The microRNA-200 family targets multiple non-small cell lung cancer prognostic markers in H1299 cells and BEAS-2B cells

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Abstract. Lung cancer remains the leading cause of cancer-related mortality for both men and women. Tumor recurrence and metastasis is the major cause of lung cancer treatment failure and death. The microRNA-200 (miR-200) family is a powerful regulator of the epithelial-mesenchymal transition (EMT) process, which is essential in tumor metastasis. Nevertheless, miR-200 family target genes that promote metastasis in non-small cell lung cancer (NSCLC) remain largely unknown. Here, we sought to investigate whether the microRNA-200 family regulates our previously identified NSCLC prognostic marker genes associated with metastasis, as potential molecular targets. Novel miRNA targets were predicted using bioinformatics tools based on correlation analyses of miRNA and mRNA expression in 57 squamous cell lung cancer tumor samples. The predicted target genes were validated with quantitative RT-PCR assays and western blot analysis following re-expression of miR-200a, -200b and -200c in the metastatic NSCLC H1299 cell line. The results show that restoring miR-200a or miR-200c in H1299 cells induces downregulation of DLC1, ATRX and HFE. Reinforced miR-200b expression results in downregulation of DLC1, HNRNPA3 and HFE. Additionally, miR-200 family downregulates HNRNPR3, HFE and ATRX in BEAS-2B immortalized lung epithelial cells in quantitative RT-PCR and western blot assays. The miR-200 family and these potential targets are functionally involved in canonical pathways of immune response, molecular mechanisms of cancer, metastasis signaling, cell-cell communication, proliferation and DNA repair in Ingenuity pathway analysis (IPA). These results indicate that re-expression of miR-200 downregulates our previously identified NSCLC prognostic biomarkers in metastatic NSCLC cells. These results provide new insights into miR-200 regulation in lung cancer metastasis and consequent clinical outcome, and may provide a potential basis for innovative therapeutic approaches for the treatment of this deadly disease.

Introduction

Lung cancer remains the leading cause of cancer-related mortality in the world, with an overall 5-year survival rate of 15%. Approximately 85% of lung cancer cases are non-small cell lung cancer (NSCLC) (1). Tumor recurrence and metastasis is the major cause of lung cancer treatment failure and death. Epithelial-mesenchymal transition (EMT) plays an important role in tumor progression and is a key feature of metastasis in many cancer types, including lung cancer (2,3). EMT characteristics include perturbations of several signaling pathways including the loss of E-cadherin expression, which is a major step in metastasis of NSCLC (4).

In the past few years, microRNAs (miRNAs) have emerged as promising molecular factors with potential for clinical applications in cancer diagnosis and therapy (5-8). MicroRNAs are small endogenous non-coding RNAs that range 19-24 nucleotides in length. MicroRNAs regulate the expression of numerous genes either via translational silencing or by inducing mRNA degradation of the targeted genes (5). Moreover, it has been estimated that one miRNA can modulate as many as 200 genes, and over 30% of human coding genes are under miRNA regulation (9,10). Increasing evidence indicates that in human cancers, miRNAs can act either as oncogenes or as tumor suppressor genes (11,12). To date, more than 1,400 miRNA sequences have been identified in human cells (13).

The microRNA-200 (miR-200) family, represented by miR-200a, -200b, -200c, -141 and -429, is a marker and powerful regulator of the EMT process. Its functions include maintaining the epithelial phenotype of tissues through suppression of the EMT-inducing transcription factors zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2) (14,15). It has been shown that suppression of ZEB1 in undifferentiated mesenchymal-like cells leads to restoration of epithelial phenotype with increased expression of epithelial...
phenotype marker, E-cadherin (16), which mediates cell-cell adhesion (14). In many cancer types, E-cadherin is somatically inactivated via mutation, truncation or epigenetic silencing, a loss that enables the cells to acquire a highly invasive phenotype with the characteristics of EMT (17). Recent studies have shown that restoring miR-200c expression decreases cell migration but does not result in E-cadherin re-expression in some cells, thus suggesting that miR-200c targets other genes involved in tumor progression and metastasis (18).

The goal of this study is to identify potential targets of miR-200 family essential in NSCLC metastasis and clinical outcome. Our previous studies identified prognostic biomarkers associated with metastasis in early stage NSCLC tumors not treated with chemotherapy (19-21). Specifically, a 35-gene signature was identified (19) and validated (20) as predictive of tumor metastasis in 434 NSCLC patients, including lung adenocarcinoma, squamous cell lung cancer and large cell lung cancer. This signature could identify more aggressive tumors from stage I A NSCLC (20). In another genome-wide DNA microarray analysis of data from the Director's Challenge study (22), 12- and 15-gene prognostic signatures were identified and validated using multi-center NSCLC patient cohorts (n=442) (21). All the identified prognostic biomarkers were confirmed with quantitative RT-PCR analysis and some were validated with western blot assays of independent snap-frozen human NSCLC tumors (20). Based on these results, we sought to identify key miRNAs that regulate multiple NSCLC prognostic marker genes, to reveal molecular regulatory events in metastasis with implications on clinical outcome. The following experimental analyses were carried out in this study. First, bioinformatics methods were used to predict miRNA regulators of the identified signature genes in NSCLC. Then, miRNAs that could potentially regulate multiple prognostic biomarkers were identified. Based on the bioinformatic prediction results, the miR-200 family was selected to further determine a regulation of the predicted target genes and to determine putative molecular networks in EMT and tumor metastasis.

