

Loss of miR-200c Expression Induces an Aggressive, Invasive, and Chemoresistant Phenotype in Non–Small Cell Lung Cancer

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Abstract

The development of metastases is the main reason for cancer-related death in non–small cell lung cancer (NSCLC). The initiation of metastasis involves an increase in cell motility mediated by the loss of cell-cell adhesion caused by E-cadherin repression, in a process commonly known as epithelial-to-mesenchymal transition. A role for microRNA-200 family members in regulating epithelial-to-mesenchymal transition has recently been indicated but data about their expression in lung tumors is still unavailable. The present study investigated the expression of miR-200c in a panel of NSCLC cell lines ($n = 9$), and a strong inverse correlation with invasion was detected. Reintroduction of miR-200c into highly invasive/aggressive NSCLC cells induced a loss of the mesenchymal phenotype by restoring E-cadherin and reducing N-cadherin expression, and inhibited *in vitro* cell invasion as well as *in vivo* metastasis formation. Moreover, miR-200c overexpression restored the sensitivity of NCI-H1299 cells to cisplatin and cetuximab. Hypermethylation of the promoter region was found to be responsible for the loss of miR-200c in invasive cells, as evaluated by 5-aza-2'-deoxycytidine treatment, methylation-specific PCR, and bisulfite sequencing. In primary tumor specimens obtained from 69 patients with consecutively resected NSCLC, lower miR-200c expression levels were found to be associated with a poor grade of differentiation ($P = 0.04$), a higher propensity to lymph node metastases ($P < 0.01$), and with a lower E-cadherin expression ($P = 0.01$). These data indicate that the loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype, and that assessment of its expression could contribute to a better clinicopathologic definition of patients with NSCLC. *Mol Cancer Res*; 8(9): 1207–16. ©2010 AACR.

Introduction

Non–small cell lung cancer (NSCLC) is the primary cause of cancer mortality in both sexes, accounting for 1.2 million deaths each year (1). Despite therapeutic advances, the high mortality of patients with NSCLC has not been substantially reduced over the past years, largely because of the potential of lung tumor cells to invade tissues and metastasize (2). Therefore, an improved understanding of the molecular mechanisms which regu-

late metastatic transformation of the cancer cells is urgently needed. Tumor metastasis involves a series of events which promote and regulate the migration of cancer cells to generate metastases at distant sites. The process is initiated in the primary tumor, where cancer cells dysregulate cell adhesion, downregulate proteins such as E-cadherin, and upregulate proteins characteristic of a more motile, mesenchymal-like phenotype such as vimentin and N-cadherin (3). This process, known as epithelial-to-mesenchymal transition (EMT), requires transcriptional reprogramming to suppress E-cadherin expression via specific transcription factors such as Snail, Slug, FOXC2, Twist, ZEB1, and ZEB2 (4, 5). MicroRNAs (miRNA) are a class of small (19–24 nucleotides) non–protein–encoding RNA molecules that regulate gene expression by modulating the activity of specific mRNA targets via direct base-pairing interactions (6). Recent evidence shows that miRNA-200 family members play a central role in the process of EMT, promoting the upregulation of E-cadherin by directly targeting the transcription factors ZEB1 and ZEB2 (7–10). The miR-200 family of miRNAs accounts for five members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) expressed in two genomic clusters, one on chromosome 1p36.33 and the other on chromosome 12p12.31. Among all other

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members, miR-200c has been shown to be the only one to regulate ZEB1 and to restore E-cadherin expression (11, 12). In initial reports, transfection of miR-200c reduced cancer cell migration and invasion, indicating a potential inhibitory role in malignant tumor progression (7). Moreover, independent studies showed that forced overexpression of miR-200c increased the sensitivity of epidermal growth factor receptor (EGFR)-blocking agents in bladder cancer (13), of doxorubicin in breast cancer cells (14), and of microtubule-binding chemotherapeutic agents in endometrial, breast, and ovarian cancer cell lines (15). However, the ability of miR-200c to suppress metastatic steps *in vivo*, molecular mechanisms leading to the suppression of miR-200c in cancer cells, and the specific role of miR-200c in NSCLC has never been explored. Therefore, we specifically investigated (a) the expression of miR-200c in a panel of NSCLC cell lines and its role in NSCLC invasion, metastasis formation, and resistance to chemotherapeutic agents; (b) the possible mechanisms regulating its expression; and (c) the possible associations between miR-200c levels and the clinicopathologic features of patients with NSCLC.

Materials and Methods

Cell lines, cultures, and drugs

Nine human NSCLC cell lines (three squamous cell carcinoma cell lines, Calu-1, NCI H520, and SKMES-1; one adenosquamous, H596; three adenocarcinomas, Calu-3, NCI-H522, and NCI-H1395; and two large cell carcinomas, NCI-H1299 and NCI-H460) were purchased from American Type Culture Collection. All cells were maintained in RPMI 1640 (except Calu-3, which was maintained in Eagle's minimum essential medium), and supplemented with 10% FCS, 2 mmol/L of L-glutamine, penicillin (25 units/mL), and streptomycin (25 µg/mL, all from Sigma-Aldrich) in a humidified atmosphere containing 5% CO₂ at 37°C. Original stock solutions of cisplatin (*cis*-diammine-dichloroplatinum; Pfizer), cetuximab (anti-EGFR antibody; Bristol-Myers Squibb), and 5-aza-2'-deoxycytidine (5-aza-dC; Sigma Chemical Co.) at a concentration of 0.5 mg/mL, 5 mg/mL, and 4 mmol/L, respectively, were stored at 4°C or -20°C (5-aza-dC) and freshly dissolved in culture medium before use.

