Pro-tumorigenic Effects of miR-31 Loss in Mesothelioma*

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The human genome encodes several hundred microRNA (miRNA) genes that produce small (21–23n) single strand regulatory RNA molecules. Although abnormal expression of miRNAs has been linked to cancer progression, the mechanisms of this dysregulation are poorly understood. Malignant mesothelioma (MM) of pleura is an aggressive and highly lethal cancer resistant to conventional therapies. We and others previously linked loss of the 9p21.3 chromosome in MM with short time to tumor recurrence. In this study, we report that MM cell lines derived from patients with more aggressive disease fail to express miR-31, a microRNA recently linked with suppression of breast cancer metastases. We further demonstrate that this loss is due to homozygous deletion of the miR-31-encoding gene that resides in 9p21.3. Functional assessment of miR-31 activity revealed its ability to inhibit proliferation, migration, invasion, and clonogenicity of MM cells. Re-introduction of miR-31 suppressed the cell cycle and inhibited expression of multiple factors involved in cooperative maintenance of DNA replication and cell cycle progression, including pro-survival phosphatase PPP6C, which was previously associated with chemotherapy and radiation therapy resistance, and maintenance of chromosomal stability. PPP6C, whose mRNA is distinguished with three miR-31-binding sites in its 3′-untranslated region, was consistently down-regulated by miR-31 introduction and up-regulated in clinical MM specimens as compared with matched normal tissues. Taken together, our data suggest that tumor-suppressive propensity of miR-31 can be used for development of new therapies against mesothelioma and other cancers that show loss of the 9p21.3 chromosome.

MicroRNAs (miRNAs) are small molecules that bind mRNA of certain genes and affect their expression (1). Recent studies have revealed that expression of miRNAs in malignant cells can be affected by chromosomal rearrangements associated with tumor progression (2, 3). Moreover, microRNAs may be directly involved in tumorigenesis via the regulation of tumor suppressors and oncogenes (4). To identify microRNAs linked with progression of pleural malignant mesothelioma (MM), an aggressive cancer that is associated with exposure to asbestos and often radiation- and chemotherapy-resistant, we performed array analysis of miRNAs expressed in MM cell lines derived from patients with poor prognosis. Here, we report that lack of miR-31 expression in MM is linked with homozygous loss in the 9p21.3 chromosome, which is as a critical event in common cancers (5–7).

In MM, the 9p21.3 deletions are often homozygous (8) and associated with poor prognosis (9, 10). The oncogenic nature of the deletion has been linked with two tumor suppressor genes that reside in this area as follows: CDKN2A that encodes cell cycle inhibitory p16INK4A and p14ARF, and the adjacent CDKN2B (p15INK4B) (5, 6, 11). In this study, we demonstrate that their neighbor MIR31, which is located ~0.5 Mb telomeric to CDKN2A, is also commonly deleted in MM. miR-31 was recently characterized as a suppressor of breast cancer metastases (12). We demonstrate that reintroduction of miR-31 into MM cells inhibits cell cycle and suppresses invasion, migration, and clonogenicity supporting critical contribution of this miRNA to adverse disease. Whole-genome profiling of miR-31 transcriptional effects identified among its prospective targets principal factors that orchestrate DNA repair, replication, and cell cycle progression.

MATERIALS AND METHODS

Cell Lines—Mesothelioma cell lines HP1, HP3, H2373, H2452, H2591, H2595, H2596, and H2461 were produced by H. I. Pass from surgical specimens derived from patients with resected MM as described previously (13). TERT-immortalized mesothelial cells LP9 (14), primary mesothelial cell culture NYU-S90.2 (passage 3), and SV40-transformed mesothelial cells Met-5A were used as controls.