Materials and methods

Patient samples and gene expression profiling. A total of 130 lung squamous cell carcinoma (SCC) samples were analyzed in this study. The patient characteristics were described in a previous publication (23). Genome-wide mRNA expression profiles of the tumor samples were quantified with the Affymetrix U133A Gene Chip (23). Microarray data were extracted and calculated using the Affymetrix MAS 5 software after global scaling of the average intensity to 600. The mRNA microarray data were available from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) with accession no. GSE4573. Out of 130 SCC tumors, 57 samples were screened for miRNA expression profiles with TaqMan miRNA assays (Applied Biosystems Inc., Foster City, CA) (24). The miRNA expression data was available from GEO with accession no. GSE16025. The mRNA and miRNA profiles of these matched 57 tumor samples were used for further analysis.

MicroRNA target prediction. The mRNA expression levels of the lung cancer prognostic markers identified in our previous studies (19-21) were retrieved from the genome-wide expression profiles of the 57 SCC tumor samples. Pearson's correlation coefficient between each mRNA and all available miRNAs was computed. Significant mRNA-miRNA pairs (\(|r| \geq 0.258; p \leq 0.05\)) were selected for target prediction. Four Bioinformatics tools were used in the study for miRNA target prediction, including TargetScan (http://www.targetscan.org/) (10), PicTar (http://www.pictar.mdc-berlin.de/) (25), miRDB (http://www.mirdb.org/miRDB/) and microRNA.org (http://www.microrna.org/microrna/home.do) (26). These computational methods use sequence complementary base-pairing between miRNA and mRNA in target prediction. Files and databases containing miRNA and predicted target genes were downloaded from the online websites of these toolsets. These files were then analyzed with in-house software script written in R to identify miRNA-mRNA gene pairs showing significant expression correlation in SCC tumor samples. In addition, TarBase (http://www.diana.cslab.ece.ntua.gr/tarbase/) (27) was used to retrieve experimentally validated miRNA targets.

The miRNA targets predicted by TargetScan are based on the presence of conserved 8mer, 7mer and 6mer sites that match the seed region of each miRNA (10). TargetScan also predicts non-conserved sites, additional types of seed matches that are preferentially conserved in different species, and sites with mismatches in the seed region that are compensated by conserved 3' pairing (28). PicTar predicts miRNA targets by searching 3' UTR alignments with predicted sites (25). The miRDB uses an algorithm, MirTarget2, based on Support Vector Machine, to predict miRNA targets. The microRNA.org uses miRanda algorithm to predict miRNA targets based on sequence and contextual features of the predicted miRNA-mRNA duplex (26).

Cell lines and cell culture. Small airway epithelial cells (SAEC) and normal human bronchial/tracheal epithelial cells (NHBE) were obtained from Lonza Walkersville Inc. (Walkersville, MD). Human non-small cell lung cancer cell line H1299 and human immortalized lung epithelial cell line BEAS-2B were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SAEC cells were cultured according to the supplier's recommendations in SABM medium supplemented with 52 µg/ml bovine pituitary extract, 0.5 µg/ml human recombinant epidermal growth factor (EGF), 0.5 µg/ml epinephrine, 1 µg/ml hydrocortisone, 0.5 µg/ml transferrin, 5 µg/ml insulin, 6.5 ng/ml triiodothyronine, 50 µg/ml gentamicin/amphotericin B (GA-1000) and 50 µg/ml fatty acid-free bovine serum albumin. NHBE cells were cultured according to the supplier's recommendations in BEBM medium supplemented with 52 µg/ml bovine pituitary extract, 0.5 µg/ml human epidermal growth factor (hEGF), 0.5 ng/ml epinephrine, 0.5 µg/ml insulin, 0.5 µg/ml transferrin, 10 ng/ml, triiodothyronine (6.5 ng/ml), gentamicin (50 µg/ml), amphotericin B (50 ng/ml), bovine serum albumin (1.5 µg/ml). H1299 and BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin,
L-glutamine and 10% fetal bovine serum. All cells were cultured at 37°C in humidified incubator with 95% air and 5% CO₂.

**Virus transduction.** Human miRIDIAN shMIMIC lentiviral miRNA particles (hsa-miR-200a: UAACACUGUCUGGAUAACGAUGU, hsa-miR-200b: UAUAACUGCCGGUAAUGAUAGA, hsa-miR-200c: UAUAACUGCCGGUAAUGAGGA), and control scrambled miRNA were purchased from Open Biosystems (Huntsville, AL) and used for infection of target cells in the presence of 4 µg/ml of polybrene.

