known miRNA (Ambion). The cell population transfected with the negative control oligonucleotide displayed a significantly greater number of metabolically active cells than cells transfected with the pre – miR-451. Values, mean ± SD of three experiments from three independent transfections. *B,* the effect of miRNA overexpression on cell proliferation was evaluated by crystal violet staining; values are reported as the percentage of incorporation in cells transfected with the miRNAs relative to control cells. Values, mean ± SD of three experiments from three independent transfections.

assessed with MTS and clonogenic assays. As shown in Fig. 4A, the proliferation rate determined by MTS at 7 days after radiation treatment was lower in cells transfected with pre – miR-451 than in control cells. Moreover, the clonogenic colony-forming ability assessed by the clonogenic assay at 10 days after radiation treatment was also reduced in cells transfected with pre – miR-451 compared with control cells (Fig. 4B and C). In conclusion, our data reveal that miR-451 overexpression reduces cell proliferation and sensitizes gastric and colorectal cancer cells to radiation therapy.

Identification of miR-451 targets. We wanted to address the molecular mechanism that allows miR-451 effects in gastric and colorectal cancers. We hypothesized that miR-451 could reduce the expression of target genes that induce cell proliferation and resistance to radiotherapy. To identify miR-451 target genes, we did mRNA expression profiling after transfection of pre – miR-451 into AGS gastric cancer cells. By microarray analysis, a total of 93 probes corresponding to 73 genes displayed a >2-fold reduction in expression in pre – miR-45 – transfected cells compared with AGS control cells. Bioinformatic analysis of the 3′UTRs of these genes identified 33 genes that had at least six nucleotides in sequence that were complementary to the miR-451 seed region (Supplementary Table S3). Among the candidates targeted, we found that the 3′UTR of human *MIF* contains a region (nucleotides 547-554; NM 00241) with perfect complementarity with the seed sequence of miR-451 (Fig. 5A). Moreover, MIF was identified as a miR-451 target with the bioinformatic prediction program miRBase Targets.[9]

We decided to study MIF further, as the overexpression of MIF has been observed in several human neoplasms (31, 32), including gastric (33) and colorectal cancer (34), and has been shown to exert effects on a variety of cell types by regulating diverse biological processes. The observed effects include inflammation, immune regulation, physiologic and pathophysiologic neovascularization, and cell replication. Thus, if MIF is a target of miR-451, miRNA down-regulation will lead to increased MIF expression and, therefore, worse prognosis.

To test this hypothesis, we analyzed whether endogenous *MIF* mRNA and protein levels fluctuated when miR-451 was overexpressed. We found that miR-451 expression in gastric and colorectal cancer cells caused a significant reduction of endogenous *MIF* mRNA (Fig. 5B) and MIF protein levels (Fig. 5C).

Moreover, to determine whether MIF was a direct target of miR-451, we constructed Renilla reporters that contained the wild-type and mutated miR-451 target sequences in the 3′UTR

[9] http://microrna.sanger.ac.uk/

of MIF. Both the wild-type and the mutant reporters were introduced into DLD1 cell lines. We found that ectopic expression of miR-451 inhibited the expression of the reporter vector containing *MIF* 3′UTR but not the reporter vector containing the mutation of the seed–miR-451 binding site (Fig. 5D). These data suggest that *MIF* is one of the direct targets of miR-451 and that miR-451 can down-regulate *MIF* at least by decreasing mRNA stability.

In vivo *regulation of MIF by miR-451*. To assess the clinical relevance of these findings, we performed quantitative real-time RT-PCR to evaluate *MIF* mRNA levels. Then, we correlated *MIF* with miR-451 expression in the same primary gastric tumors. As shown in Fig. 6, when *MIF* mRNA levels were plotted against miR-451 expression, an inverse significant correlation was found (two-tailed Spearman test: $P = 0.018$; $r_{Spearman} = -0.408$). These results support the premise that miR-451 down-regulation increased levels of *MIF* gene in gastric tumoral cells from patients.

Finally, to examine whether MIF expression could mediate miR-451 regulation of cell proliferation, siRNAs against MIF or siRNA negative control were transfected in AGS gastric and DLD1 colorectal cancer cells. MIF siRNA efficiently decrease MIF expression as detected by quantitative RT-PCR (Supplementary Fig. S4A) or by western blot analysis (Supplementary Fig. S4B). MIF siRNA down-regulation resulted in a decrease of cell proliferation as compared with control cells (Supplementary Fig. S5). These results indicate that down-regulation of miR-451 and, consequently, MIF overexpression are involved in the abnormal proliferation of cancer cells.