RNA Isolation and miRNA Array Analysis—MicroRNA-containing RNA was isolated from cultured cells using mirVana kit (Ambion) and assessed for integrity in agarose gel. Custom miRNA arrays were prepared as described previously (15). Briefly, ~900 DNA oligonucleotide probes representing microRNAs were spotted in triplicates on coated microarray slides (Nexterion® Slide E, Schott, Mainz, Germany). 3–5 μg of total RNA were labeled by ligation of an RNA linker, p-CrU-Cy/dye (Eurogentec S.A., Cy3 or Cy5), to the 3′-end. Slides were

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incubated with the labeled RNA for 12–16 h at 42 °C and then washed twice. Arrays were scanned at a resolution of 10 μm, and images were analyzed using SpotReader software (Niles Scientific, Portola Valley, CA). Microarray spots were combined and signals normalized as reported previously (15).

Microarray Data Analysis and Statistics—The expression of all microRNAs was normalized as described previously (15). Each comparison was between two groups. Only microRNAs that had a median signal higher than signal background levels (normalized fluorescence signal of ~300) in at least one of the two groups were tested. Expression levels between each two groups of samples were compared using the two-sided Student’s t test. To correct for multiple hypothesis testing, the Benjamini-Hochberg false detection rate method was used, with false detection rate = 0.1. Any microRNA that had a p value below the false detection rate-moderated threshold was taken to be significantly differentially expressed. A microRNA was, however, only reckoned truly differential if the fold-change between group medians was at least five.

Introduction of miR-31 Mimic and Inhibitor in MM Cells—MicroRNA mimic (miR-31), miR-31 inhibitor, and oligonucleotide negative control were purchased from Ambion (Austin, TX). For transfection of these oligonucleotides, MM cells were plated at 60–70% confluency in 10-cm dishes. Lipo-fectamine-2000 and the protocol recommended by Invitrogen were used with 0.4 nmol of miR-31 mimic or negative control and with 0.8 nmol of miR-31 inhibitor added to 10 ml of media. Transfection media were removed after 4 h of incubation and replaced with fresh Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum medium. 48 h post-transfection, miR-31-transfected cells were trypsinized, counted, and assayed for proliferation, colony formation in soft agar, wound closure, and Matrigel invasion in triplicate experiments as described previously (16, 17).

Cell Cycle Analysis—Cell cycle analysis was performed in triplicates on permeabilized cells using a routine propidium iodide staining protocol. Cells plated at ~25–30% confluency were transfected with miRNA mimic or inhibitor as described above and grown for 48 h until ~80% confluency. Trypsinized cells were then washed with phosphate-buffered saline and fixed with cold 70% ethanol. RNase treatment (0.2 mg/ml) of above and grown for 48 h until iodide staining protocol. Cells plated at 80% confluency. Trypsinized cells were trypsinized, counted, and assayed for proliferation, colony formation in soft agar, wound closure, and Matrigel invasion in triplicate experiments as described previously (16, 17).

RT-PCR Detection of Endogenous or Transfected miR-31—To detect miR-31, we used an end point modification of the loop RT-PCR technology described previously (18). In the first stage, a reverse primer and Superscript II reverse transcriptase kit (Invitrogen) were used to produce a chimeric cDNA product on miR-31 (Suppl. Fig. S1). To increase the efficiency of reverse transcription and PCR, we used modifications introduced by Varkonyi-Gasic et al. (19). The product of looped RT-PCR was used to prepare cRNA following the Affymetrix 3’IVT Express kit labeling protocol and standardized array processing procedures recommended by Affymetrix, including hybridization, fluidics processing, and scanning of the Affymetrix HG-U133 Plus 2.0 arrays. The raw data (Affymetrix CEL files) were normalized using Robust Multichip Average algorithm (20) in GeneSpring GX software (Agilent Technologies, Santa Clara, CA). Differentially expressed genes were determined by comparison between miR-31 mimic-transfected HP-1 cells and two controls as follows: mock oligonucleotide-transfected cells and Lipofectamine-treated nontransfected cells. Genes that showed reproducible reduction in their expression levels (ranging from 1.33- to 10-fold) in miR-31 transfectants were used for further analysis. Gene Ontology analysis of differentially expressed genes was performed using GeneCodis portal (21, 22).

