Epigenetic inactivation of microRNA-34b/c predicts poor disease-free survival in early stage lung adenocarcinoma

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Statement of Translational Relevance

MiR-34b/c are members of microRNA (miR) 34 family that target relevant genes in lung adenocarcinoma (AC) involved in cell cycle, apoptosis, and invasion including *CCDE2, MYC, BCL2, NOTCH1* or *MET*. We analyzed the DNA methylation status of miR-34b/c in lung AC cell lines and in primary tumors and correlated to miR expression. Interestingly, early stage lung AC with increased miR-34b/c methylation had worse outcome. Ectopic expression of miR-34b/c in lung AC cell lines decreased cell proliferation, migration and invasion. These results suggest that miR-34b/c methylation is an independent prognostic marker in early stage lung AC patients and potential therapeutic target.

Abstract

Purpose: The microRNA-34b/c (miR-34b/c) is considered a tumor suppressor in different tumor types and a transcriptional target of *TP53*. The main objectives of this study were to investigate the clinical implications of miR-34b/c methylation in early stage lung adenocarcinoma (AC) patients and to determine the functional role of miR-34b/c re-expression in lung AC cell lines.

Experimental Design: Aberrant methylation and expression of miR-34b/c were assessed in 15 lung AC cell lines and a cohort of 140 early stage lung AC. Lung AC cell lines were transfected with miR-34b/c and the effects upon cell proliferation, migration, invasion and apoptosis were investigated.

Results: Aberrant methylation of miR-34b/c was detected in 6 (40%) of 15 lung AC cell lines and 64 out of 140 (46%) primary lung AC. Expression of miR-34b/c was significantly reduced in all methylated cell lines and primary tumors, especially with *TP53* mutations. Patients with increased miR-34b/c methylation had significantly shorter disease-free and overall survival as compared to patients with unmethylated or low level of miR-34b/c methylation. Ectopic expression of miR-34b/c in lung AC cell lines decreased cell proliferation, migration and invasion.

Conclusions: Epigenetic inactivation of miR-34b/c by DNA methylation has independent prognostic value in early stage lung AC patients. Re-expression of miR-34b/c leads to a less aggressive phenotype in lung AC cell lines.

Introduction

Lung cancer is the second most common cancer and the leading cause of cancer death in industrialized countries (1, 2). Non-small cell lung cancer is a heterogeneous disease, with adenocarcinomas (AC) and squamous cell carcinomas the most common subtypes (3). These histological subtypes have diverse clinical outcomes, different treatments, revealing heterogeneity in both disease aggressiveness and underlying molecular alterations (4). Complete resection gives the highest probability of long-term remission and cure. Even among surgically-treated early stage patients, the 5-year survival rate is only 52% (2). Postoperative adjuvant chemotherapy is the standard of care for resected stage II and III and provides an absolute benefit of 4-5% in 5-year survival rates (5, 6). However a subset of stage I patients also have poor prognosis and it is important to identify these high-risk patients who might benefit from an additional therapeutic intervention.

Molecular biomarkers incorporated with clinicopathological factors might improve NSCLC patient management (7). *KRAS* and *TP53* mutations (8, 9), and gene-expression signatures can classify surgically-resected patients with different outcomes (10). MiRs are small non-coding regulatory RNAs that are upstream regulators of gene-expression and contribute to cancer development and progression by acting as oncogenes or tumor suppressor genes (11-13). MiRs are promising biomarkers and involved in regulating diverse biological processes such as cell proliferation, apoptosis, adhesion, migration, invasion and angiogenesis.

MiR-34b and miR-34c, two members of the miR-34 family, are encoded by a bicistronic transcript from chromosome 11q23 (14). Their expression may be induced by *TP53* in response to DNA damage or cell stress (15) as well as regulated by DNA methylation. The promoter regions of miR-34b/c are hypermethylated in several tumor types resulting in silencing of miR-34b/c expression (16-21). MiR-34b/c methylation was prognostic in NSCLC stage I patients (22), but this result has not been validated in an independent cohort. In addition, miR-34 family

acts as a tumor-suppressor among different tumor types, inducing a less aggressive phenotype (16, 20, 23, 24), however the functional role of miR-34b/c has not been examined in lung AC.