Discussion

In this study, we identified miR-451 as a potential suppressor miRNA targeting proliferation pathways in gastric and colon cancers. We provide evidence that miR-451 was down-regulated in gastric and colon cancers, and this alteration was associated with a reduction of DFS and overall survival in gastric cancer patients. In addition, *in vitro* overexpression of miR-451 in cancer cells lines inhibited cell growth and increased radiation response. We identified MIF as a direct target of miR-451, and we detected an inverse significant correlation between *MIF* and miR-451 expression in gastric biopsies.

miRNAs have been shown to be important in the development and maintenance of normal cellular function, and

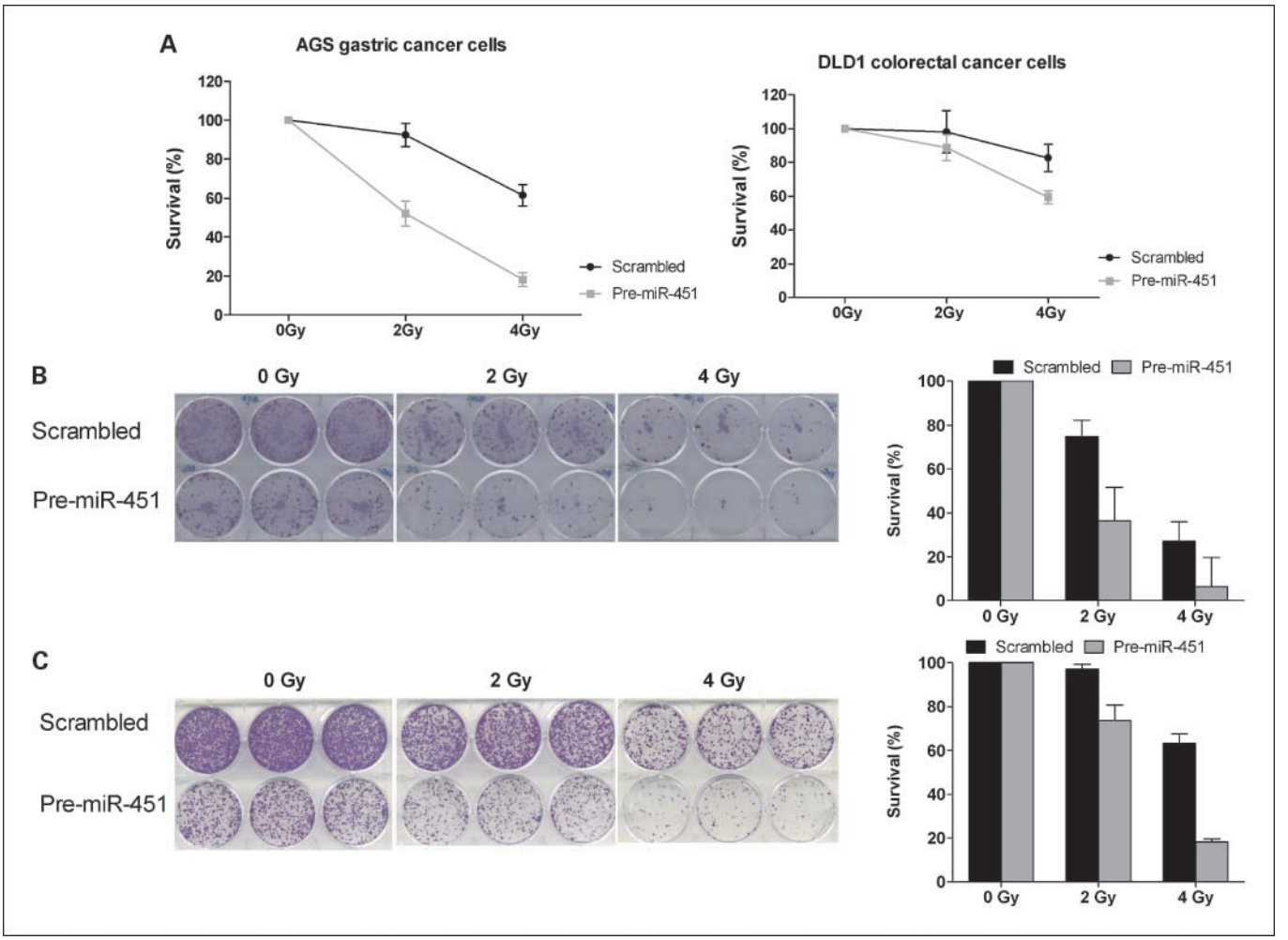

Fig. 4. MicroRNA-451 overexpression affected the response to radiotherapy assessed by MTS and clonogenicity assays. *A,* cell proliferation was assessed 7 d post treatment, and the proliferation index was derived. Values, mean ± SD of three experiments from three independent transfections. *B* and *C,* clonogenicity was evaluated as the percentage of colonies formed in cells treated with radiotherapy and transfected with specific pre–miR-451 or control relative to untreated cells.