Validation of Putative miR-31 Targets—For clinical validation of putative miR-31 targets, we used our U133 Plus 2.0 Affymetrix expression array data from 32 MM and 7 normal peritoneal specimens as described previously (10, 16). Western analysis was performed with antibodies to PPP6C (NB100-2883) and MCM2 H00004171-M01) from Novus Biologicals (Littleton, CO). RT-PCR validation of gene expression on clinical MM specimens was performed using SingleTube Superscript II kit (Invitrogen) and Kodak Image station and software for reading and processing signal intensities as reported previously (10). RNA specimens were isolated from resected tumors and matched healthy peritoneum specimens as described previously (7). We used PPIA, an invariantly expressed gene to reassure equal loading. The following primers were used: PPP6CF, 5′-AGTATGTGGAAATAGCGCGG-3′ (position 91–110); PPP6CR, AAAATGGTGC-AGCAGGATG (position 1016–997, GenBank™ reference sequence NM_002721.3); PPIAF, 5′-TCTGAGCACTGGAG-GAGAAAGG-3′ (position 197–217); and PPIAR, 5′-GGAAAACATGGAAAACCCCA-3′ (position 717–697, GenBank™ reference sequence NM_021130.3).

Mapping of MIR31 in the 9p21.3 Deletion in Clinical MM Specimens—To this end, we used our whole-genome representation oligonucleotide microarray analysis data produced as described previously (10). Briefly, we extracted DNA from archived 22 MM specimens with complete survival and progression information. Nine patients who had tumor recurrence/progression for less than 12 months were defined as short term recurrence group (STR), and 13 patients who progressed after more than 12 months were defined as long term recurrence group. The coordinates of the deletion in clinical specimens were assessed via processing of the representation oligonucleotide microarray analysis data using CGH-Explorer software (23). Mapping the boundaries of homozygous 9p21.3 deletions in DNA specimens isolated from MM cell lines was done via genomic PCR on the CDKN2A-flanking areas using primers listed in supplemental Table S1.

RESULTS

Loss of miR-31 Expressed in Most MM Cell Lines—To identify microRNAs whose expression is associated with MM progression, we performed array analysis of microRNAs expressed in
cell lines established from eight patients with advanced pleural mesothelioma (13) comparing them with three benign controls of mesothelial origin as follows: SV40-transfected mesothelial MeT-5A cells, a tert-immortalized cell line LP9, and primary culture NYU-590.2 produced from a healthy peritoneum. The subject of this study is miR-31, which was unique in showing no detectable signals in seven out of our eight malignant cell lines and an ∼5–15-fold lower signal intensity in the remaining cell line H2461 as compared with benign controls (Fig. 1A). The fact that all control cell lines, including 20-year-old MeT-5A, were miR-31-positive argued against cell culture artifacts suggesting that loss of this microRNA expression is linked to the disease.

**Co-deletion of the MIR31 Gene with CDKN2A and -2B in Vivo and in Vitro**—MIR31, the miR-31-encoding gene, is located in the 9p21.3 region proximal to two tumor suppressor genes CDKN2A and -2B frequently deleted in MM and other malignancies (8, 9, 24–28). We recently characterized this deletion as one of the most frequent events in MM tumors and associated it with poor prognosis (10). We therefore asked if loss of miR-31 expression is due to the 9p21.3 deletion. To this end, we re-analyzed our previous DNA copy number variation representation oligonucleotide microarray analysis data produced on 22 patients with the goal to map the deletion accurately. As a result, we localized MIR31 in the common 9p21.3 deletion area (Fig. 1B) and showed that all seven patients from the STR group that lack CDKN2A/2B are also devoid of MIR31 (Fig. 1C). The fact that 7 out of 13 STR but none of the long term recurrence patients showed loss of MIR31 supported our hypothesis of its clinical importance.

