Loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype in non-small cell lung cancer.

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Running title: Mir-200c expression in NSCLC.
Key-words: non-small cell lung cancer; microRNA-200c, epithelial-to-mesenchymal transition; invasion.

ABSTRACT

Purpose: The development of metastases is the main reason for cancer-related death in non-small cell lung cancer (NSCLC). 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 (EMT). A role for microRNA-200 family members in regulating EMT has been recently indicated, but data about their expression in lung tumors are still unavailable.

Experimental design and results: 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. Re-introduction 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 to cisplatin and cetuximab of NCI-H1299 cells. Hyper-methylation of the promoter region was found to be responsible for the loss of miR-200c in invasive cells, as evaluated by 5-aza-dC treatment, methylation-specific PCR and bisulfite sequencing. In primary tumor specimens obtained on June 25, 2017. © 2010 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from mcr.aacrjournals.org on June 25, 2017. © 2010 American Association for Cancer Research.
from 69 consecutively resected NSCLC patients, lower miR-200c expression levels were found 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).

**Conclusion:** These data indicate that the loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype, and that assessment of its expression can contribute to a better clinico-pathological definition of NSCLC patients.

**INTRODUCTION**

Non-small cell lung cancer (NSCLC) is the first cause of cancer mortality in both sexes, accounting for 1.2 million deaths each year (1). Despite therapeutic advances, the high mortality of NSCLC patients 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, improved understanding of the molecular mechanisms which regulate metastatic transformation of the cancer cells are urgently needed. Tumor metastasis involves a sequential series of events which promote and regulate the migration of cancer cells to generate metastases at distant sites. The process initiates 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, like 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 messenger RNA targets via direct base-pairing interactions (6). Recent evidences demonstrate that microRNA-200 family members play a central role in the process of EMT, promoting the up-regulation of E-cadherin by direct 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 members miR-200c has been shown to be the only one to be regulate ZEB1 and to restore E-cadherin expression (11, 12). In first 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 over-expression of miR-200c increased the sensitivity to EGFR-blocking agents in bladder cancer (13), to doxorubicin in breast cancer cells (14) and in endometrial, breast, and ovarian cancer cell lines to microtubule-binding chemotherapeutic agents (15). However, an ability of miR-200c to suppress metastatic step in vivo, molecular mechanisms leading to a suppression of miR-200c in cancer cells, and a specific role of miR-200c in NSCLC has never been explored. Therefore we specifically investigated 1) 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 2) the possible mechanisms regulating its expression and, 3) the possible associations between miR-200c levels and the clinical-pathological features of NSCLC patients.
MATERIAL AND METHODS

Cell lines, cultures and drugs. Nine human NSCLC cell lines (three squamous cell carcinoma cell lines, Calu-1, NCI-H520, and SK-MES-1, one adeno-squamous, H596, three adenocarcinoma, Calu-3, NCI-H522 and NCI-H1395, and 2 large-cell carcinomas, NCI-H1299 and NCI-H460) were purchased from American Type Culture Collection (Manassas, VA). All cells were maintained in RPMI-1640, except Calu-3 maintained in Eagle’s Minimum Essential Medium, and supplemented with 10% FCS, 2mM 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., St. Louis, MD) at a concentration of 0.5 mg/ml, 5 mg/ml and 4mM, respectively, were stored at 4°C or -20°C (5-aza-dC) and freshly dissolved in culture medium before use.

MicroRNA transfection and invasion assay. Cells were transfected with 50 nM control-miR (scrambled) or pre-miR-200c (Ambion) with RNAiFect Transfection Reagent (Qiagen). Twenty-four hours later cells were trypsinized, and 1X10⁵ plated on transwell chambers (Costar) precoated with 10µg Matrigel (BD Biosciences). Medium containing 10% FBS in the lower chamber served as chemo-attractant. After 16/18 hours, non-invading 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 100x magnification.
Cell viability assay. Both scrambled and transfected H1299 cells were plated in 96-well plates for 24 hours and then treated with cisplatin 10 or 20μM for 48 hours, cetuximab 4 or 8μM for 72 hours, or with drug-free medium. The growth inhibition was evaluated using 10 μL/well of Cell-Titer 96 AQueous One Solution (Promega/WI/USA). The absorbance at 490nm was measured using an automated plate reader (Milenia-Kinetic-Analyzer).