**Western blot analysis.** Cells were lysed in 1X SDS lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Total protein was quantified by the BCA method, β-mercaptoethanol was added to lysates to a final concentration 100 mM. Equal amounts of total protein were separated by 4-12% SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked 1 h with 5% non-fat milk in 1X PBS containing 0.05% Tween-20. Membranes were then incubated for 1 h at room temperature with primary antibodies. After incubation with the primary antibody, membranes were washed thrice in 1X PBS with 0.05% Tween-20 for 5 min each and blocked for 7 min in blocking solution. Membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP) conjugated donkey anti-mouse IgG or donkey anti-rabbit IgG in 1X PBS with 0.05% Tween-20. Membranes were then washed five times for 5 min in PBS-Tween-20 and finally developed with HyGLO Western Blotting Substrate (Denville Scientific) according to the instructions of the manufacturer. Protein band intensity was determined using FluorChem™ Q software (AlphaInnotech, Santa Clara, CA). Relative protein level was determined after normalization to tubulin and relative to negative control (miR-scr) samples. The following antibodies were used: ATRX (Santa Cruz Biotechnology, catalog no. SC-55584), DLD1 (BD Biosciences, catalog no. 612020), HFE (Santa Cruz Biotechnology, catalog no. SC-130375), ZEB1 (Sigma, catalog no. HPA027524), HNRNPA3 (Santa Cruz Biotechnology, catalog no. SC-133665), E-cadherin (BD Biosciences, catalog no. 610181), GAPDH (Millipore, catalog no. MAB374) and tubulin (Sigma, catalog no. T9026).

**RNA isolation.** Total RNA was extracted using the mirVana™ kit (Ambion Inc., Austin, TX) according to the manufacturer's protocol. To ensure a good RNA quality, the quality and integrity of the total RNA was evaluated using 28S/18S ratio and a visual image of the 28S and 18S bands were evaluated on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA isolated using this method yielded a very good quality, with a RIN number ≥9. Concentration of the total RNA was assessed using the NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Germany).

**Quantitative real-time RT-PCR.** Complementary DNA (cDNA) was generated using total RNA according to the TaqMan™ MicroRNA Reverse Transcription protocol (Applied Biosystems Inc.). Quantitative RT-PCR for microRNA was performed using TaqMan MicroRNA assays (Applied Biosystems Inc.). Human U47 small nuclear RNA was used as an endogenous control. The expression levels of miRNAs were quantified using ABI 7500 quantitative real-time instrument and SDS software (Applied Biosystems Inc.). The abundance of miRNA is expressed as Ct (threshold fluorescence) which gives the number of cycles required to reach threshold fluorescence. Real-time PCR for target genes was determined using total RNA and cDNA was generated using a High-Capacity cDNA Reverse Transcription kit and TaqMan gene expression assays (Applied Biosystems Inc.). E-cadherin (CDH1) mRNA was measured using SYBR-Green Master mix and CDH1 specific primers according to manufacturer's protocol (Applied Biosystems Inc.). All qRT-PCR reactions were performed on 7500 instrument (Applied Biosystems Inc.). In the qRT-PCR analysis of E-cadherin, the dissociation curve showed the absence of a secondary peak, indicating no presence of primer dimer. Specificity of the PCR product obtained from SYBR-Green reactions was verified by sequencing. The expression level of each gene was determined by following formulas: fold change = 2^(-∆∆Ct), where ∆Ct (cycle threshold) = Ct_target gene - Ct_endogenous control gene and ∆∆Ct = ∆Ct treated sample - ∆Ct control sample. The expression level of the analyzed genes is reported as fold change relative to negative miR-scrambled (-src) infected samples. The human UBC gene was used as an endogenous control gene.

In this study, a predicted gene was considered a confirmed target if the mRNA level was significantly downregulated or the protein level was downregulated at least 15% relative to negative control samples. Not all of the predicted targets were analyzed at the protein level due to the lack of specificity of commercially available antibodies.

**Functional pathway analysis.** Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA) was used to derive curated molecular interactions reported in the scientific literature. These interactions included both physical and functional interactions, as well as interactions representing pathway relevance. In this study, in order to delineate molecular networks of genes interacting with the miR-200 family and novel molecular targets, a core analysis was employed to identify the most relevant canonical pathways, biological functions and physiological processes from the interactions reported in the IPA database. We then selected pathways that were statistically significant with a p<0.05 in adjusted Benjamini-Hochberg tests.

**Statistical analysis.** The statistical significance of the difference between groups was determined by un-paired t-tests at p≤0.05. The qRT-PCR expression data are presented as mean ± SEM.

**Results**

**Prediction of miR-200 novel molecular targets.** To screen for potential miRNA regulators of our previously identified lung cancer prognostic gene signatures (19-21), the correlation between the mRNA expression of these biomarkers and all available human miRNA expression profiles in 57 squamous cell lung cancer tumors was computed. For all miRNA-mRNA gene pairs showing significant correlation (r>0.258, p≤0.05, Pearson's correlation analysis) in the lung SCC tumors, 4 bioinformatics toolsets (TargetScan, PicTar, miRDB and microRNA.org) were used to determine whether or not a given gene is a predicted target of the corresponding miRNA (Fig. 1A). A total of 233 miRNA-mRNA gene pairs were predicted as a target pair by at least one bioinformatic method. The correla-
tion analysis and the prediction results may be viewed on our website (http://www.wvucancer.org/guoLab/Publications). Due to alternative splicing, each gene may have multiple probes in DNA microarray data. This will lead to discrepant gene expression among different probes for the same gene and discordant correlation between mRNA and miRNA. The mRNA-miRNA pairs with a negative correlation for at least one probe set were selected for further analysis.