To answer if homozygous MIR31 loss can account for the observed lack of miR-31 expression in all seven MM cell lines assessed in this study, we performed PCR on the
CDKN2-flanking regions. Genomic PCR was paralleled by loop-RT-PCR to validate expression array data represented in Fig. 1A. As shown in Fig. 2A, all seven cases of miR-31 loss were linked to homozygous deletions of MIR31. Also, all MM cell lines under study were completely devoid of the CDKN2 genes in line with their origin from adverse tumors. miR-31-expressing H2461 was the only MM cell line that possessed at least one copy of MIR31 and showed miR-31 expression. Based on the PCR mapping shown in Fig. 2B and Suppl. Fig. S2, the size of the homozygous deletion area was estimated as highly variable spanning from less than 0.7 Mb in H2461 to ∼2 Mb in H2452 and HP-3, 3–8 Mb in H2591, and to up to more than 6 MB in HP-1, H2373, H2596, and H2595.

miR-31 Suppresses Invasion, Migration, Proliferation, and Clonogenicity of MM Cells—Based on the fact that miR-31 is frequently co-deleted with the CDKN2 genes, we asked if it possesses tumor suppressor properties or is a mere “bystander” with no particular function in tumorigenesis. To this end, we transfected miR-31 mimic into H2595 and HP-1 cells and analyzed its effect on their protumorigenic properties. In this and other assays, miR-31 levels were monitored with looped RT-PCR (see below). Our in vitro functional studies showed that invasive, migratory, proliferative, and colony formation capacities of both cell lines were significantly (∼1.5–2-fold) reduced upon miR-31 introduction as compared with the Lipofectamine only or negative oligonucleotide controls, suggesting that miR-31 may serve as a tumor suppressor (Fig. 3). The inhibiting effect of miR-31 on cell proliferation (Fig. 3C) led to a more detailed investigation of the

![FIGURE 1. Co-deletion of MIR31 with cell cycle inhibitors CDKN2A and CDKN2B in MM. A, expression of miR-31 in MM cell lines and control nonmalignant mesothelial cells as determined by comparative miRNA microarray study. The diagram compares signal intensities in arbitrary units. B, location of the MIR31 and CDKN2 genes in the vicinity of the telomeric (TEL) boundary of the deletion. CEN, centromeric. Losses are shown below the base line; gains are shown above the base line. The frequency of the deletion is ∼32%. C, 7 out of 13 patients with poor prognosis (STR group) show the 9p21.3 deletion (dashed line) that includes CDKN2 genes and MIR31. STR, short term recurrence (<12 months to relapse); LTR, long term recurrence (≥12 months to relapse).](image-url)

![FIGURE 2. Homozygous co-deletions of MIR31 and CDKN2A in MM cell lines. A, comparison of looped RT-PCR assessment of miR-31 expression with genomic PCR on the CDKN2A-neighboring areas. Only one out of eight MM cell lines, H2461, shows miR-31 expression. None of MM cell lines expresses CDKN2A. B, map of homozygous 9p21.3 deletions in MM cell lines shows homozygous co-deletion of MIR31 with CDKN2A in seven out of eight MM cell lines. Homozygous CDKN2A deletion in H2461 is the smallest and does not include MIR31.](image-url)

![FIGURE 3. Suppressive effects of miR-31 on tumorigenic cell behaviors. H2595 and HP-1 MM cells were transfected with miR-31 mimic or negative control oligonucleotide and assessed on invasion, migration, cell proliferation, and anchorage-independent growth. p values are shown above bars.](image-url)
possible link between this miRNA and cell cycle regulation.