Apoptosis assay. Both scrambled and transfected H1299 cells were seeded in 6-well plates at appropriate density and then treated for 24 hours with 20μM cisplatin or 4μM cetuximab. Cells were harvested and stained with Annexin V and Propidium iodide and then analyzed on a cytofluorimeter by FACS can (BD Biosciences, San Jose, CA). Propidium positive cells were considered as necrotic, Annexin positive/Propidium negative as apoptotic and the double negative as alive.

Fluorescence-activated cell sorter (FACS) analysis. Cells were harvested with 0.5mM EDTA 48 hours after transfection and washed twice with ice-cold PBS buffer supplemented with 5% FCS. Cells were then stained with monoclonal anti-human E-cadherin allophycocyanin (APC)-conjugated and N-Cadherin fluorescein-conjugated (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 (CAM) assay. In brief, 2x10^6 cells were placed on the upper chorion-allantoic-membrane (CAM) of 10-day-old chicken embryos. Specific pathogen-free eggs were from Charles River Laboratories, Wilmington, MA. Eggs were incubated for
other 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 performed as previously described (16).

5-Aza-2'-Deoxycytidine (5-aza-dC) treatment of cells, bisulfite conversion of DNA, methylation specific PCR and bisulfite sequencing. Cells were seeded at a density of 1x10^5/wells in 6-well plates, cultured for 24 hours, and then treated with 0.2μM, or 4μM of 5-aza-dC for 5 days, replacing drug-containing medium daily, before RNA extraction and miR-200c quantification. Bisulfite conversion of DNA was performed by EpiTect Bisulfite Kit (Qiagen) according to manufacturer’s instructions. Methylation specific PCR was performed as previously described (17). Primers were designed in the CpG island in the 12p13.31 with Methprimer software (http://www.urogene.org/methprimer) approximately 400 bp upstream of miR-200c. Sequences were as follows: Methylated forward: GAATTTGGGGTTTTAAAGTTTTTC; methylated reverse: CACCCTAAATCGCTAATCACG; unmethylated forward: GAATTTGGGGTTTTAAAGTTTTTT; unmethylated reverse: CACCCTAAATCGCTAATCACAAA. PCR products were detected by electrophoresis on a 1.5% agarose gel with ethidium bromide staining (Sigma). A bisulfite sequencing PCR was performed for the 290 bp-length, GC-rich, region 59 bp upstream to the miR200c, which includes the predicted transcription start site. Converted DNA was amplified by PCR (primer sequences are shown in Supplementary figure 3b) 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 phosphate-buffered saline and lysed in extraction-buffer (Biosource, Camarillo, CA, USA). Protein concentration was determined by BCA (Pierce, Rockford, IL, USA). 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, Santa Cruz, CA) antibody followed by horseradish–peroxidase-linked immunoglobulin G, and visualized by chemiluminescence (ECL, Amersham, Germany).

**DNA/RNA isolation and cDNA synthesis from cells and fresh snap-frozen NSCLC specimens.** DNA isolation and purification from cells was performed with DNeasy Blood & Tissue Kit (Qiagen). Total RNA was isolated from cell lines and from lung specimens with Trizol reagent (Invitrogen) according to manufacturer’s instructions. Expression of mature miRNA 200c was determined by the TaqMan miRNA-assay (Applied Biosystems, Foster City, CA, USA), and normalized using the 2−ΔΔCt method relative to U6-snRNA (RNU6B). All TaqMan-PCRs were performed in triplicates 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 of 69 NSCLC patients 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 pre-surgical...
chemo/radiation therapy. All cases were reviewed and classified according to the WHO classification by one of the investigators (MP), using anonymized samples; none of the researchers conducting gene expression and statistical analyses had access to disclosed clinical-pathological data. The study was approved by the institutional review board of the University Hospital.