Several miRNAs, including the miR-200 family, were predicted to target multiple prognostic biomarkers. We focused on the miR-200 family because of its reported role in tumor metastasis. The miRNA-200 family is represented by miR-200a, miR-200b, miR-200c, miR-141 and miR-429, based on their genomic location and primary sequence. Based on sequence similarity, the miR-200 family is divided into two subclasses: one class includes miR-200b, -200c and -429, and the other class includes miR-200a and miR-141 (29). The members in each subclass share the same seed sequence. We analyzed the expression levels of the miR-200s in squamous cell lung cancer patient primary tumors and normal lung tissues in the cohort from Raponi et al (24). The results show that miR-200a had a 2.56-fold overexpression (p<1.35e-7; unpaired t-tests) in the tumors, miR-200b exhibited a 2.94-fold overexpression (p<1.32e-6; unpaired t-tests) and miR-200c showed a 3.16-fold overexpression (p<0.001; unpaired t-tests) in the patient tumors (Fig. 1B). However, during metastasis, previous studies showed that the miR-200 family expression is lost in mesenchymal subtypes of epithelial cancers and negatively correlates with cancer cell invasion (15,16).

The predicted targets for hsa-miR-200a include deleted in liver cancer 1 gene (DLC1), E2F transcription factor 4 (E2F4), and AHNAK nucleoprotein (desmoyokin) (AHNAK); for hsa-miR-200b: DLC1, ubiquitin-like modifier activating enzyme 6 (UBA6), ubiquitin-conjugating enzyme E2I (UBE2I) and heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3); for hsa-miR-200c: alpha thalassemia/mental retardation syndrome X-linked gene (ATRX), hereditary hemochromatosis (HFE), DLC1 and thrombospondin 1 (THBS) (Tables I and II). To confirm the regulation of miR-200a, -200b, -200c on the predicted targets, expression of these prognostic biomarkers genes at the mRNA and protein levels were examined following re-expression of miR-200a, -200b, -200c in H1299 and BEAS-2B cells.

Restoring miR-200 expression in H1299 cells. We analyzed the expression levels of miR-200a, -200b and -200c in a metastatic human NSCLC model, H1299 cells. Normal human small airway epithelial cells (SAEC) were used as control cells. The expression level of miR-200a, -200b and -200c in H1299 cells was at the detection limit (Fig. 2). In contrast, SAEC expressed higher levels of miR-200a, -200b and -200c (data not shown; http://www.wvucancer.org/guoLab/Publications) consistent with their normal epithelial phenotype. In order to identify potential molecular targets of miR-200a, -200b, -200c, H1299 cells were stably infected with lentiviral vectors expressing miR-200a, -200b, -200c or negative control miRNAs. The infected H1299 cells expressed high levels of exogenous miR-200a, -200b, -200c, which were comparable with the levels exhibited in normal lung epithelial SAEC cells (data not shown; http://www.wvucancer.org/guoLab/Publications).

miR-200 regulation on predicted molecular targets. ZEB1 and ZEB2 genes are the most extensively characterized targets of the miR-200 family (14,15) and they were used as positive controls in the qRT-PCR experiments. In H1299 cells over-expressing miR-200a, -200b and -200c, ZEB1 and ZEB2
Table I. The predicted target genes analyzed in this study.

| Gene symbol | Gene name | Assay ID | Function | Pathway | Remarks |
|-------------|-----------|----------|----------|---------|---------|
| AHNAK       | AHNAK nucleoprotein (desmoyokin) | Hs00225285_m1 | Protein-protein binding | Signal transduction | NSCLC prognostic biomarker (19,20) |
| ATRX        | Alpha thalassemia/mental retardation syndrome X-linked | Hs00230877_m1 | Transcriptional regulator, chromatin remodeling | Transcription | NSCLC prognostic biomarker (19,20) |
| DLC1        | Deleted liver cancer 1 | Hs00183436_m1 | Regulation of small GTP-binding proteins | Signal transduction | Tumor suppressor gene (60) and NSCLC prognostic marker (21) |
| E2F4        | E2F transcription factor-4 | Hs00608098_m1 | Transcriptional factor, cell cycle, apoptosis | Transcription | NSCLC prognostic maker (19,20) |
| HFE         | Hemochromatosis | Hs00373474_m1 | Regulation of body iron metabolism | Iron metabolism | NSCLC prognostic biomarker (21) |
| HNRNPA3     | Heterogeneous nuclear ribonucleoprotein-A3 | Hs00864845_s1 | Cytoplasmic RNA binding and trafficking, protein binding | Signal transduction | NSCLC prognostic biomarker (19,20) |
| THBS1       | Thrombospondin 1 | Hs00962914_m1 | Extracellular adhesive glycoprotein | Protein interaction | NSCLC prognostic biomarker (21) |
| UBE2I       | Ubiquitin-conjugating enzyme E2I | Hs00163336_m1 | Ubiquitin-activating protein | Protein degradation | NSCLC prognostic biomarker (19,20) |
| UBA6        | Ubiquitin-like modifier activating enzyme-6 | Hs00414964_m1 | Ubiquitin-conjugation for protein degradation | Protein degradation | NSCLC prognostic biomarker (21) |