MiRNA transfection and invasion assay

Cells were transfected with 50 nmol/L of control-miR (scrambled) or pre-miR-200c (Ambion) with RNAiFect Transfection Reagent (Qiagen). Twenty-four hours later, cells were trypsinized, and 1×10^5 cells were plated on transwell chambers (Costar) precoated with 10 µg of Matrigel (BD Biosciences). Medium containing 10% fetal bovine serum in the lower chamber served as a chemo-attractant. After 16 to 18 hours, noninvading cells were removed with cotton swabs. Invading cells were trypsinized and counted using CellTiter-Glo luminescent cell viability assay (Promega). Light microscopy pictures of transfected cells were taken at $\times 100$ magnification.

Cell viability assay

Both scrambled and transfected H1299 cells were plated in 96-well plates for 24 hours and then treated with 10 or 20 µmol/L of cisplatin for 48 hours, 4 or 8 µmol/L of cetuximab for 72 hours, or with drug-free medium. Growth inhibition was evaluated using 10 µL/well of CellTiter 96 AQueous One Solution (Promega). The absorbance at 490 nm was measured using an automated plate reader (Millenia Kinetic Analyzer).

Apoptosis assay

Both scrambled and transfected H1299 cells were seeded in six-well plates at an appropriate density and then treated for 24 hours with 20 µmol/L of cisplatin or 4 µmol/L of cetuximab. Cells were harvested and stained with Annexin V and propidium iodide, and then analyzed on a cyto-fluorimeter by FACScan (BD Biosciences). Propidium-positive cells were considered necrotic, Annexin-positive/propidium-negative cells were considered apoptotic, and the double negative cells were considered alive.

Fluorescence-activated cell sorter analysis

Cells were harvested with 0.5 mmol/L of EDTA 48 hours after transfection and washed twice with ice-cold PBS supplemented with 5% FCS. Cells were then stained with monoclonal anti-human E-cadherin allophycocyanin-conjugated and N-cadherin fluorescein-conjugated antibodies (R&D Systems) and analyzed with a FACSort flow cytometer (BD Biosciences). Data were processed using CellQuest and WinMDI 2.8 software and expressed as histograms of the fluorescence intensity versus cell number.

Chicken embryo metastasis (chorion allantoic membrane) assay

In brief, 2×10^6 cells were placed on the upper chorion allantoic membrane of 10-day-old chicken embryos. Specific pathogen-free eggs were from Charles River Laboratories. Eggs were incubated for 7 days, after which, the amount of lung or liver metastasis was determined by harvesting the organs and processing them using the Puregene DNA purification system (Qiagen). The detection of human DNA by quantitative Alu PCR was done as previously described (16).

5-Aza-dC treatment of cells, bisulfite conversion of DNA, methylation-specific PCR, and bisulfite sequencing

Cells were seeded at a density of 1×10^5 /wells in six-well plates, cultured for 24 hours, and then treated with 0.2 µmol/L or 4 µmol/L of 5-aza-dC for 5 days, replacing drug-containing medium daily, before RNA extraction and miR-200c quantification. Bisulfite conversion of DNA was done by EpiTect Bisulfite Kit (Qiagen) according to the instructions of the manufacturer. Methylation-specific PCR was done as previously described (17). Primers were designed in the CpG island in the 12p13.31 region with MethPrimer software (<http://www.urogene.org/methprimer>) approximately 400 bp upstream of miR-200c. Sequences

were as follows: methylated forward, GAATTTGGGGTTT-TAAAGTTTTTTC; methylated reverse, CACCCTAAATCGCTAATCACG; unmethylated forward, GAATTTGGGGTTTTAAAGTTTTTTC; unmethylated reverse, CACACCCTAAATCACTAATCACAAA. PCR products were detected by electrophoresis on a 1.5% agarose gel with ethidium bromide staining (Sigma). A bisulfite-sequencing PCR was done for the 290-bp-long GC-rich region 59 bp upstream of miR-200c, which includes the predicted transcription start site. Converted DNA was amplified by PCR (primer sequences are shown in Supplementary Fig. S3B) and the products used for pGMT-easy cloning (Promega). Ten clones were sequenced from each cell line using T7 primers.

Preparation of cell lysates: Western blots

Cells were washed with PBS and lysed in extraction buffer (Biosource). Protein concentration was determined by bicinchoninic acid (Pierce). Aliquots (30 µg) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with the E-cadherin (clone G-10) and β-actin (AC-15, Santa Cruz Biotechnology) antibody followed by horseradish peroxidase-linked IgG, and visualized by chemiluminescence (Amersham).

DNA/RNA isolation and cDNA synthesis from cells and fresh snap-frozen NSCLC specimens

DNA isolation and purification from cells was done with DNeasy Blood and Tissue Kit (Qiagen). Total RNA was isolated from cell lines and from lung specimens with Trizol reagent (Invitrogen) according to the instructions of the manufacturer. Expression of mature miRNA-200c was determined by the TaqMan miRNA assay (Applied Biosystems), and normalized using the $2^{-\Delta\Delta C_t}$ method relative to U6-snrRNA (RNU6B). All TaqMan PCRs were done in triplicate with a fluorescence-based, real-time detection method (ABI PRISM 7900 Sequence Detection System; Applied Biosystems).