miR-31 Inhibits Progression of MM Cells to S-phase—To provide a better insight into the anti-proliferative propensity of miR-31, we analyzed its impact on cell cycle progression using flow cytometry analysis of propidium iodide-stained cells. To modulate miR-31 levels in both directions, we used two different cell lines. HP-1 cells devoid of miR-31 were used for miR-31 transfection, whereas H2461 cells that retain endogenous miR-31 expression (see Fig. 2A) were transfected with an miR-31 inhibitor. As shown in Fig. 4A (representative data of three experiments), re-introduction of miR-31 into HP-1 cells produced a significant reduction in the amount of cells in S-phase (from 18.8 to 13.8%), whereas transfection of the miR-31 inhibitor into H2461 cells increased the S-phase cell ratio from 25.1 to 34.4%. miR-31 also caused a 7.2% increase in the G1 ratio in HP-1 cells, although its inhibitor produced a 20.2% decrease in G1 in H2461. Finally, H2461 cells showed a significant increase in the G2/M cell fraction caused by the miRNA inhibitor. In summary, our cell cycle analysis demonstrated that both exogenous and endogenous miR-31 profoundly and reproducibly affected cell cycle progression in MM cells.

Re-introduction of miR-31 into HP-1 Suppresses Predicted miR-31 Targets—To provide insight into molecular mechanisms of miR-31 activity in mesothelioma cells, we then asked if genes modulated by miR-31 can be identified. Computational prediction of miRNA-binding sites normally generates hundreds of potential targets that need validation. Thus, we used HP-1 cells transfected with miR-31 for gene expression array studies and GenoCodis 2.0 portal for the whole-genome search for potential miR-31 targets and subsequent statistical analysis of gene annotations. Proper delivery of the miRNA into the cells was validated by looped RT-PCR (data not shown). Microarray-assisted analysis identified 1145 Affymetrix probes that showed an ~1.33–10-fold down-regulation upon miR-31 delivery into HP-1 cells as compared with negative control oligonucleotide and Lipofectamine-treated cells. Search for predicted miRNA targets using Gene Ontology (GO) analysis revealed among these probes 650 GO annotated genes that appeared to be highly enriched with computationally predicted miR-31 targets. Specifically, among these 650 genes, we identified 60 predicted miR-31 targets with a hypergeometric $\text{Hyp}^*$ value of $\approx 1.9 \times 10^{-22}$ (Fig. 4B). miR-31 targets were on the top of the list significantly outperforming miR-200a (47 genes), miR-583 (42 genes), miR-873 (42 genes), and other predicted miRNA targets. With this observation, we were assured that our experiment on miR-31 transfection produced biologically relevant information that can be further analyzed to reveal possible roles of the 650 GO annotated candidate miR-31 targets in tumor growth.

Re-introduction of miR-31 Suppresses Genes Involved in DNA Repair, Replication, and Cell Cycle Regulation—Using the same approach and software, we then analyzed biological annotations of the 650 genes suppressed in HP-1 cells upon miR-31 mimic introduction to identify affected molecular processes. GO analysis identified among these genes DNA Replication (21 genes), Cell Cycle (33 genes), DNA Repair (21 genes), and Response to DNA Damage Stimulus (24 genes) categories as groups that show statistically significant $p$ values. In the next step, we used KEGG GO profiling to highlight genes whose specific functions are well established. This approach produced “Cell Cycle” (18 genes, $\text{Hyp}^* = 1.7 \times 10^{-11}$), “DNA Replication” (11 genes, $\text{Hyp}^* = 7.2 \times 10^{-10}$), and “p53 Signaling Pathway” (13 genes, $\text{Hyp}^* = 3.7 \times 10^{-9}$) groups as statistically sound and suggested that miR-31 may simultaneously control different components of the cellular machinery that coordinately regulate biological processes associated with cell proliferation (Table 1 and data not shown). Similar results have been generated using a different algorithm (david.abcc.ncifcrf.gov).