Table II. Genes regulated by miR-200a, -200b and -200c in H1299.

| Genes    | miR-200a | miR-200b | miR-200c |
|----------|----------|----------|----------|
|          | Predicted Downregulated mRNA Protein | Predicted Downregulated mRNA Protein | Predicted Downregulated mRNA Protein |
| AHNAK    | •        | ✓        | ✓        |
| ATRX     |          |          |          |
| DLC1     | •        | ✓        | ✓        | •        | ✓        |
| E2F4     | •        |          |          |
| HFE      |          | ✓        |          | •        | ✓        |
| HNRNPA3  |          | •        | ✓        |          |
| THBS1    |          |          |          |
| UBE2I    |          |          |          |
| UBA6     |          |          |          |
| ZEB1     | •        | ✓        | ✓        | •        | ✓        | ✓        |
| ZEB2     | •        | ✓        | ✓        | •        | ✓        | ✓        |

*, The gene is a predicted target for the corresponding miRNA; ✓, downregulation at mRNA level; ✓✓, downregulation at protein level.
miR-200 family members according to the microRNA.org database (26), except for ATRX/miR-200a and HFE/miR-200a. The 3’ UTR region of DLC1 contains 2 binding sites for miR-200a, 3 for miR-200b and 3 for miR-200c. ATRX contains 1 binding site for miR-200c. HFE contains 1 binding site for miR-200b and 1 for miR-200c. HNRNPA3 contains 3 binding sites for miR-200b (Fig. 3A). HFE is a predicted target of miR-200c, not miR-200a or -200b, in NSCLC. Although miR-200b and -200c have the same seed sequence, the correlation between HFE and miR-200b was not statistically significant (p=0.057) in SCC patient samples. Therefore, it was not initially selected as a predicted target of miR-200b in NSCLC. Nevertheless, HFE was shown to be regulated by miR-200a, -200b and 200c in metastatic H1299 cells. AHNK and E2F4 are predicted targets of miR-200a. However, miR-200a did not suppress the expression of these two genes in H1299 cells (Fig. 2A). Similarly, UBA6 and UBE2I were not regulated by miR-200b in metastatic lung cancer cells as they were predicted to be. These results were summarized in Table II.

To further substantiate the regulatory effects of miR-200 on these lung cancer prognostic markers, SCC patient samples (n=57) (23,24) were screened to investigate the correlation between the miR-200 family and the mRNA expression of its predicted target genes as well as ZEB1 and ZEB2. The results showed that all the downregulated genes had a significant negative correlation with the corresponding miR-200 family member in SCC patient tumor tissues, except ATRX/miR-200a and HFE/miR-200a (Table III). The results in the patient samples further strengthened the in vitro findings.

The regulation of miR-200 on these predicted target genes was also evaluated in human immortalized lung epithelial cells BEAS-2B. The overexpression of miR-200b in these cells resulted in significantly downregulated mRNA level of HFE (Fig. 4A). The overexpression of miR-200a, -200b and -200c in BEAS-2B cells caused approximately 60, 40 and 70% downregulation of HNRNPR3 protein, respectively (Fig. 4B and C). The overexpression of miR-200b in BEAS-2B resulted in a 20% downregulation of ATRX at the protein level and the re-expression of miR-200c resulted in a 70% downregulation of HFE at the protein level. These results indicate that miR-200 family downregulates HNRNPR3, HFE and ATRX in normal lung epithelial cells. Together, these results substantiate the role of miR-200 family and its regulated genes in lung cancer initiation and progression.

E-cadherin and miR-200. After the potential molecular targets of the miR-200 family were shown in the present study,
we sought to explore the effect of miR-200s on EMT in the metastatic NSCLC cells. Re-expression of miR-200c induced a 1.53-fold upregulation of E-cadherin (CDH1) through the downregulation of the E-cadherin repressor transcriptional factors ZEB1 and ZEB2, which is consistent with previous studies in breast cancer or NSCLC cells (16,30,31) (Fig. 2B).

E-cadherin protein was highly expressed in normal lung SAEC cells, but not in metastatic NSCLC H1299 cells (data not shown; http://www.wvucancer.org/guoLab/Publications). These results indicate that re-expression of miR-200 may reverse EMT process.

Molecular network analysis. Molecular network interactions and significant canonical signaling pathways associated with miR-200s and their predicted molecular targets were retrieved using IPA. The molecular network map shows

Table III. Correlation between the expression of miR-200 and its regulated genes in squamous cell lung cancer patient tumors (n=57).

| Genes    | miR-200a | miR-200b | miR-200c |
|----------|----------|----------|----------|
| ATRX     | -0.0629  | NA       | -0.301*  |
| DLC1     | -0.313*  | -0.374*  | -0.496*  |
| HFE      | -0.193   | -0.253*  | -0.393*  |
| HNRNPA3  | NA       | 0.264*   | NA       |
| ZEB1     | -0.426*  | -0.458*  | -0.484*  |
| ZEB2     | -0.395*  | -0.379*  | -0.382*  |

*Statistically significant at p<0.05. NA, gene not regulated by miR-200 in H1299. *Borderline significant at p=0.057.
interactions between the miR-200s and their known target genes, ZEB1 and ZEB2, as well as potential targets identified from the present study (Fig. 5; the regulation identified in the present study is shaded in orange font). Among the genes in the molecular network, Histone H3 and E-cadherin (CDH1) were major focal points in the miR-200 network. Histone H3 is a component of the nucleosome and CDH1 is a cell adhesion protein and epithelial phenotype marker. These results suggest that miR-200s and their potential target genes participate in molecular interactions involved in gene transcription regulation, either during the regulation of gene expression at the chromatin level or in the regulation of cell-cell interactions as mediated by E-cadherin. Increasing evidence indicates that chromatin remodeling induced by DNA damage or epigenetic changes are responsible for carcinogenesis.