In the present study, we sought to determine the role of miR-34b/c methylation and expression in lung AC cell lines and primary tumors, the relationship to patient prognosis in two independent cohorts of early stage resected lung AC and the functional impact of miR-34b/c ectopic expression on lung AC invasion and proliferation.

Material and Methods

Tissue samples

Frozen primary tumors and associated nonmalignant lung tissue of 140 patients with stage I-II lung ACs who underwent surgical resection were collected at the Bellvitge Hospital in Barcelona (2001-2007) and the University of Michigan Health System, Ann Arbor (1991-2007). Informed consent, approved by the respective Institutional Review Board, was obtained and specimens collected, immediately frozen and stored at -80°C. Regions containing >70% tumor cellularity were used for nucleic acid isolation. Patients receiving preoperative radiation or chemotherapy were not included. Clinical data was retrospectively collected and all cases were staged according to the revised 7th TNM classification and provided in **Suppl. Table S1**. Patients from the University of Michigan were older and included more females and former-smokers than the Bellvitge Hospital set. These differences reflected specific patterns of lung AC according to patient's site (25).

Lung cancer cell lines

Fifteen human lung AC cell lines (SK-LU-1, NCI-H2228, NCI-H1838, NCI-H1563, NCI-H2347, NCI-H1395, Calu-3, A549, NCI-H2087, NCI-H1299, NCI-H838, NCI-H23, NCI-H1792,

HCC4006 and HCC827) were purchased from American Type Culture Collection (Manassas, VA) and authenticated by genotyping using the Identifier Plus kit (Applied Biosystems). All cells were maintained in RPMI-1640 (except Calu-3 and A549, which used Eagle's minimum essential medium and DMEM-F12 medium respectively), and supplemented with 10% FBS, 1% Gibco® Antibiotic-Antimycotic (Life Technologies) in an atmosphere containing 5% CO_2 at 37°C. Cell line mutational status was obtained from the IARC *TP53* database (26) and Sanger Institute Catalogue of Somatic Mutations In Cancer web site (27) with nine harboring *TP53* mutations or *TP53* deletion and listed in **Suppl. Table S2**.

Chemicals and demethylation treatment of lung AC cell lines

Stock solutions of 1mM cis-diammine-dichloroplatinum (Sigma-Aldrich), 1mM pemetrexed (Lilly), 10mM erlotinib hydrochloride (Selleckchem) and 25mM 5-aza-2'-desoxycytidine (5-aza-dC, Sigma-Aldrich) were stored at -20°C and freshly dissolved in culture medium before use. SK-LU-1, NCI-H2228, NCI-H1838, NCI-H23 and HCC4006 cells were seeded in 6 well plates, cultured for 24 hours, and treated with 0.5 μmol/L of 5-aza-dC for 5 days in triplicate, replacing drug-containing medium daily. The cells were subjected to DNA and RNA isolation for direct sequencing and miR-34b/c quantification, respectively.

Bisulfite genome sequencing (BGS)

DNA was isolated using the proteinase K/phenol extraction method. Bisulfite conversion was carried out using 1 μg of DNA using an EZ-DNA Methylation Gold kit (Zymo Research). The PCR was performed using Immolase DNA Polymerase (Bioline) and the following primers: 5'-GGTTGGGAATTGAAGTTTG-3' (F) and 5'-TTAATAATTATAACCACCACAATACAA-3' (R). The reactions were cycled at 95°C for 10 minutes, then 30 cycles of 30 seconds (sec) at 95°C, 30 sec at 58°C and 30 sec at 72°C, including a final extension step for 15 minutes. PCR products were gel-purified using QIAquick Gel Extraction kit (Qiagen) and cloned into pCR®4-

TOPO® Vector (Invitrogen) using the TOPO® TA Cloning® kit (Invitrogen). Five individual clones were sequenced using M13 primers at the University of Michigan DNA Sequencing Core. The region assessed by BGS included 45 CpG sites from the miR-34b/c promoter and average methylation from individual clones was calculated as a percentage of the number of methylated CpG sites over the number of total CpG sites sequenced.