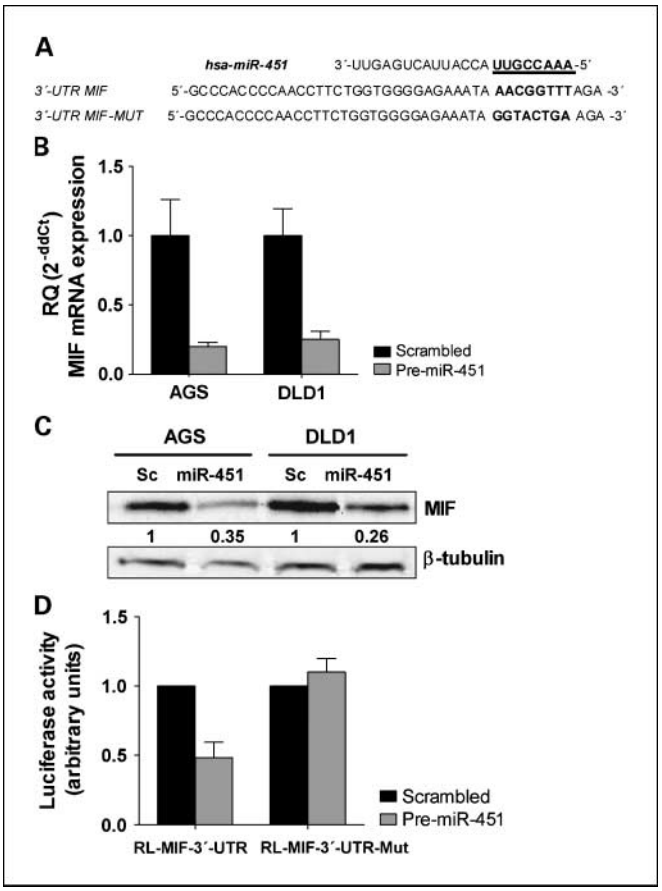

Fig. 5. miR-451 negatively regulated *MIF* by binding to the *MIF* 3′UTR. *A,* sequence alignment of human miR-451 with the 3′UTR of *MIF*. The seed sequence of miR-451 (*top*) matches the 3′UTR of *MIF* (*middle*). Bottom, mutations of the 3′UTR of *MIF* for creating the Renilla reporter constructs. *B,* pre – miR-451 reduced *MIF* mRNA levels in AGS gastric and DLD1 colorectal cancers. Bar, mRNA expression normalized to 18s RNA ± SD. *C,* pre – miR-451 reduced MIF protein levels in AGS gastric and DLD1 colorectal cancers. Protein expression was quantified and normalized to β-tubulin. *D,* pre – miR-451 inhibited wild-type, but not mutated, *MIF*-3′UTR reporter activity. Bar, Renilla luciferase activity normalized to firefly luciferase activity ± SD. Each reporter plasmid was transfected at least twice (on different days), and each sample was assayed in triplicate.

alteration in expression of miRNAs can result in human cancer initiation and tumor progression. However, little information is available on the impact of altered miRNA expression on patient survival. It seems clear that patients with identical clinicopathologic characteristics or the same stage of gastric cancer have a very different clinical outcome. Our initial expression profiling experiments identified that down-regulation of miR-451 was significantly associated with low DFS and low overall survival in stage III gastric cancer patients (Fig. 1A). These data were confirmed with different normalization processes (global median and RNAU6B) and was confirmed including a group of patients who received similar treatment with different stages (Fig. 1B). The multivariate analysis showed that low miR-451 expression was an independent prognostic factor associated with poor prognosis (Table 1). Moreover, the continuous quantitative data of miR-451 was significantly lower in patients that experienced recurrence of disease than in patients that did not show recurrence (data not shown). The results of this retrospective analysis suggest that miR-451 could be a prognostic marker for relapse in gastric cancer after curative surgery that was uniformly treated with adjuvant chemo-radiotherapy. Although additional studies are necessary to confirm these results, our study describes a valuable approach to begin to understand the biological significance miR-451 in gastric cancer.

Previous research indicated that miR-451 is involved in the specific differentiation of erythrocytes (30, 35). However, high expression of miR-451 has been detected in lung tissues, suggesting that several cell types, including erythrocytes and pulmonary epithelial cells, may require miR-451 for their maintenance and/or development (36). Our results showed that miR-451 was expressed in the cytoplasm of epithelial cells of normal gastric mucosa. This suggests that the decreased levels of miR-451 observed in tumor tissues correspond to a decrease of this miRNA in epithelial cells rather than a decrease in the amount of miR-451 – expressing erythrocytes invading the tumor. These data were not in accordance with those published by Sempere et al., which suggested that miR-451 expression changes observed in breast cancer samples correspond to a cell type of nonepithelial origin. In their study, miR-451 expression was detected exclusively in mature RBCs and was not detected in any of the breast cancer cell lines studied (37). However, our negative controls (omission of the probe and the use of a scrambled probe) and our positive control (the detection of a strong signal in erythrocytes and the detection of a nuclear signal for the RNAU6B probe) showed the specificity of the technique.