The IPA functional analysis found a total of 69 canonical pathways associated with the miR-200 network, of which 13 canonical pathways were statistically significant (adjusted p<0.05 with Benjamini-Hochberg tests; Table IV). The top signaling pathways include virus entry via endocytic pathways, allograft rejection signaling, OX40 signaling pathway, caveolar-mediated endocytosis signaling, communication between innate and adaptive immune cells, chronic myeloid leukemia signaling, molecular mechanisms of cancer and DNA double-strand break repair by homologous recombination, among others (Table IV). Furthermore, the IPA functional analysis found 25 significant diseases and disorders related to the miR-200 network. The top 3 diseases included genetic disorders, metabolic diseases and cancer (Table V). At the molecular level, beta-2-microglobulin (B2M), CDH1, ZEB1, ZEB2, ATRX, HFE and the miR-200s are involved in genetic disorders; B2M, transferrin receptor 2 (TFR2), HFE and angiotensin II receptor (AGTR1) are involved in metabolic diseases; B2M, E2F, miR-200s, ATRX, DLC1, ZEB1, ZEB2, CDH1 and caveolin 1 (CAV1) are involved in cancer (Table V). These results indicate that miR-200 network involves complex signaling pathways and mechanisms, and has implications in numerous human diseases and disorders.

Discussion

Lung cancer is a dynamic and diverse disease and is associated with numerous somatic mutations, deletions and amplification events. Tumor recurrence and metastasis causes lethality and failure in lung cancer treatment. About 35-50% of stage I NSCLC patients will develop and die from tumor recurrence within 5 years following surgery (32,33) and adjuvant
chemotherapy of stage II and stage III disease has resulted in very modest survival benefits (34). Epithelial-mesenchymal transition (EMT) is a key process in tumor metastasis. Novel therapeutic approaches targeting EMT are needed to effectively prevent tumor recurrence and metastasis.

miRNAs are small non-coding RNAs that regulate gene expression via degradation or translational inhibition of target mRNAs. Importantly, one miRNA can regulate the expression of multiple genes because it can bind to its mRNA targets regardless of whether there is perfect seed sequence complementarity (5).

Our previous studies identified prognostic marker genes for NSCLC (19-21). The expression of these prognostic biomarkers was associated with metastatic potential in early stage NSCLC tumors. Identification of miRNAs that regulate multiple prognostic biomarker genes could shed light on the mechanisms underlying tumor metastasis and potentially provide the basis for the development of novel therapeutic targets to improve the clinical outcome.

Table IV. Top 13 significant canonical pathways related to the miR-200 molecular network in Ingenuity pathway analysis.

| Canonical pathways                                           | P-value | Molecules          |
|--------------------------------------------------------------|---------|--------------------|
| Virus entry via endocytic pathways                           | 0.0002  | B2M, CAV1, TFRC    |
| Allograft rejection signaling                                | 0.0019  | B2M, IL2           |
| OX40 signaling pathway                                       | 0.0025  | B2M, IL2           |
| Caveolar-mediated endocytosis signaling                      | 0.0044  | B2M, CAV1          |
| Communication between innate and adaptive immune cells       | 0.0056  | B2M, IL2           |
| Chronic myeloid leukemia signaling                           | 0.0071  | CTBP1, E2F4        |
| Molecular mechanisms of cancer                               | 0.0102  | DAXX, CDH1, E2F4   |
| DNA double-strand break repair by homologous recombination   | 0.0191  | ATRX               |
| Lipid antigen presentation by CD1                            | 0.0257  | B2M                |
| Antiproliferative role of TOB in T cell signaling            | 0.0355  | IL2                |
| Colorectal cancer metastasis signaling                       | 0.0407  | CDH1, E2F4         |
| Role of CHK proteins in cell cycle checkpoint control        | 0.0447  | E2F4               |
| Cell cycle regulation by BTG family proteins                | 0.0468  | E2F4               |

Figure 5. Molecular network analysis of the miR-200 family and potential molecular targets with Ingenuity pathway analysis (IPA).
Deregulated expression of miR-200 family members has been observed in multiple cancer types (15,16,29,30). Numerous studies showed that miR-200 family members regulate the EMT and cancer cell invasion by suppressing the expression of ZEB1 and ZEB2 genes (15,35). ZEB1 and ZEB2 are key transcription factors regulating EMT by binding to an E box upstream of their target genes, which include E-cadherin, and repressing their expression. Moreover, ZEB1 and ZEB2 can repress the transcription of miR-200 genes via negative feed-back loop mechanism (15,36). Absence of E-cadherin in cell junctions renders loss of cell-cell communication, thereby allowing cancerous cells to acquire an aggressive, invasive phenotype. Thus, downregulation of E-cadherin is associated with increased lymph node metastasis and poor-prognosis of NSCLC (37). Numerous studies have reported a stringent control of E-cadherin expression by the miR-200s via suppression of ZEB1 and ZEB2 (16,30,31). The miR-200 family expression is lost in mesenchymal subtypes of epithelial cancers and negatively correlates with cancer cell invasion (15,16) and metastasis in NSCLC (38).