Real-time PCR temperature dissociation (melting curve analysis, MCA)

Genomic DNA treated with *Sss*I methylase (New England Biolabs) was used as positive control to amplify fully methylated DNA for the dissociation curve. Whole genome amplified DNA obtained by REPLI-G kit (Qiagen) was used as a negative control to amplify fully unmethylated DNA for dissociation curve. Methylation standards (100%, 75%, 50%, 25%, 10%, 5%, 1% and 0%) were prepared by mixing the positive and negative controls accordingly. All primers used for BGS and MCA were designed using the Methyl Primer Express v1.0 and did not target CpG dinucleotides in order to specifically amplify the bisulfite-modified sequences. Melting curve analysis was carried out as described before (28). Bisulfite converted DNA was first amplified in a 20 cycle external PCR reaction using the primers and conditions as described for BGS. One µl of amplified DNA was used as a template for a nested PCR using a LightCycler 480 II (Roche Applied Science) in the presence of LightCycler 480 SYBR Green I Master (Roche) and the following oligonucleotides: 5'-GTATTTTTGGGGGTTATGG-3' (F) and 5'-TCAACTAATAACACTACCTACAAACC-3' (R). The reactions were cycled for 30 cycles of 10 sec at 95°C, annealing at 67°C for 20 sec and extension at 72°C for 15 sec. After the amplification, temperature was gradually increased from 65°C to 95°C to obtain the melting curves. Lightcycler480 software (Roche) plotted the melting peaks by calculating the negative derivative of fluorescence over temperature and quantified the area under the curve (AUC) for the melting peak corresponding to the unmethylated and methylated alleles. AUC values for the melting peak corresponding to the methylated alleles were highly correlated with the percentage

of methylated DNA of the methylation standards, shown in **Suppl. Fig. S1**. In addition, the percentage of DNA methylation was estimated based upon the linear regression between the AUC values and the methylation standards. The estimated value of DNA methylation was normalized within each cohort by subtracting the mean and dividing by the standard deviation. Samples were classified as methylated when the estimated methylation value was higher than 5%.

RNA isolation and miR and mRNA quantification in cell lines and tumors

Total RNA was isolated using miRNeasy kits (Qiagen) following manufacturer's instructions. Expression of mature miR-34b and miR-34c was assessed by real-time RT-PCR (qRT-PCR) using TaqMan Micro-RNA assays (Applied Biosystems) and the 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Expression of reported miR-34b/c targets (*AXL, BCL2, HMGA2, MET, NOTCH1 and NOTCH2*) was assessed by qRT-PCR in miR-34b/c transfected cells (14, 29). cDNA was prepared from total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following manufacturer's instructions and using Power SYBR Green and the 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Primers, designed using Primer-BLAST (30), are shown in **Suppl. Table S3**. Data were analyzed using the SDS 2.2.2 software (Applied Biosystems) with threshold setting at 0.2 and manual baseline from 3 to 18 cycles. Relative quantification was performed by using the $2^{-\Delta\Delta Ct}$ method, using endogenous RNU48 and ß-Actin expression as controls for miR and mRNA quantification, respectively.

Plasmid construction and stable transfection

The miR-34b/c was subcloned into pSilencer 4.1-CMV puro Expression Vector (Ambion) containing the flanking regions of the mature miR-34b/c (16). Ten µg of the constructed plasmid and the empty vector were introduced into H1838 and SK-LU-1 cells using FUGENE 6

Transfection Reagent (Promega). 48 hours post-transfection, cells were cultured in selection media with puromycin (Sigma-Aldrich). Resistant clones were selected for further cell culture and experiments.

Western blot

Cells were grown to 60% confluence and harvested in RIPA buffer supplemented with proteinase inhibitor to extract protein. 10-20 µg of protein were separated by SDS-PAGE, transferred to PVDF membranes, and incubated overnight at 4°C with the primary antibodies: anti-MET (25H2, Cell Signaling), anti-PARP antibody (Cell Signaling), anti-BCL2 (clone 10, Millipore) and ß-actin (AC-15, Abcam) followed by goat anti-rabbit (Cell signaling) or anti-mouse (Southern Biotech) IgG-conjugated horseradish peroxidase (HRP) and detected by chemiluminescence using Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare Life Sciences).