The elucidation of the modulatory miRNA effects on cancer cell lines is essential to determine the role of the prognostic value assigned to the miRNA expression in the tumor. Our results suggest that miR-451 regulation of cell proliferation may contribute to the mechanism that drives tumor progression. Furthermore, the role that miR-451 plays in the response to radiation suggests a novel approach for the exploration of new markers that can predict treatment responsiveness.

Each miRNA has the potential to target hundreds of genes that harbor in their 3′UTRs sequences complementary to the seed region of the miRNA (3). *MIF* modulation by miR-451

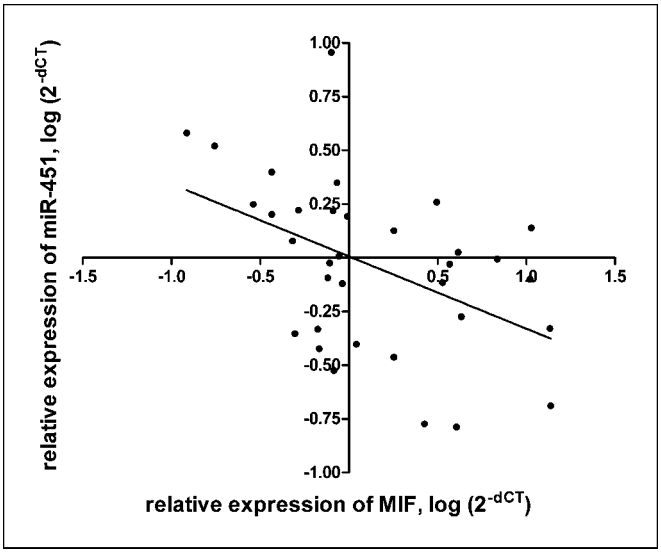

Fig. 6. Inverse correlation between miR-451 expression and *MIF* mRNA levels in gastric cancer samples. A statistically significant correlation between miR-451 and *MIF* mRNA levels ($P < 0.05$) was observed with the use of two-tailed Spearman's test.

was shown by *in vitro* transfection experiments in gastric-derived and colon-derived cell lines and by proving the direct interaction between miR-451 and *MIF* 3′UTR target sites with the use of a reporter assay. Many studies have reported that *MIF* expression is increased in premalignant, malignant, and metastatic tumors. Several reports also indicate that *MIF* expression closely correlates with tumor aggressiveness and metastatic potential, suggesting that *MIF* may make an important contribution to disease severity (38). *MIF* has been indirectly implicated in promoting tumor growth and progression, and certain tumors possess a functional requirement for *MIF* expression to maintain optimal growth and progression (39, 40). Takahashi et al. showed that depleting endogenous MIF resulted in a >40% reduction of tumor cell growth in colon cancer (41) In this sense, we also proved the existence of an inverse correlation between miR-451 and *MIF* expression in primary gastric cancer. Moreover, we showed that *MIF* inhibition reduced cancer cell proliferation. These data, taken together, suggest that MIF up-regulation represents one of the molecular mechanisms for which miR-451 down-regulation could promote tumorigenesis.

However, miRNAs may function according to a combinatorial circuits model, in which a single miRNA may target multiple mRNAs, and several coexpressed miRNAs may target a single mRNA. Recent studies have suggested that the biological concept of 'one hit–multiple targets' could be used in clinical therapeutics (42). If the primary molecular defect of a disease is in the expression of a miRNA, the expression of several critical protein targets could be deregulated. In that case, one might recover the normal phenotype of the cells by normalizing the miRNA expression. Although individual targets responsible for observed phenotypes have been proposed for many miRNAs, it is likely that a specific miRNA may function through cooperative down-regulation of multiple targets. Thus, other target genes of miR-451 may also contribute to tumorigenesis. Some genes with reduced expression in the microarray analysis (Supplementary Table S4) may be targeted and contribute also in increasing oncogenicity. Furthermore, miRNAs function also by suppressing the translation of their target genes, which is not always associated with mRNA decay. Therefore, only a subset of relevant targets can be identified on the basis of mRNA expression analysis. To explore the full impact of a miRNA, genome-wide proteomic studies should be done.

In conclusion, expression profiling and functional studies suggest that miR-451 has a tumoral suppression function in gastrointestinal cancer, at least in part through direct suppression of MIF. Our experiments documented the lower expression of miR-451 in gastric cancer specimens with worse prognosis. Reduction of cell proliferation by a pre–miR-451 oligonucleotide indicates that such strategy may serve as a basis for the development of new potential therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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