On the other hand, overexpression of miR-200 was also found in cholangiocarcinoma malignant cells compared to non-malignant cells (39), melanoma cell lines (29), ovarian cancer (40), colorectal cancer (41) and NSCLC (Fig. 1B). These results indicate that expression of miR-200 family during tumorigenesis is rather complex, and may adopt a bimodal pattern with elevated levels in primary tumors and dramatic downregulation in metastatic cells. Alternatively, the role of miR-200 may differ depending on cancer type and stage.

### Table V. Top 25 significant disease and disorder functions related to the miR-200 molecular network in Ingenuity pathway analysis.

| Disease and Disorders | P-value | Molecules |
|-----------------------|---------|-----------|
| Genetic disorder      | 0.00004 | B2M, CDH1, IL2, TFR2, ZEB2, ATRX, mir-200, CAV1, ZEB1, HFE, AGTR1 |
| Metabolic disease     | 0.00004 | B2M, TFR2, HFE, AGTR1 |
| Cancer                | 0.00005 | B2M, E2F4, mir-200, ATRX, ZEB1, DLC1, CTBP1, CDH1, BMI1, IL2, ZEB2, CAV1, TFRC, AGTR1 |
| Reproductive system disease | 0.00005 | B2M, CDH1, BMI1, IL2, ATRX, mir-200, CAV1, TFRC, DLC1, AGTR1 |
| Gastrointestinal disease | 0.00021 | B2M, CDH1, BMI1, IL2, mir-200, ZEB2, CAV1, TFRC, AGTR1 |
| Hepatic system disease | 0.00021 | B2M, BMI1, IL2, mir-200, DLC1, AGTR1 |
| Organismal injury and abnormalities | 0.00021 | IL2, AGTR1 |
| Infection mechanism   | 0.00053 | CTBP1, E2F4, IL2, CAV1, TFRC, ZEB1 |
| Infectious disease    | 0.00053 | CTBP1, B2M, IL2, CAV1, TFRC, AGTR1 |
| Dermatomal disease and conditions | 0.00122 | B2M, CTBP1, E2F4, IL2, ATRX, CAV1, DLC1, AGTR1, HFE |
| Inflammatory response | 0.00138 | CAV1, ZEB1, HFE |
| Ophthalmic disease    | 0.00138 | B2M, CDH1, IL2, ZEB1, AGTR1 |
| Respiratory disease   | 0.00186 | CTBP1, B2M, CDH1, IL2, mir-200, CAV1, AGTR1 |
| Immunological disease | 0.00247 | B2M, DAXX, E2F4, CDH1, HNRNPA3, IL2, TFRC, DLC1, AGTR1 |
| Antimicrobial response | 0.00276 | IL2 |
| Cardiovascular disease | 0.00276 | B2M, IL2, CAV1, AGTR1 |
| Inflammatory disease  | 0.00401 | B2M, DAXX, CDH1, HNRNPA3, IL2, ZEB2, TFRC, ZEB1, DLC1, AGTR1 |
| Connective tissue disorders | 0.00501 | B2M, DAXX, CDH1, HNRNPA3, IL2, TFRC, DLC1, AGTR1 |
| Neurological disease  | 0.00550 | BMI1, ZEB2, CAV1, AGTR1 |
| Renal and urological disease | 0.00550 | B2M, CDH1, AGTR1 |
| Skeletal and muscular disorders | 0.00733 | B2M, DAXX, CDH1, HNRNPA3, BMI1, IL2, TFRC, DLC1 |
| Developmental disorder | 0.00961 | ATRX, ZEB2, AGTR1 |
| Nutritional disease   | 0.01230 | IL2 |
| Endocrine system disorders | 0.04720 | AGTR1|
Figure 6. Proposed mechanisms of the miR-200 regulation in tumor initiation and metastasis.

Despite strong evidence that miR-200s inhibit EMT and suppress cancer cell invasion, several functional overexpression studies have yielded conflicting results on the role of miR-200s in metastasis, supporting both their anti-metastatic (18,30,42-44) and pro-metastatic (45,46) potential.

The present study sought to determine whether some of our previously identified human lung cancer prognostic markers are potential molecular targets of miR-200a, -200b, -200c, and -200d. The study goal was to explore whether biomarkers associated with NSCLC poor prognosis are functionally involved in EMT and metastasis through miR-200 regulation. In order to identify new molecular targets of the miR-200 family, we used the H1299 NSCLC cell line. This cell line is p53-deficient, has metastatic characteristics and is devoid of miR-200 family and E-cadherin expression. We chose this cell model in order to identify new molecular targets following re-expression of miR-200a, -200b, -200c, and -200d, independent of p53 regulation (47-49). p53 is a tumor suppressor protein that regulates the expression of a myriad of genes and miRNAs including the miR-200 family (47-50). The present study shows an increased expression of E-cadherin in H1299 cells re-expressing miR-200c, consistent with other published results (16,30). It has been shown that E-cadherin promoter is methylated in NSCLC cells (51) and re-expression of E-cadherin may not be solely dependent on suppression of ZEB1 but also dependent on other processes such as promoter demethylation (52). The modest re-expression level of E-cadherin in the present study is in agreement with other reports (16,18,52), suggesting that more significant downregulation of ZEB1 and ZEB2 might be required for full re-activation of E-cadherin.