Patients and samples

Fresh snap-frozen surgical specimens of tumor tissues from 69 patients with NSCLC completely resected between 2005 and 2006 at the San Luigi Hospital, University of Turin were consecutively collected. The main patients' characteristics are reported in Table 1. None of the patients received presurgical chemotherapy/radiation therapy. All cases were reviewed and classified according to the WHO classification by one of the investigators (M. Papotti), using anonymized samples; none of the

Table 1. Mir-200c expression in fresh-frozen tumor tissues from 69 patients with consecutively resected NSCLC according to patient and tumor characteristics

	Total	mir-200c low	mir-200c high	Proportions		P
Sex						
M	51	26	25	51%	49%	ns
F	18	8	10	44%	56%	
Age						
<Median	34	19	15	56%	44%	ns
>Median	35	21	14	60%	40%	
Histotype						
ADC	50	26	24	52%	48%	ns
SQCC	19	8	11	42%	58%	
Grade of differentiation						
Well	11	3	8	27%	73%	0.04
Moderate	28	12	16	43%	57%	
Poor	30	19	11	63%	37%	
pT						
1	14	7	7	50%	50%	ns
2	41	19	22	46%	54%	
3	14	8	6	57%	43%	
pN						
0	41	16	25	39%	61%	<0.01
1	13	5	8	38%	62%	
2	15	13	2	87%	13%	

NOTE: Tumors were divided into "low" and "high" mir-200c expression according to the median level. Significant associations between the patients' clinicopathologic features and miR-200c expression were evaluated by Fisher's exact test.

Abbreviations: ADC, adenocarcinoma; SQCC, squamous cell carcinoma; pT, pathologic tumor classification; pN, pathologic lymph node status; ns, not significant.

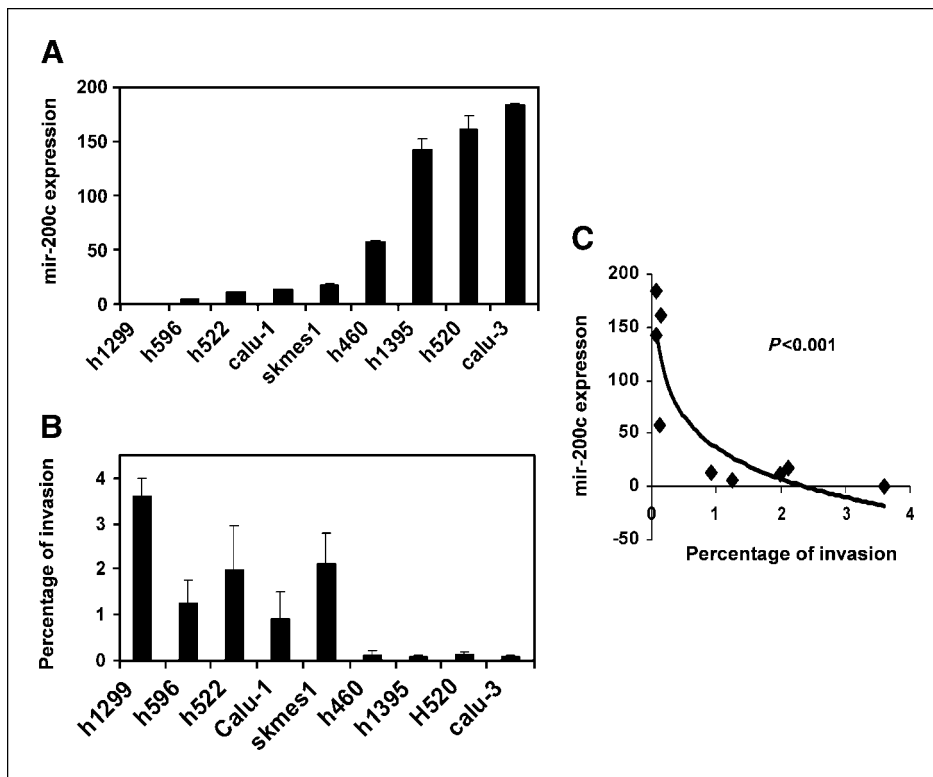


FIGURE 1. Correlation between endogenous miR-200c levels and the invasive ability of NSCLC cell lines. A, relative miR-200c levels in the panel of NSCLC cell lines evaluated by real-time PCR. B, invasive ability of the cells evaluated by Matrigel assays. Data are expressed as the percentage of invading cells compared with noninvading cells. Columns, mean of four replicate estimations; bars, SD. C, significant correlation between the data from A and B (P value calculated by Spearman's correlation).

researchers conducting gene expression and statistical analyses had access to disclosed clinicopathologic data. The study was approved by the Institutional Review Board of the University Hospital.

Immunohistochemistry

Expression levels of E-cadherin protein were detected by using a mouse E-cadherin antibody (36 BS; Novocastra) diluted 1:40, with 40 minutes of incubation at room temperature, followed by anti-mouse horseradish peroxidase-conjugated secondary antibody at room temperature for 30 minutes. Immunoreactions were revealed by a biotin-free, dextran chain detection system (Envision, DakoCytomation), and developed using diaminobenzidine as the chromogen. Before primary antibody incubation, antigen retrieval was done with a Pascal pressure chamber (Dako) heated in an EDTA buffer solution (pH 8; 5 min at 125°C). Slides were counterstained with 10% hematoxylin. For each tumor, both the percentage of expression and the subcellular localization of E-cadherin were evaluated. Twelve out of the 69 consecutive samples (17%) were excluded from immunohistochemistry analysis due to tissue unavailability.

Statistical analysis

In cell line experiments, all data were expressed as mean \pm SD. In Matrigel experiments, data were presented as the percentage of invading cells compared with noninvading cells, whereas the correlation with miR-200 expression levels was estimated by Spearman's rank correlation

method. Differences between groups were calculated with Student's t test which was done using GraphPad software version 5.0. Relative miR-200c levels in tumor tissues were estimated with the Δ Ct method and normalized by subtracting all Δ Ct values by the highest value (the sample with the lowest expression) and converted in a linear scale. miR-200c expression levels were dichotomized into two groups of "high" and "low" expression, according to the median value. Significant associations between the patients' clinicopathologic features and miR-200c expression were evaluated by Fisher's exact test. In all experiments, statistical significance was set at $P < 0.05$.