Cell proliferation assay

Empty vector and miR-34b/c stably transfected H1838 and SK-LU-1 cells were plated in 96-well plates for 24 hours. Chemosensitivity was tested by treating miR-34b/c stably transfected cells with cisplatin, pemetrexed and erlotinib for 48 hours or with drug-free medium. The cell proliferation and viability was assessed using 10µl/well of WST-1 reagent (Roche). Absorbance at 450 nm and reference at 630 nm were measured with an automated plate reader (ELx808 Bio-Tek) at different time-points. Relative proliferation rates were calculated as a percentage of the initial T_0 reading within each sub-cell line.

Apoptosis assay

Both empty vector and miR-34b/c stably transfected SK-LU-1 cells were plated in 60 mm plates, treated for 48 hours with 5 μmol/L of cisplatin, harvested and processed for Western Blot.

Wound-healing assay

Empty vector and miR-34b/c stably transfected cells were grown to confluence. A wound was made through the monolayer using a p20 tip and measurements taken over time to calculate the migration rate according to the equation: percentage wound healing = ((wound length at 0 h) - (wound length at 4, 6, 20 or 27 h)) / (wound length at 0 h) x 100. Two independent experiments were performed.

Trans-well invasion assay

Cancer cells were re-suspended in media without growth factors then seeded at 25,000 cells per well into matrigel-coated (BD Matrigel), growth-factor-reduced, invasion chambers (8 μm pore size, BD Biosciences). The bottom chamber contained 20% FBS media as chemo-attractant and incubated overnight in a humidified incubator at 37°C, 5% CO_2 atmosphere. The top non-invading cells were removed with a cotton swab moistened with medium and the lower surface of the membrane was stained with Diff-Quick Stain Set (Siemens). The number of cells migrating to the basal side of the membrane was visualized with an Olympus microscope at 20x magnification. Pictures of five random fields from replicate wells were obtained and the number of cells stained quantified relative to the migration of cells through the uncoated membrane.

Statistical analysis

Differences between both cohorts used in the methylation analysis were calculated with Student's t-test and Fisher's exact tests. Non-parametric tests were used for differences in miR-34b/c expression or methylation between different clinical variables. Survival curves were

plotted using the Kaplan-Meier method and survival differences were assessed using the log-rank test. The univariate and multivariate Cox proportional hazards model were used to assess survival results. Disease-free survival (DFS) was measured from the date of surgery to the time of recurrence, death or censoring. Overall survival (OS) was measured from date of surgery to the time of death or censoring.

Results

MiR-34b/c methylation and underexpression are a frequent alteration in lung AC cell lines

MCA was used to assess miR-34b/c methylation in lung AC cell lines and BGS performed to confirm results in selected cell lines and primary tumors. Representative examples of MCA are shown in **Fig. 1**. An excellent concordance between the results obtained by both methods was observed. Based upon BGS, NCI-H2087 and NCI-H1838 cells had 3% and 78% of 45 CpG sites methylated respectively whereas the estimated percentage of methylation by MCA was 0.5% and 77%. The concordance was also optimal for primary tumors with BGS demonstrating 59% methylation of CpG sites, whereas the estimated percentage of methylation by MCA was 53% **(Fig. 1).** MCA is a suitable alternative for reliable, efficient and quantitative assessment of DNA from both cell lines and clinical samples.

Six of the lung AC cell lines (40%) had miR-34b/c promoter methylation: H2228, SK-LU-1, H1838, H1563, H2347 and H1395. Expression of miR-34b/c was lower in cell lines with miR-34b/c methylation relative to unmethylated cell lines (P = 0.001 for both miRs) and not different according to *TP53* status. Interestingly, a significant difference in miR-34b/c expression was detected when the cells were classified based upon the promoter methylation status and their *TP53* mutational status (P = 0.011 and 0.015, for miR-34b and miR-34c respectively). As shown

in **Fig. 2A**, the cells with lowest expression contained miR-34b/c promoter methylation and *TP53* mutation.

The miR-34b/c expression was significantly increased from 29- to >500 fold when cells with hypermethylated miR-34b/c promoter (SK-LU-1, H2228 and H1838) were treated with 5-aza-2'-deoxycytidine (5-aza-dC) for 5 days (**Suppl. Fig. S2A**). A relative change in the methylation level was detected by direct bisulfite sequencing in cell lines treated with 5-aza-dC (**Suppl. Fig S2B**).