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

**Statistical analysis.** In cell lines experiments, all data here expressed as mean ± standard deviation. In Matrigel experiments, data were represented as the percentage of invaded cells compared to non-invading cells, while the correlation with miR-200 expression levels was estimated by the Spearman's rank correlation method. Differences between groups were calculated with the Student's t-test, performed using GraphPad.
software version 5.0. Relative miR-200c levels in tumor tissues were estimated with the ΔCt method and normalized by subtracting all ΔCt 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’ clinical-pathological features and miR-200c expression were evaluated by the Fisher’s exact test. In all experiments, statistical significance was set at p<.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 the nine NSCLC cell lines. The data are reported in figure 1A. H1299, H596 and H522 were the cells with relatively low miR-200c expression, while H520, H1395 and Calu-3 showed higher levels of miR-200c (>150 fold compared to H1299). To test the impact of miR-200c expression on the invasive ability, a comparative Matrigel assay was performed with the different cell lines. The results are plotted in figure 1B as the percentage of invading cells after 18 hours. A statistically significant inverse correlation between miR-200c expression and the invasiveness of the cells was found (Rₛ=0.9, p<0.001), which was evaluated by the Spearman’s rank correlation method. A correlation between miR-200c expression and cell motility was also visible from the morphological inspection of the cells. As shown in the supplementary figure 1A, H1299, H596 and H522 cells were able to grow in vitro dissociated from each other adopting a flat and well spread morphology, while
H520, H1395 and Calu-3 were polygonal, tightly-joined, and showing the typical cobblestone morphology of epithelial cells. To confirm these morphological 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, highly expressing miR-200c expressing H520, H1395 and Calu-3 cells (supplementary figure 1B).

**Forced re-introduction 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 figure 2A). Supplementary figure 2A shows light microscope pictures of H1299 cells seeded at a higher density to better show the morphological 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 figure 2B). To directly address the morphological changes observed with the mesenchymal to epithelial transition, a FACS analysis of H1299 cells was performed 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 (92 to 34%) expression was found.
miR-200c inhibits *in vitro* invasion and *in vivo* metastasis. Invasion assays were performed on H1299, H596 and H522 cells transfected with pre-miR-200c. The results, as reported in figure 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, an MTT assay was performed 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 figure 2C. Then, in order to determine the *in vivo* relevance of miR-200c in suppressing cancer cell metastasis, a CAM assay of the chicken embryo was performed with H1299 cells transfected with scrambled or with pre-miR-200c (see methods). The number of H1299 cells that had metastasized into embryonic chicken liver and lungs, plotted in figure 3B, were significantly reduced in miR-200c over-expressing cells.

miR-200c restores the sensitivity of resistant NSCLC cells to cisplatin and cetuximab. In order to explore the impact 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 MTT assays. The results, as reported in figure 4A and 4B, showed an increased cytotoxicity of both cisplatin (10 and 20µM) and cetuximab (4 and 8µM) in miR-200c overexpressing cells, as compared to those transfected with control miRNA (all p-values <.05). Annexin V/Propidium iodide apoptosis assays confirmed a significantly higher cisplatin-induced cell death in the cells transfected with pre-miR-200c, the population of apoptotic cells being increased from 4 to 17% after 24 hours of treatment.
with cisplatin 20µM. Less changes were observed with cetuximab treatment in terms of apoptotic rate (figure 4C).