Results

miR-200c expression correlates with the invasive potential of NSCLC cell lines *in vitro*

The endogenous expression of miR-200c was evaluated by real-time PCR in all nine NSCLC cell lines. The data are reported in Fig. 1A. H1299, H596, and H522 cells had relatively low miR-200c expressions, whereas H520, H1395, and Calu-3 showed higher levels of miR-200c (>150-fold compared with H1299). To test the effect of miR-200c expression on invasive ability, a comparative Matrigel assay was done using different cell lines. The results are plotted in Fig. 1B as the percentage of invading cells after 18 hours. A statistically significant inverse correlation between miR-200c expression and invasiveness of the cells was found ($R_s = 0.9$, $P < 0.001$), and evaluated

with Spearman's rank correlation method. A correlation between miR-200c expression and cell motility was also visible from the morphologic inspection of the cells. As shown in Supplementary Fig. S1A, H1299, H596, and H522 cells were able to grow dissociated from each other *in vitro*, adopting a flat and well-spread morphology, whereas H520, H1395, and Calu-3 were polygonal, tightly joined, and showed the typical cobblestone morphology of epithelial cells. To confirm these morphologic observations, a Western blot analysis was conducted for the epithelial marker E-cadherin and its expression was found to be restricted to the epithelial-like, miR-200c expressing high amounts of H520, H1395, and Calu-3 cells (Supplementary Fig. S1B).

Forced reintroduction of miR-200c into cells induces a loss of the mesenchymal phenotype by restoring E-cadherin and reducing N-cadherin expression

To test the *in vitro* effects of exogenous introduction of miR-200c into invasive cancer cells, H1299, H596, and H522 cells were transfected with scrambled control or with pre-miR-200c. Light microscope pictures taken 48 hours after transfection showed that the cells clearly lost their elongated phenotype, displaying a more round morphology with reduced or absent cytoplasmic extensions (see Fig. 2A). Supplementary Fig. S2A shows light microscope pictures of H1299 cells seeded at a higher density to better show the morphologic changes associated with pre-miR-200c transfection. The efficiency and intensity of transfection was found to be very high and stable up to 72 hours

(see Supplementary Fig. S2B). To directly address the morphologic changes observed with EMT transition, a fluorescence-activated cell sorter analysis of H1299 cells was done 48 hours after transfection, staining the cells with E-cadherin and N-cadherin (mesenchymal marker)-specific antibodies. As a result, a significant increase of E-cadherin (from 8% to 30%) and a strong reduction of N-cadherin (from 92% to 34%) expression were found.

miR-200c inhibits *in vitro* invasion and *in vivo* metastasis

Invasion assays were done on H1299, H596, and H522 cells transfected with pre-miR-200c. The results, as reported in Fig. 3A, showed that all cells significantly reduced their migration ability *in vitro*. To define whether these effects were influenced by an alteration of cell cycle regulation caused by miR-200c, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done 24, 48, and 72 hours after transfection, and the results showed no significant changes in terms of percentage of proliferating cells, as shown in Supplementary Fig. S2C. Then, to determine the *in vivo* relevance of miR-200c in suppressing cancer cell metastasis, a chorion allantoic membrane assay of the chicken embryo was done with H1299 cells transfected with scrambled or pre-miR-200c control cells (see Materials and Methods). The number of H1299 cells that had metastasized into embryonic chicken liver and lungs, plotted in Fig. 3B, was significantly reduced in miR-200c-overexpressing cells.