MiR-34b/c methylation and underexpression are a frequent event in lung AC tumors

MiR-34b/c methylation was assessed by MCA in 140 lung AC tumors and 10 nonmalignant lung tissues. Fifty-nine tumors (42%) showed more than 5% DNA methylation (**Suppl. Fig. S3A**) and only one nonmalignant lung tissue was methylated, which was confirmed by bisulfite sequencing. Expression of miR-34b and miR-34c were quantified in a subset of 49 early stage lung AC tumors and 10 matched nonmalignant lung samples. Nonmalignant lung samples had a higher expression of both miRs in non-tumoral samples (median: 3.67 for miR-34b and 2.83 for miR-34c) as compared with lung AC tumors (median: 0.95 for miR-34b and 0.86 for miR-34c; P = 0.002 and 0.003, respectively). 30 tumors (61%) were methylated and had lower expression levels of miR-34b and miR-34c (median: 0.73 and 0.62, respectively) as compared to unmethylated tumors (median: 4.46 and 2.57, P = 0.002 and 0.003, respectively, **Suppl. Fig. S3B**).

MiR-34b/c methylation/expression and clinicopathological correlations

The level of methylation according to AUC values was correlated with clinical characteristics and survival (**Table 1**). MiR-34b/c methylation was associated with higher tumor stage among the 140 stage I and II patients examined (P = 0.033), recurrence (P = 0.017) and

death (*P* = 0.019). Tumors from patients with a smoking history had a trend of higher levels of miR-34b/c methylation.

We determined the correlation between miR-34b/c expression with clinical and molecular variables for the 49 cases analyzed (**Suppl. Table S4**). Interestingly, lower expression of miR-34b was detected among smokers compared to nonsmokers (*P* = 0.025). Tumors harboring *TP53* mutation had a trend of lower miR-34b and 34c expression (*P* = 0.043 and 0.060, respectively). A significant difference in the expression of miR-34b/c was observed in primary tumors when classified based upon the promoter methylation status and the *TP53* mutational status (*P* = 0.0007 and 0.001, respectively, **Fig. 2B**). MiR-34b/c methylation appears to be the predominant mechanism of regulation of miR-34b/c expression in cooperation with *TP53* function. Indeed, tumors with methylated miR-34b/c consistently expressed low levels of miR-34b/c even when *TP53* was not mutated. Of note, higher miR-34b/c expression was associated with *EGFR* mutation (*P* = 0.007 and 0.013, respectively, **Suppl. Table S4**). MiR-34b/c expression was not associated with outcome as previously reported by others (31).

MiR-34b/c methylation as independent prognostic marker in early stage resected lung AC

We determined the prognostic value of miR-34b/c methylation in the training set and we found that patients with higher AUC for miR-34b/c methylation (top third) had a significantly shorter DFS (median: 28.5 months) compared to patients with low AUC levels (bottom two thirds) (median: not reached, Log-rank test *P* = 0.016, **Fig. 3A**). We used two thirds cut-off (AUC = 1.34, estimated methylation = 20%) instead of the median, because it provided a higher percentage of events within the group of high level of DNA methylation. In the multivariate Cox model, higher levels of miR-34b/c methylation (top third) were independently associated with shorter DFS (HR = 3.04, 95% CI 1.26 - 7.03, *P* = 0.013) as compared to low-medium levels, after adjusting for age, sex and disease stage. In the test set, high (top third) also had a shorter DFS (median: 18.6 months) compared to patients with low AUC (bottom two thirds) (median: not

reached, Log-rank test *P* = 0.005, **Fig. 3B**). In the multivariate analysis, high levels of miR-34b/c methylation (top third) were associated with shorter DFS (HR = 2.05, 95% CI 1.09 - 3.84, *P* = 0.025).