This study identified a regulation of miR-200 family on their potential novel molecular targets. The results show that DLC1, ATRX, and HFE genes are regulated by miR-200a and miR-200c; DLC1, HFE, and HNRP3 are regulated by miR-200b. Although, the changes in the expression of these genes at the mRNA and protein levels after re-expression of miR-200s in H1299 cells were relatively small (<2-fold), such small changes in the expression of microRNA targets are very common (16,53). Despite restoration of normal miR-200 levels, some other components of the post-transcriptional gene silencing pathway in H1299 cells might be in limiting amounts, for example Dicer, which is commonly downregulated in cancer cells (54). These findings are consistent with the model that miR-200s regulate their targets differentially either by targeting mRNA for degradation or/and by inhibiting its translation. All of these potential novel molecular targets of the miR-200 family are prognostic biomarkers for NSCLC (19-21). These genes showed overexpression in metastasis-prone NSCLC cells (H1299) compared with normal lung epithelial cells (NHBE and SAEC; Fig. 4B). HFE is important in iron metabolism disorder and oxidative stress (55). HFE polymorphism is associated with multiple cancer types and chemoresistance (56). The complex of beta-2-microglobulin (B2M) and its receptor HFE activates EMT and promotes metastases in human prostate, breast, lung and renal cancer cells both in vivo and in vitro, through the modulation of iron responsive pathways (57). Inhibition of either B2M or HFE reverses EMT (57). Our results show that HFE is regulated by all three of the studied miR-200 family members, indicating new mechanisms in EMT induction and lung cancer metastasis. AHNAK, a pseudopod-specific protein, also controls EMT in metastatic cancer cells (58). AHNAK knockdown in metastatic cells causes reduced cell migration and induces mesenchymal-epithelial transition (MET). Consistent results were observed in clinical cohorts, in which overexpression of AHNAK was associated with poor prognosis of NSCLC (19). Overexpression of ATRX (19) and HNRP3 (59) was observed in NSCLC tumors and poor prognosis patients. Collectively, loss of miR-200 could lead to overexpression of HFE, AHNAK, ATRX and HNRP3, which in turn is associated with poor prognosis of NSCLC (Fig. 6).

DLC1, a tumor suppressor gene, is frequently silenced in various types of human cancer (60). DLC1 was first identified in primary human hepatocellular carcinoma, with an inhibitory effect on the growth of breast and liver tumors (61). Downregulation of DLC1 by miR-200a in primary human liver cells has been previously reported (62). In the present study, re-expression of miR-200a, -200b, or -200c in metastatic human NSCLC cells resulted in DLC1 downregulation at both mRNA and protein expression levels, with miR-200a exerting the most significant repression of DLC1. The overexpression of miR-200 family observed in primary tumors (Fig. 1B) could downregulate DLC1, which is involved in tumorigenesis (Fig. 6). Downregulation of DLC1, in turn, is associated with poor prognosis of NSCLC (21). These results, again, indicate potential pleiotropic regulatory mechanisms of miR-200 in lung cancer development and progression. Together, these results indicate that deregulation of miR-200 induces aberrant expression of multiple genes involved in lung cancer carcinogenesis, EMT, cell migration and metastasis, with significant implications on NSCLC clinical outcome. The proposed mechanisms of miR-200 regulation in carcinogenesis and metastasis are illustrated in Fig. 6.

IPA functional pathway analyses found that the miR-200 molecular network involved canonical pathways of immune response, molecular mechanisms of cancer, metastasis signaling transduction, cell-cell communication, proliferation and DNA repair. These results indicate that miR-200 is essential in regulating signaling pathways responsible for many biological functions and complex molecular mechanisms (Table IV). Moreover, the miR-200 related molecular network is implicated in at least 25 human diseases and abnormalities, including genetic disorders, metabolic disease, cancer and reproductive system disease, among many others (Table V).
In conclusion, this study combined computational predictions and quantitative experimental validations to demonstrate that the miR-200 family regulates multiple NSCLC prognostic marker genes. The identified regulation, direct or indirect, provides important insights of possible microRNA regulatory mechanisms in EMT and lung cancer metastasis and lays a foundation for future functional analysis. These potential molecular targets, each with significant prognostic value in NSCLC patients, are involved in the regulation of gene transcription and signal transduction pathways. The findings of the miR-200 downregulation of DLC1, ATRX, HNRPA3, AHNAK and HFE in metastatic human NSCLC cells and the proposed regulatory mechanisms in tumorigenesis and metastasis could provide the basis for the development of novel therapeutic approaches for the treatment of this deadly disease. In our future research, reporter luciferase assays with mutations at specific seed sequences will be carried out to validate the direct interaction of these genes downregulated by miR-200 family.

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