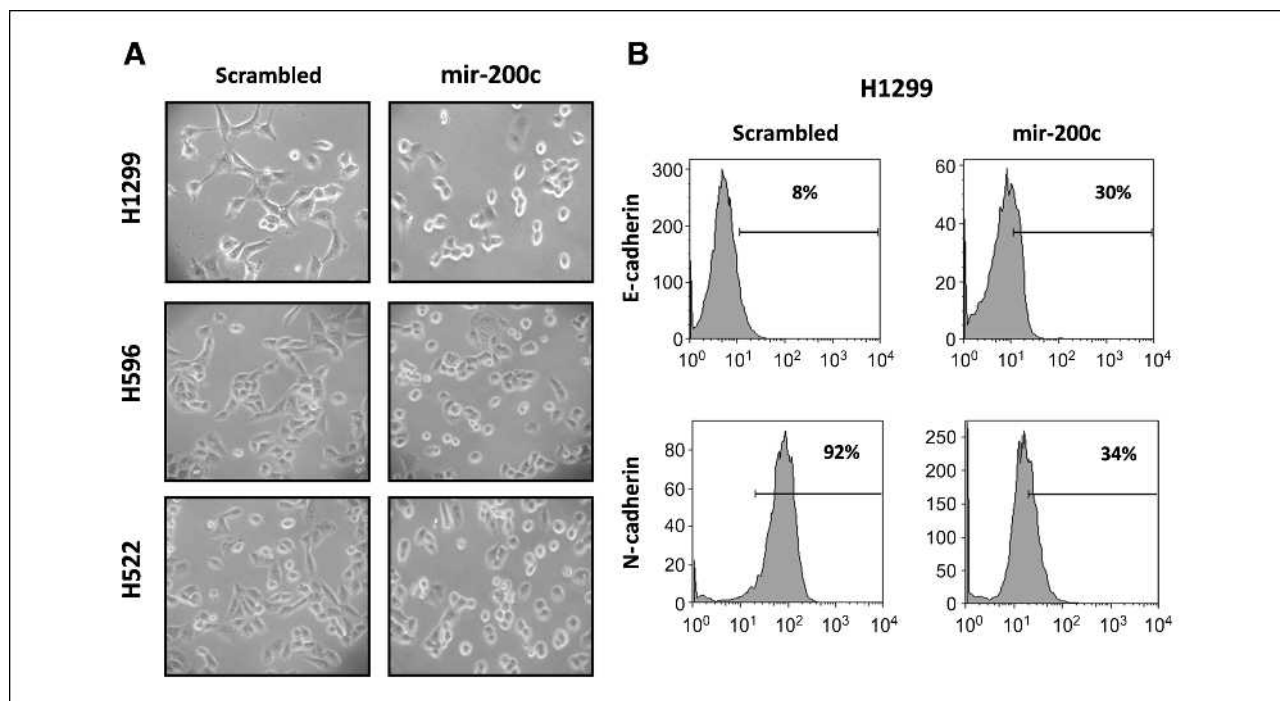


FIGURE 2. Forced re-introduction of miR-200c into invasive NSCLC cells induces a loss of the mesenchymal phenotype. A, H1299, H596, and H522 cells (all with relatively low levels of miR-200c) were transfected with scrambled (control miR) or pre-miR-200c. Light microscope pictures were taken 48 h after transfection (magnification, $\times 100$). B, fluorescence-activated cell sorter analysis of H1299 cells stained with E-cadherin (epithelial marker) and N-cadherin (mesenchymal marker)-specific antibodies 48 h after transfection. Data are expressed as the percentage of positive cells.

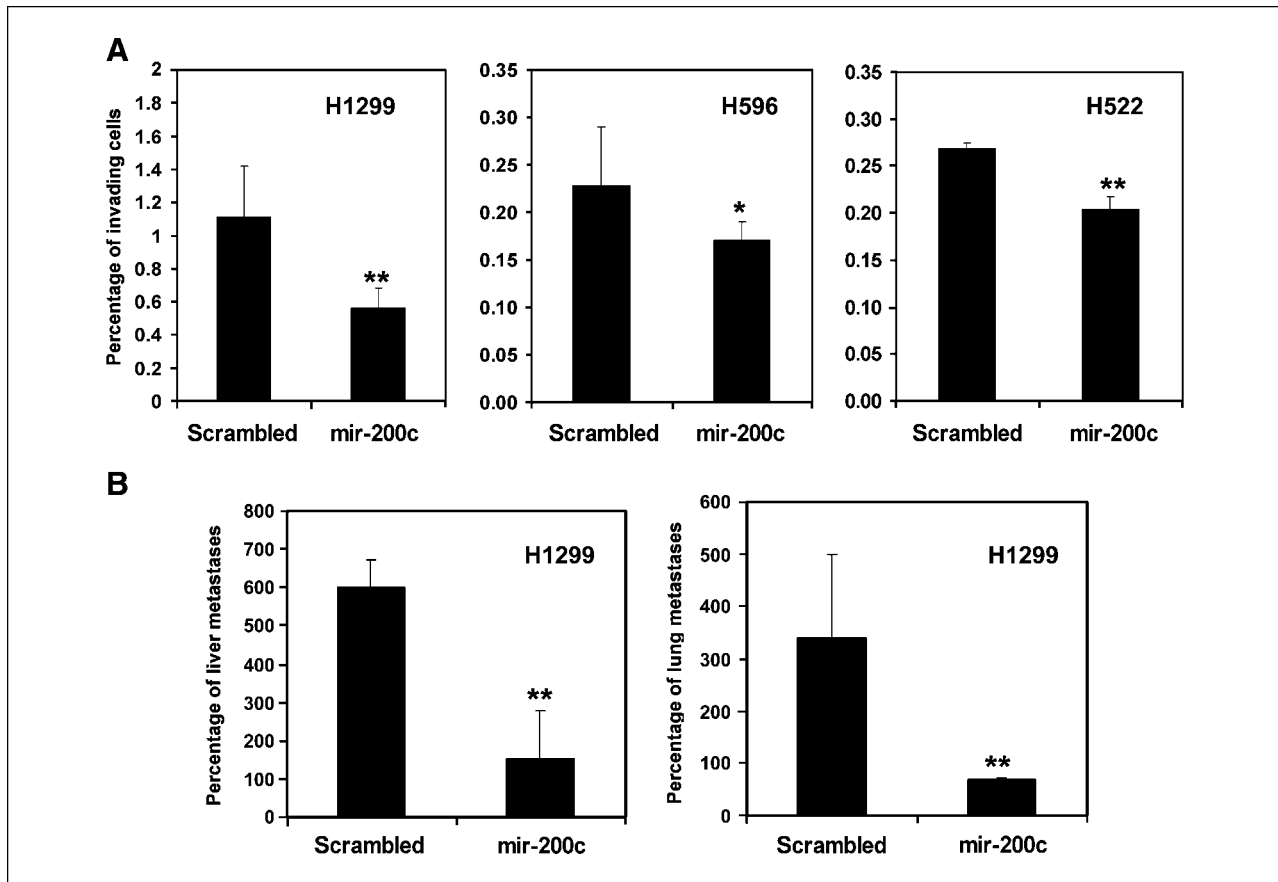


FIGURE 3. Mir-200c mitigates *in vitro* invasion and *in vivo* metastasis formation. A, H1299, H596, and H522 cells transfected with pre-miR-200c showed reduced invasion ability into Matrigel. Data are presented as the percentage of invading cells. Columns, mean of four replicates; bars, SD. B, the chicken embryo metastasis (chorion allantoic membrane) assay was done with H1299 cells transfected with scrambled control or with pre-miR-200c. The number of metastatic cells into the liver (left) and into the lungs (right) are plotted \pm SD of eight replicates (*, $P < 0.05$ and **, $P < 0.01$ according to *t* tests).

miR-200c restores the sensitivity of resistant NSCLC cells to cisplatin and cetuximab

To explore the effect of miR-200c on drug resistance of lung cancer cells, the *in vitro* sensitivity of transfected H1299 cells to cisplatin and cetuximab treatment was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The results, as reported in Fig. 4A and B, showed an increased cytotoxicity of both cisplatin (10 and 20 $\mu\text{mol/L}$) and cetuximab (4 and 8 $\mu\text{mol/L}$) in miR-200c-overexpressing cells, as compared with those transfected with control miRNA (all $P < 0.05$). Annexin V/propidium iodide apoptosis assays confirmed a significantly higher cisplatin-induced cell death in cells transfected with pre-miR-200c, the population of apoptotic cells increasing from 4% to 17% after 24 hours of treatment with 20 $\mu\text{mol/L}$ of cisplatin. Fewer changes, in terms of apoptotic rate, were observed with cetuximab treatment (Fig. 4C).