**Loss of miR-200c in invasive NSCLC cells is due to the hyper-methylation 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 hyper-methylation of its promoter region, four cell lines (H1299, Calu-1, H520 and H1395) were exposed to 5-aza-dC de-methylating agent, and the expression of miR-200c evaluated after 5 days of continuous treatment. As a result, a strong increase in miR-200c expression was found in H1299 and in Calu-1 cells (both with low expression and high invasive ability), while no changes were found in H520 and H1395 cells (see figure 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), while absent or poor methylation was observed in less invasive H1395 and Calu-3 cells (2 positive clones out of 10), respectively (figure 5B). Then, DNA was isolated from eight cell lines (4 with low and 4 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 figure 3A 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, while among the cells with higher expression none or poor methylation was found.
Lower miR-200c expression in NSCLC patients 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 tissues of 69 consecutively resected NSCLC patients. miR-200c expression was detected in all tumor tissues, normalized and converted into a linear scale (see methods). Tumors were divided into two groups of “low” and “high” expression, according to the median level, and Fisher’s exact tests were performed to compare miR-200c expression levels with the patients’ clinical-pathological 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, while 8 out of 11 (73%) of the patients with a well-differentiated tumor belonged to the group of “high” expression, this percentage decreased to 57% and 37% in patients with moderate and poor grade of differentiation, respectively (p=0.04). Similarly, while 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 tumor cells spread to numerous regional or most distant lymph nodes, p<0.01).

In order to investigate potential correlation between miR-200c and E-Cadherin levels, an immunohistochemical analysis of E-cadherin was performed in 57 samples. As a result, 24 tumors (42%) showed no staining and 6 (10%) were found with a cytoplasmatic pattern of expression, and were grouped together as “negative” tumors (n=30), while 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% (±19%, range 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 4).

DISCUSSION

An overwhelming amount of molecular data have been generated in the last years regarding the impact of regulation of gene expression by micro-RNAs in cancer, covering all the aspects of tumorigenesis, including tumor proliferation, migration, angiogenesis and, more recently, epithelial-to-mesenchymal transition (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 been recently 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 paper, we demonstrated 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 morphological 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 \textit{in vitro} invasion ability, as evaluated by Matrigel assay, and \textit{in vivo} metastasis formation, as evaluated by CAM assay. Transfected cells displayed an altered morphology, losing their elongated phenotype. In order to correlate these morphological changes with the mesenchymal to epithelial transition, FACS analysis was performed and showed the 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 chemo-resistance is a research priority to improve the effectiveness of anticancer agents in this deleterious tumor disease. Interestingly, miR-200c transfection restored the sensitivity to two therapeutic agents currently used in the treatment of NSCLC, the alkylating agent cisplatin and the EGFR antagonist cetuximab, in chemo-resistant H1299 cells (23, 24). EMT and related molecules have already been shown to be involved in the development of resistance to radio- and chemotherapy (25, 26) and E-cadherin expression has been proposed as a novel biomarker predicting clinical activity of the EGFR inhibitor erlotinib in NSCLC patients (27). The over-expression of miR-200c increases the
sensitivity to EGFR-inhibiting agents of bladder cancer (13), and restores the sensitivity to anti microtubule drugs in several types of cancer (15). According to our present observations, in cetuximab-treated cells, the sensitizing effects of miR-200c transfection seems to be more related to the block of cell proliferation, rather than to the induction of cancer cell death, while a relevant impact on the cytotoxicity induced by cisplatin was observed both in proliferation and apoptosis assays. Since cisplatin-based chemotherapy represents the standard of care for most of the cases of NSCLC, our data support further translational efforts to explore the predictive impact 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, in order 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 de-methylating agent 5-aza-dC and methylation-specific PCR analysis, confirmed by bisulfite sequencing, revealed that the miR-200c promoter region was hyper-methylated in the 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 gene, ZEB1, in a feedback loop of regulation (7). However, the data of 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 microRNA that controls epithelial differentiation and invasion. Noteworthy, the presence of mir-200c gene in a chromosomal region (12p12) frequently altered in lung cancer (30) could represent an additional mechanism of inactivation.

In order to confirm some of the observations obtained in cell line experiments, an expression analysis was performed in primary tumor specimens from a case-series of consecutive NSCLC patients. Correlation analysis with the patients' main clinical-pathological 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 resected NSCLC patients. Moreover, translational efforts investigating the clinical impact of miR-200c methylation analysis in lung and other cancer tissues are clearly warranted.
In conclusion, all these findings highlight a pivotal role of miR-200c in various aspects of NSCLC progression. Translating into clinical practice, the determination of its expression in tumors could be used, for example, for the stratification of the patients into different prognostic groups, or for the treatment with anti-metastatic therapies, cisplatin or cetuximab. According to the emerging concept of cancer stem cell, 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 hypothesize a critical function of miR-200c, whose suppression initiates an aggressive/dedifferentiated, invasive, metastatic, and chemoresistant phenotype of NSCLC.