When the survival analysis was carried out using the whole study population (*n* = 140), miR-34b/c methylation not only was associated with shorter DFS, but also with shorter OS (Kaplan-Meier plots are shown in **Suppl. Fig. S4**). In the multivariate Cox regression analysis, higher levels of miR-34b/c methylation (top third) was independently associated with a shorter DFS (HR = 2.16, 95% CI 1.32 - 3.52, *P* = 0.002, **Table 2**) as compared to low to medium levels (bottom two-thirds). MiR-34b/c remained an independent prognostic marker when considered as continuous variable in the Cox regression (**Suppl. Table S5**). Higher levels of miR-34b/c (top third) were also independently-associated with shorter OS (HR = 1.79, 95% CI 1.07 - 3.02, *P* = 0.027, **Suppl. Table S6**) as compared to low-medium levels. Remarkably, methylation of miR-34b/c was also a prognostic marker for stage I patients and patients with high AUC for miR-34b/c had a shorter DFS (median: 43.1 months) as compared to those with low to medium levels (median: not reached, Log-rank test *P* = 0.009, **Suppl. Fig. S5**).

MiR-34b/c ectopic expression reduced cell proliferation, migration and invasion

Two cell lines with low levels of miR-34b/c (H1838 and SK-LU-1) were transfected with miR-34b/c precursors that mimics endogenous miRs, or alternatively with empty vector. Expression of miR-34b/c determined by qRT-PCR in the stable transfectants was significantly higher compared to empty vector and parental cells (**Suppl. Fig. S6A**). Several genes reported as putative targets of miR-34b/c were examined and a significant reduction of the transcript levels were found for *AXL*, *BCL-2, HMGA2, MET, NOTCH1* and *NOTCH2* in both transfected cell lines (**Suppl. Fig. S6B**). At the protein level significant reduction of MET expression was observed (**Suppl. Fig. S6C**).

Cells expressing miR-34b/c showed a significantly lower proliferation rate as compared to empty vector ($P < 0.001$, **Fig. 4A**). Accordingly, stable transfection with miR-34b/c mimics resulted in significantly increased PARP cleavage (**Suppl. Fig. S7A**). We treated the stable cell lines with cisplatin (CDDP), pemetrexed or erlotinib, all of which are currently used for treating lung AC. A modest sensitization effect to CDDP was observed after restoring miR-34b/c expression in SK-LU-1 cells (**Suppl. Fig. S7B**). Migration and cell invasion was significantly suppressed in miR-34b/c stable transfectants of SK-LU-1 (**Fig. 4B, 4C and Suppl. Fig. S8**) as compared to empty vector or parental cells.

In summary, the restoration of miR-34b/c expression in lung AC lines can reduce cell proliferation, cell migration and invasion, conferring a less aggressive phenotype. Re-expression thus might be a novel potential therapeutic strategy in patients expressing low levels of miR-34b/c.

Discussion

MiRs play an important role in tumorigenesis and cancer progression. The discovery of their regulatory function has added a new level of complexity in our understanding of cancer genetics (32). Interestingly, miR expression is often widely down-regulated in cancer cells relative to normal tissues (33), and forced reduction of global miR expression promotes transformation (34). MiR expression is deregulated in human cancer, including NSCLC and several regulatory mechanisms have been identified: DNA copy abnormalities (35), mutation (36), failure of post-transcriptional regulation (37), regulation by transcription factors (38), and a defective miR biogenesis pathway (34). Additional epigenetic mechanisms such as methylation of the 5' regulatory regions associated with specific miR down-regulation in tumors were observed (16, 39, 40).

The miR-34 family consists of miR-34a, located at the chromosome 1, and miR-34b and miR-34c, located at the chromosome 11 as a bicistronic cluster and primarily expressed in non-tumoral lung (23). The putative promoter of miR-34b/c resides 4.5 kb upstream of the miR-coding sequence and includes a dense CpG island and the starting transcription site of *BTG4* gene (21). We found that hypermethylation of miR-34b/c is frequent in lung AC tumors and cell lines (40-46%), which is similar to the frequency reported previously by other groups in NSCLCs (22). Interestingly, we observe patients with higher levels of miR-34b/c methylation show a worse outcome. A recent study reported that miR-34b/c methylation might be prognostic for stage I NSCLC patients (22). In this study, DNA methylation was assessed by Methylation-Specific PCR from formalin-fixed paraffin tissue samples. We used frozen samples and DNA methylation was assessed using MCA, which achieved an excellent analytical sensitivity. We feel that MCA is a useful technique for detecting and estimating DNA methylation by simultaneous assessment of multiple CpG residues, rendering the technique less vulnerable to the behaviour of specific residues. When analyzing other markers using MCA, it is important to determine the analytical sensitivity for any specific gene to define a cut-off for classifying the samples as either methylated or unmethylated. While focusing on lung AC, we validated the prognostic value of miR-34b/c methylation in an independent cohort of these patients.