In the next step, we asked if miR-31 can potentially bind to the 3’-UTR of its prospective targets that control cell cycle pro-
Using one of the most efficient predicting algorithms, RNA22 MicroRNA Target Detection interface (29), we screened 18 genes from the KEGG Cell Cycle group for miR-31-binding sites. As shown in Table 1, half of these genes possess miR-31-binding sites in their mRNAs, which show favorable thermodynamics. This prediction, although not comprehensive, suggests that miR-31 may directly control a large fraction of its potential targets. Altogether, these data imply that miR-31 may directly or indirectly control genes that orchestrate DNA replication, DNA repair, and cell proliferation.

**Clinical Validation of Potential miR-31 Targets Linked with Cell Cycle Regulation**—To evaluate the clinical significance of KEGG Cell Cycle genes suppressed by miR-31 introduction, we assessed their expression in our collection of 32 specimens of MM and 7 healthy peritoneum controls, which were used in our previous studies (10, 16). This comparison showed that half of the proposed miR-31 targets are, indeed, up-regulated in MM (see column 5 in Table 1) as compared with normal mesothelium and may therefore be important for tumor progression. The most prominent of these genes were cyclin B2 (CCB2), which was 12-fold overexpressed in MM, followed by CHEK1, SKP2, MCM2, CDC6, and CDC2. We also took advantage of the Oncomine portal to answer if these genes are up-regulated in other cancers. Table 1 shows that 13 out of 18 KEGG Cell Cycle genes may serve as reliable discriminators between different breast cancer grades. This conclusion is

### Table 1

| GENE ID   | Description                                  | Positions of putative miR-31 binding site/Folding energy | miR-31 alignment with target | Validation in MM (fold up-regulation) | Validation in breast carcinoma** |
|-----------|-----------------------------------------------|--------------------------------------------------------|------------------------------|---------------------------------------|---------------------------------|
| CCNA1     | Cyclin-A1                                     | NM_000914.3, pos. 1511–1531; –33.3 Kcal/mol           | AGCTGAACGACTTTTGTCT          | 12.4, p=0.02                         | Prognostic, 8 studies; p=1E-50   |
| CCK2      | G2/mitotic-specific cyclin-B2                | NM_004701.2, pos. 1076–1096; –27.1 Kcal/mol           | GCAACT---GTCTCTGTTTTCTCT     |                                       | Prognostic, 6 studies; p=1E-9    |
| CCK4      | G1/S-specific cyclin-B2                      | Not found                                              |                              |                                       | Prognostic, 6 studies; p=1E-9    |
| CDC2      | Cell division control protein-2 homolog       | NM_001786.3, pos. 1607–1627; –25.6 Kcal/mol           | AGACG-ATCCGAA-ATTTCTCTA      | 1.9, p=0.03                          | Prognostic, 9 studies; p=1E-50   |
| CDC6      | Cell division control protein-6 homolog       | Not found                                              |                              |                                       | Prognostic, 7 studies; p=1E-13   |
| CDC2      | Cell division protein kinase-2 homolog        | NM_001798.3, pos. 1649–1669; –33.5 Kcal/mol           | ACCCTA-GTTAGGGTTTTGCTCT     | 3.2, p=0.04                          | Prognostic, 6 studies; p=4E-6    |
| CHEK1     | Serine/threonine-protein kinase Chk1           | Not found                                              |                              | 7.1, p=0.001                         | Prognostic, 8 studies; p=1E-50   |
| GADD45A   | Growth arrest and DNA-damage-inducible protein GADD45 alpha | Not found                                              |                              |                                       | Prognostic, 6 studies; p=1E-50   |
| GADD45B   | Growth arrest and DNA-damage-inducible protein GADD45 beta | Not found                                              |                              |                                       | Prognostic, 6 studies; p=1E-50   |
| MCM2      | DNA replication licensing factor MCM2          | NM_004526.2, pos. 2994–3014; –27 Kcal/mol              | TGTT---TGCTGACATCTGCTCA      | 4.8, p=0.002                         | Prognostic, 8 studies; p=1E-50   |
| MCM3      | DNA replication licensing factor MCM3          | Not found                                              |                              | 1.57, p=0.00                         | Prognostic, 6 studies; p=1E-50   |
| MCM5      | DNA replication licensing factor MCM5          | NM_006739.3, pos. 1610–1630; –28 Kcal/mol              | TTC-AGGCCACACAGCTTCTCCG      | 1.67, p=0.03                         | Prognostic, 7 studies; p=1E-11   |
| MCM6      | DNA replication licensing factor MCM6          | Not found                                              |                              | 1.87, p=0.01                         | Prognostic, 9 studies; p=1E-50   |
| MCM7      | DNA replication licensing factor MCM7          | NM_005916.3, pos. 725–745; –26.8 Kcal/mol              | GCTACGGAAGCTTCTGCTGCTG      | 6, p=0.002                           | Prognostic, 6 studies; p=2E-9    |
| ORC4L     | Origin recognition complex subunit 4          | NM_018714.2, pos. 1580–1600; –33 Kcal/mol              | TGTTCACTGATGGCAGCAGATCTGCTG |                                       | Prognostic, 5 studies; p=1E-14   |
| SFN       | 14-3-3 protein sigma                          | Not found                                              |                              |                                       | Prognostic, 5 studies; p=1E-14   |
| SKF2      | S-phase kinase-associated protein 2 (F-box protein 8k) | Not found                                              |                              | 4.1, p=0.001                         | Prognostic, 8 studies; p=1E-50   |
| TWAHA     | 14-3-3 protein eta (Protein A81)              | NM_003405.3, pos. 1506–1526; –27.7 Kcal/mol            | AGTAAGCT---CTCTGTTTTTCTGCTG |                                       | Prognostic, 5 studies; p=1E-14   |