Loss of miR-200c in invasive NSCLC cells is due to the hypermethylation of its promoter region

To understand whether the mechanisms behind the lack of miR-200c expression in invasive NSCLC cell lines was

attributable to the hypermethylation of its promoter region, four cell lines (H1299, Calu-1, H520, and H1395) were exposed to 5-aza-dC demethylating agent, and the expression of miR-200c was evaluated after 5 days of continuous treatment. As a result, a strong increase in miR-200c expression was found in H1299 and Calu-1 cells (both with low expression and high invasive ability), whereas no changes were found in H520 and H1395 cells (see Fig. 5A). Bisulfite sequence analysis of miR-200c promoter methylation confirmed that invasive H1299 and Calu-1 cells were totally or widely methylated in the predicted CpG sites ($n = 15$), whereas absent or poor methylation was observed in less invasive H1395 and Calu-3 cells, respectively (Fig. 5B). Then, DNA was isolated from eight cell lines (four with low and four with high invasive potential) and was subjected to bisulfite conversion and subsequent methylation-specific PCR, with “methylated” and “unmethylated” pairs of primers designed in the CpG island promoter region of miR-200c. Supplementary Fig. S3A shows the PCR products on an agarose gel. As expected, in H1299, Calu-1, and in all the other cells with lower levels of miR-200c, a “methylated” band was consistently observed, indicating the total or partial silencing of

this miRNA by promoter methylation in these cells with higher motility, although among the cells with higher expression, no methylation or poor methylation was found.

Lower miR-200c expression in patients with NSCLC is significantly associated with a poor grade of differentiation, a higher degree of spread to lymph nodes, and with a lower E-cadherin expression

The level of expression of miR-200c was quantified in the tissues of 69 patients with consecutively resected NSCLC. miR-200c expression was detected in all tumor tissues, normalized, and converted into a linear scale (see Materials and Methods). Tumors were divided into two groups of “low” and “high” expression according to the median level, and Fisher's exact tests were done to compare miR-200c expression levels with the patients' clinicopathologic features. All the results are reported in Table 1. Among all the information available, a significant association was found between low miR-200c expression and a poor grade of differentiation and higher degree of spread to lymph nodes (pN-stage). In fact, although 8 out of 11

(73%) patients with a well-differentiated tumor belonged to the “high” expression group, this percentage decreased to 57% and 37% in patients with moderate and poor grades of differentiation, respectively ($P = 0.04$). Similarly, although among tumors with pathologic lymph node status 0 (tumor cells absent from regional lymph nodes, pN0) or 1 (spread to closest or to a small number of regional lymph nodes, pN1), 61% and 62% had a “high” expression of miR-200c, the proportions significantly decreased to 13% for those patients with pN2 (indicating that tumor cells spread to numerous regional or more distant lymph nodes; $P < 0.01$).

To investigate the potential correlation between miR-200c and E-cadherin levels, an immunohistochemical analysis of E-cadherin was done in 57 samples. As a result, 24 tumors (42%) showed no staining and 6 (10%) were found with a cytoplasmic pattern of expression, and were grouped together as “negative” tumors ($n = 30$), whereas 27 cases (47%) were found positive with a membrane-staining pattern. The median percentage of positive cells within the group of positive cases was 70% ($\pm 19\%$; range,