ACKNOWLEDGMENTS

Work concerning Cetuximab of this paper was supported by a grant given from Merck KGaA, Darmstadt, Germany. HA was supported by Alfried Krupp von Bohlen und Halbach Foundation (Award for Young Full Professors), Essen, Hella-Bühler-Foundation, Heidelberg, Dr. Ingrid zu Solms Foundation, Frankfurt/Main, the Hector Foundation, Weinheim, Germany, the FRONTIER Excellence Initiative of the University of Heidelberg, and the Walter Schulz Foundation, Munich, Germany.
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FIGURES AND TABLES LEGEND

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 invaded cells as compared to non-invading cells. All data are represented as mean ± SD of four replicate estimations. C) Significant correlation between the data from A) and B) (p-value calculated by Spearman’s correlation).

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 at 100x magnification were taken 48 hours after transfection. B) FACS analysis of H1299 cells stained with E-cadherin (epithelial marker) and N-cadherin (mesenchymal marker) specific antibodies 48 hours 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 represented as the percentage of invading cells, as mean ± SD of four replicates. B) The chicken embryo metastasis (CAM) assay was performed 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 ± SD of eight replicates. * are t-tests with p<.05 and ** <.01.

**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 20µM) and B) cetuximab (4 and 8µM) treatment for 48 and 72 hours, respectively, as evaluated by MTT assay. Data are represented as the percentage of proliferating cells as compared to untreated cells (NT, treated with medium only), mean ± SD of six replicates. * t-tests with p<.05. C) Apoptosis assay of H1299 transfected cells treated with cisplatin and cetuximab at indicated doses for 24 hours. Percentage of living (lower left), apoptotic (right) and dead (upper right) cells are displayed.

**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 (5-aza) for 5 days and the expression of mir-200c was evaluated by Real Time PCR. Data are represented as relative expression levels in a logarithmic scale; ** t-tests with p<.01. 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 the samples were subcloned and each 10 clones analyzed by the use of bisulfite sequencing. For each clone the methylation status of analyzed CpG sites is shown (solid circles: methylation, open circles: no methylation).

**Table 1. Mir-200c expression in fresh-frozen tumor tissues from 69 consecutively resected NSCLC patients according to patient and tumor characteristics.**
were divided into “low” and “high” mir-200c expression according to the median level. Significant associations between the patients' clinical-pathologic features and miR-200c expression were evaluated by the Fisher's exact test. ADC=adenocarcinoma. SQCC=squamous cell carcinoma. Diff=differentiation. pT= Pathologic tumor classification. pN= Pathologic lymph node status.
Figure 1

a) mir-200c expression

b) Percentage of invasion

c) p<0.001
Figure 2

(a) scrambled vs. mir-200c in H1299, H596, and H522 cell lines.

(b) Flow cytometry analysis of E-Cadherin and N-Cadherin expression in H1299 cells under scrambled and mir-200c conditions.

8% vs. 30% for E-Cadherin
92% vs. 34% for N-Cadherin
Figure 4

(a) H1299 (48 h)
- scrambled
- mir200c

Cell number (percentage)

NT CDDP 10 μM CDDP 20 μM

(b) H1299 (72 h)
- scrambled
- mir200c

Cell number (percentage)

(c) Scrambled Mir-200c

- Cisplatin 20 μM
  5% 88% 4% 73%
  7% 17%

- Cetuximab 4 μM
  5% 90% 1% 84%
  8% 4%
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Loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype in non-small cell lung cancer.

Paolo Ceppi, Giridhar Mudduluru, Regalla Kumarswamy, et al.

Mol Cancer Res  Published OnlineFirst August 9, 2010.

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