* Data produced on 32 MM specimens were collected by H. I. Pass.

** Results are from the profiling of multiple sets of Oncomine breast cancer gene expression data.
based on multiple independent studies that all show extremely low p values for the null hypothesis. Most of these KEGG genes were also overexpressed in multiple other cancers such as lung, ovarian, central nervous system, head-and-neck, gastrointestinal, bladder tumors, and sarcomas (data not shown). Overall, our analysis suggests that miR-31 deficiency may up-regulate a set of genes principal for cell cycle regulation and tumor survival that may serve as important clinical markers for MM and other cancers.

**Validation of Protein Phosphatase 6 as an miR-31 Target and a Diagnostic Marker for MM**—Among the predicted miR-31 targets, protein phosphatase PPP6C is one of the most intriguing due to its ability to trigger stress-related response and establish links with cell cycle regulation (30), apoptosis, and chemoresistance (31), resistance to radiation (32), and regulation of chromosome segregation (33). Because all these PPP6C properties suggest a principal role for the gene in progression of MM, a cancer distinguished with multiple chromosomal instability (10) and resistance to common therapies, we asked if PPP6C is indeed a direct miR-31 target. PPP6C possesses in its 3’-UTR three predicted binding sites for miR-31 (Fig. 5A). In our expression microarray study on HP-1, PPP6C was consistently down-regulated by miR-31 introduction (Fig. 5B). Our RT-PCR and Western analyses of MM cells transfected with miR-31 or its inhibitor showed that expression of endogenous PPP6C was responsive to the miR-31 status in both cell lines on both mRNA and protein levels. Suppression of endogenous miR-31 in H2461 caused up-regulation of PPP6C suggesting that the observed relation between miR-31 and PPP6C is highly reproducible and biologically relevant (Fig. 5, C and D). We also demonstrated that another potential miR-31 target, MCM2, is suppressed by miR-31 on the protein level (Fig. 5D).