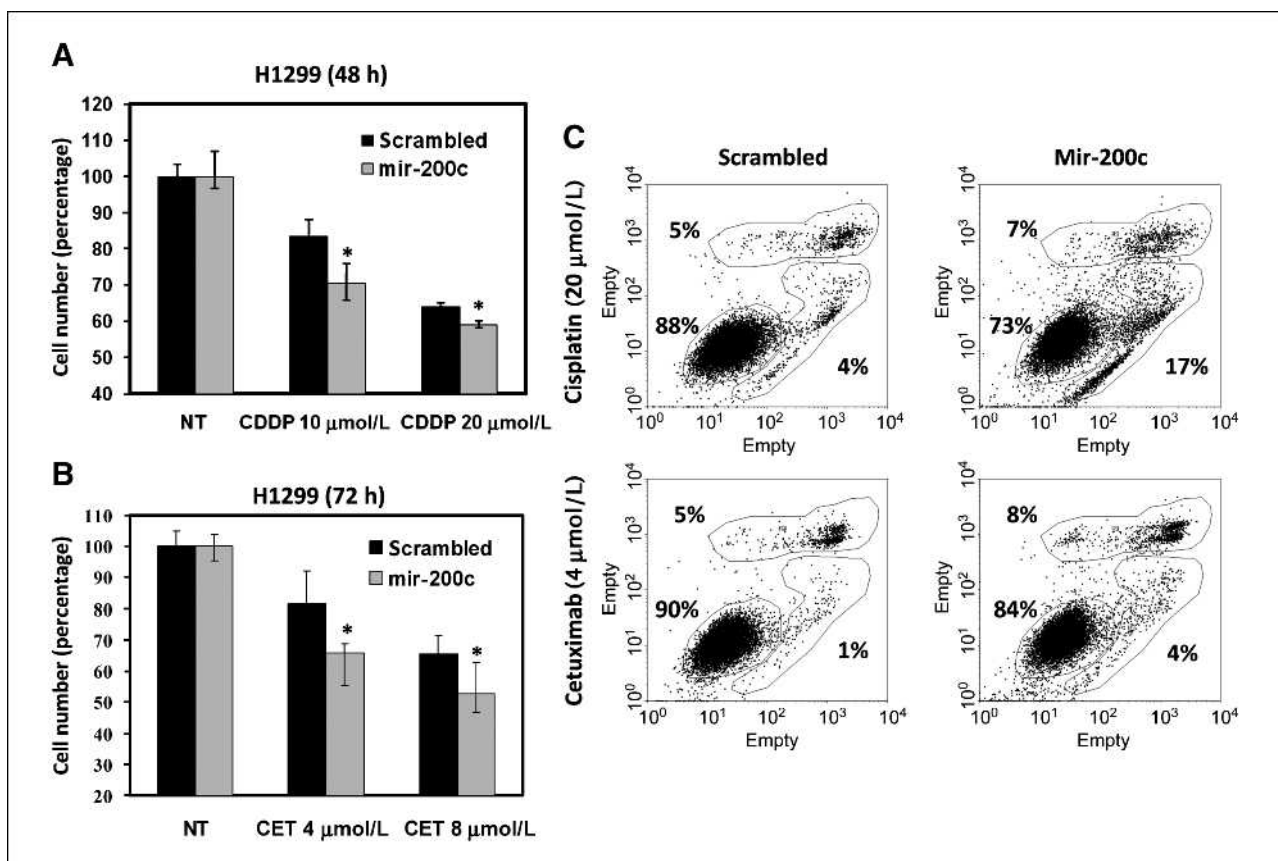


FIGURE 4. Mir-200c restores cisplatin and cetuximab sensitivity in H1299 cells. Sensitivity of H1299 cells transfected with scrambled control or pre-mir-200c to (A) cisplatin (10 and 20 $\mu\text{mol/L}$) and (B) cetuximab (4 and 8 $\mu\text{mol/L}$) treatment for 48 and 72 h, respectively, as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are presented as the percentage of proliferating cells as compared with untreated cells (NT, treated with medium only). Columns, mean of six replicates; bars, SD (*, $P < 0.05$, according to t tests). C, apoptosis assay of H1299-transfected cells treated with cisplatin and cetuximab at the indicated doses for 24 h. Percentages of living (bottom left), apoptotic (right), and dead (top right) cells.

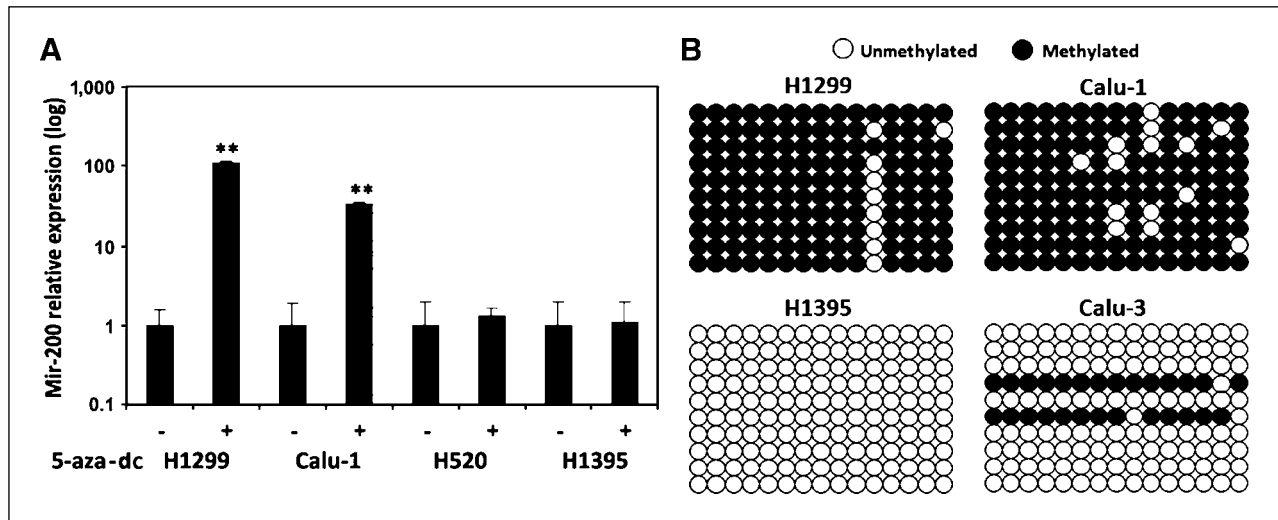


FIGURE 5. The promoter of miR-200c is methylated in NSCLC cell lines with invasive ability. A, H1299, Calu-1, H520, and H1395 cells were treated with 5-aza-dC for 5 d and the expression of miR-200c was evaluated by real-time PCR. Data are presented as relative expression levels in a logarithmic scale (**, $P < 0.01$ according to t tests). B, bisulfite sequence analysis of miR-200 promoter methylation in H1299, Calu-1, H1395, and Calu-3 cells. MiR-200 promoter amplicons obtained from all samples were subcloned and 10 clones each were analyzed with the use of bisulfite sequencing. For each clone, the methylation status of analyzed CpG sites is shown (●, methylation; ○, no methylation).

20-100%). A significant association was found between high expression of miR-200c (according to the median level) and a positive E-cadherin staining ($P = 0.01$, see Supplementary Fig. S4).

Discussion

An overwhelming amount of molecular data has recently been generated regarding the effect of regulation of gene expression by miRNAs in cancer, covering all the aspects of tumorigenesis, including tumor proliferation, migration, angiogenesis, and more recently, EMT (18, 19). EMT, originally described by embryologists in many developmental processes, plays a crucial role in metastasis formation, where cancer cells acquire their invasive phenotype by undergoing a change to a more dedifferentiated state (20). The miR-200 family has recently been shown to prevent EMT by suppressing the expression of ZEB1 and ZEB2, two transcriptional repressors of E-cadherin (8, 10). miR-200c downregulation has been shown to regulate stem cell functions such as self-renewal, proliferation, and EMT by targeting the expression of BMI1, while, on the other hand, its overexpression inhibits the clonal expansion of breast cancer and suppresses the *in vitro* growth of embryonal carcinoma cells (21).

We tested the role of miR-200c expression in NSCLC, a tumor type with high metastatic potential. In the present article, we showed a highly significant inverse correlation between relative miR-200c expression and the invasive ability of a panel of NSCLC cells, indicating that its expression is a critical determinant for the establishment of a more aggressive phenotype. The correlation between expression levels and cell motility was also evident from the simple

morphologic inspection of the cells and from the quantification of E-cadherin protein levels. Furthermore, to explore its functional relevance in NSCLC, miR-200c was transiently overexpressed in highly invasive/aggressive cells. As a result, we found that miR-200c was able to significantly reduce *in vitro* invasion ability, as evaluated by Matrigel assay, and *in vivo* metastasis formation, as evaluated by chorioallantoic membrane assay. Transfected cells displayed an altered morphology, losing their elongated phenotype. To correlate these morphologic changes with EMT transition, fluorescence-activated cell sorter analysis was done and showed an increase of E-cadherin and the simultaneous reduction of N-cadherin expression.

Resistance to chemotherapeutic agents represents a major problem for the treatment of patients with NSCLC. Despite several critical biomarkers of drug resistance that have been identified in the last decade (22), additional knowledge on the molecular determinants of chemoresistance is a research priority to improve the effectiveness of anticancer agents in this deleterious tumor disease. Interestingly, miR-200c transfection restored the sensitivity of two therapeutic agents currently used in the treatment of NSCLC, the alkylating agent cisplatin and the EGFR antagonist cetuximab, in chemoresistant H1299 cells (23, 24). EMT and related molecules have already been shown to be involved in the development of resistance to radiotherapy and chemotherapy (25, 26), and E-cadherin expression has been proposed as a novel biomarker predicting the clinical activity of the EGFR inhibitor erlotinib in patients with NSCLC (27). The overexpression of miR-200c increases the sensitivity of bladder cancer to EGFR-inhibiting agents (13) and restores the sensitivity of several types of cancer to antimicrotubule drugs (15). According

to our present observations, in cetuximab-treated cells, the sensitizing effect of miR-200c transfection seems to be related to the blockage of cell proliferation rather than to the induction of cancer cell death, whereas a relevant impact on the cytotoxicity induced by cisplatin was observed both in proliferation and apoptosis assays. Because cisplatin-based chemotherapy represents the standard of care for most cases of NSCLC, our data supports further translational efforts to explore the predictive effect of miR-200c expression. Moreover, novel determinants of cisplatin sensitivity (such as DNA repair enzymes or proteins with signaling transduction activity) specifically targeted by miR-200c could be potentially identified in future studies.

New lines of evidence indicate that epigenetic silencing of miRNAs with tumor suppressor features is a common hallmark of tumors and contributes to the development of metastasis (28). Therefore, to determine the possible causes explaining the lower expression of miR-200c in aggressive NSCLC, we hypothesized a potential regulation by DNA methylation. Treatment with the demethylating agent 5-aza-dC and methylation-specific PCR analysis, confirmed by bisulfite sequencing, revealed that the miR-200c promoter region was hypermethylated in cells with higher invasive capacity. The transcriptional repression of miR-200c and miR-141 in invasive cancer cells has already been shown to be mediated by one of their known target genes, ZEB1, in a feedback loop of regulation (7). However, the data in the present study, together with those recently reported in normal and cancer cells (29), indicate that promoter methylation is an additional mechanism regulating the expression of miR-200c, a critical miRNA that controls epithelial differentiation and invasion. It is noteworthy that the presence of the miR-200c gene in a chromosomal region (12p12) frequently altered in lung cancer (30) could represent an additional mechanism of inactivation.

To confirm some of the observations obtained in cell line experiments, an expression analysis was done in primary tumor specimens from a case-series of patients with consecutive NSCLC. Correlation analysis with the patients' main clinicopathologic features showed that the loss of miR-200c was significantly associated with a poor grade of differentiation ($P = 0.04$), and that the frequency of lymph node metastases in tumors with impaired expression of miR-200c was significantly higher ($P < 0.01$). Moreover, a significant association with E-cadherin protein expression

($P = 0.01$), as evaluated by immunohistochemistry, was observed. This is in line with previous reports which showed an association between the expression of EMT molecules and the tumor's grade of differentiation and/or with the extent of lymph node involvement (31, 32). These results contribute to further indicate the central role of miR-200c in the regulation of the epithelial-versus-mesenchymal phenotype and in the malignant progression of lung cancer cells. In light of these data, future prospective investigations should be undertaken to determine the efficacy of miR-200c expression as a predictor for lymph nodes or distant metastasis, and/or as a prognostic factor for patients with resected NSCLC. Moreover, translational efforts investigating the clinical effect of miR-200c methylation analysis in lung and other cancer tissues are clearly warranted.

In conclusion, all these findings highlight the pivotal role of miR-200c in various aspects of NSCLC progression. Translated into clinical practice, the determination of its expression in tumors could be used, for example, for the stratification of patients into different prognostic groups or for treatment with antimetastatic therapies, cisplatin, or cetuximab. According to the emerging concept of cancer stem cells, which are hypothesized to be responsible for the formation, growth, and metastasis of neoplastic tissue and are naturally resistant to chemotherapy (33), the present data hypothesizes a critical function of miR-200c, the suppression of which initiates an aggressive/dedifferentiated, invasive, metastatic, and chemoresistant phenotype of NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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