Because PPP6C expression has not been yet associated with cancers (most likely due to the lack of specific Affymetrix probes), we developed an end point RT-PCR assay for analysis of PPP6C expression in clinical MM specimens supplemented with matched controls. This study showed a profoundly elevated expression of this gene in ~86% of MM specimens as compared with matched normal healthy peritoneum (Fig. 5E).
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DISCUSSION

Association of the 9p21.3 deletion with poor prognosis in MM brings attention to the genes that reside in this area and may potentially contribute to tumor growth and its radiation and chemotherapy resistance. Although CDKN2 genes from 9p21.3 have been already characterized as tumor suppressors, experimental evidence suggests that recurrently deleted chromosomal regions may contain clusters of genes suppressive for tumor growth (34). For the first time, the results of our study associate the 9p21.3 deletion in MM with loss of miR-31, a microRNA that has been recently associated with suppression of breast metastases in a mouse model (12). We show that co-deletion of MIR31 with the CDKN2 genes may be beneficial for tumor progression because miR-31 inhibited proliferation, migration, invasion, and colony formation in MM cell lines. The suppressive effect of miR-31 on progression from G1 to S was confirmed in two cell lines with one of them expressing miR-31 endogenously. Genome-wide search for potential miR-31 targets showed, with robust p values, that this microRNA may modulate multiple DNA replication, cell cycle progression, and DNA damage response and repair factors. Recently, a similar link between miR-16 and cell cycle regulators has been reported (35). We noticed, however, that miR-31 may control a distinct and much broader set of genes associated with cell survival. The fact that these molecular effects of miR-31 were in line with its suppressive effect on the cell cycle stimulated a more detailed investigation into specific roles of potential miR-31 targets. Analysis of gene annotations suggested that miR-31 may target different subunits of the same protein complex, such as most of the six MCM factors (MCM2–7) that cooperate as a multiprotein subunit with CDC6 during DNA replication origin and licensing. A substantial part of these genes possess miR-31-binding sites in their 3′-UTRs (Table 1). Our clinical assessment of potential miR-31 targets showed that most of them are suppressed in patients with poor prognosis.

Among the possible miR-31 targets that we have identified, there exist factors that have the capacity to trigger a regulatory signaling cascade and could principally contribute to chemotherapy and radiation therapy resistance of cancer cells. Although biological activities of these proteins have been established in yeast, their relation to cancer is not yet fully understood. Some of such proteins, phosphatase PPP6C/PP6C, is a human ortholog of the yeast protein Sit4 that functions downstream of the yeast protein Sit4 during DNA replication origin and licensing. The data suggest that PPP6C/pp1 with control of chromosome segregation in yeast (33), it is tempting to speculate that dysregulated PPP6C expression in MM as a result of miR-31 loss may not only contribute to chemoresistance but also increase chromosomal instability, which, as we demonstrated earlier, is especially pronounced in patients with poor prognosis (10). Suppression of this phosphatase may therefore contribute to treatment of such patients.

Collectively, our findings prompt us to hypothesize that one of the major miR-31 functions in mammalian cells is coordination of cell division with DNA repair, replication, and chromosome segregation. Association of miR-31 loss with the 9p21.3 lesion in cancer may have widespread clinical implications. Deletions that may potentially contain MIR31 have been recently reported in urothelial carcinomas (37), and down-regulation of this microRNA has been observed in gastric cancer (38). Also, 9p21.3 deletions were found in 31% of non-small lung cancers (39), ~85% of pancreatic adenocarcinomas (40), 35% of biliary tract tumors (41), 37.5% of osteosarcomas (42), 38% of soft tissue sarcomas (43), 75% of gliomas (44), 33–39% of T-cell acute lymphoblastic leukemias, 67% of primary cutaneous diffuse large B-cell lymphoma of the leg type (45), and ~17% of breast cancers (46). In a study on solid tumors that metastasize in brain, 9p21.3 loss was among four most frequent losses detected in 70% of cases (47). In conclusion, our observation that recurrent miR-31 loss in MM is linked with the 9p21.3 deletion and enhanced capacity of cells to proliferate, migrate, and invade opens new opportunities for treatment of MM and other tumors associated with the 9p21.3 deletion.

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