According to previous data (23), global association between *TP53* mutational status and miR-34b/c expression was not found in lung AC lines. Indeed, although miR-34b/c are *bona fide* transcriptional targets of *TP53* and their promoters contain TP53-canonical binding sites, miR-34 family appeared to be not necessary for *TP53* function using a miR-34 deficient mouse (41). A potential interaction between DNA methylation status and *TP53* status was found in lung AC cell lines and primary tumors. In addition, the restoration of miR-34b/c expression with 5-aza-dC suggests that DNA methylation plays a role in the transcriptional regulation of these miRs in lung AC cells.

Interestingly, miR-34b expression was higher in nonsmoking patients and both miRs were significantly overexpressed in *EGFR* mutant cell lines and primary tumors. Although nonsmoking patients had lower levels of miR-34b/c expression as compared to smokers, we did not find a significant correlation between tobacco use history and DNA methylation. Similarly, during bronchial carcinogenesis of squamous cell carcinomas, expression of miR-34c progressively was reported to decrease from normal epithelium of nonsmokers to invasive bronchial lesions of smokers (42).

The role of miR-34b/c expression as a prognostic marker is controversial. Landi et al (43) found that miR-34b/c expression measured by microarray technology was associated with outcome in surgically-resected lung NSCLC patients. However miR-34b/c measured by qRT-PCR was not prognostic in a large cohort of early stage NSCLC (31). In our study, we could not find an association between miR-34b/c expression and outcome potentially due to the limited number of cases analyzed.

We used stable transfectants expressing miR-34b and 34c to study the pathogenic role of these miRs in lung AC lines. Using this *in vitro* model, we found that the restoration of miR-34b/c expression suppressed cell proliferation, migration and invasiveness. These findings suggest that these miRs might act as a tumor suppressor in lung AC, which is consistent with its role in other human cancers (20, 24, 44, 45).

MiR-34b/c restoration did not considerably modify the sensitivity to CDDP, pemetrexed or erlotinib in lung AC lines. Correspondingly, miR-34b/c expression was previously not found to have a predictive effect on survival in lung cancer patients treated with adjuvant chemotherapy after tumor resection (31). However, miR-34c appears to be significantly overexpressed in erlotinib-sensitive NSCLC cell lines (46) and was associated with a signature predictive for response to erlotinib. In our study miR-34b/c transfectants did not show higher sensitivity to erlotinib (data not shown).

Several strategies have been proposed to restore the function of miRs with tumor suppressor properties that are down-regulated in cancer (47), for example DNA demethylating agents and histone deacetylase inhibitors, which have demonstrated anti-tumor activity in chemo-refractory NSCLC (48). However a limitation of this strategy is that these agents lack specificity. A more promising strategy may be miR replacement therapy using delivery vehicles (49, 50), which are able to restore loss of function activity and to reactivate cellular pathways in cancer that drive a therapeutic response.

In conclusion, our findings show that miR-34b/c is frequently inactivated by promoter DNA methylation in lung AC and restoration of miR-34b/c expression induces a less aggressive and invasive phenotype. In addition, miR-34b/c methylation might be an independent prognostic marker in early stage lung AC and is potentially useful for selecting a subset of stage I tumors with higher risk of recurrence or death after lung resection that would benefit from an additional therapeutic intervention. MiR replacement therapy might be a potential strategy for treating those tumors with hypermethylated miR-34b/c, although further investigation in this area is needed.

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Table 1. Correlation between miR-34b/c DNA methylation and clinicopathological characteristics (*n* = 140).

Clinical covariates	Total	miR-34b/c methylation status		*P-value*
		AUC, mean (±SD)	AUC (mean rank)	
Age				
<65 years	60	1.82 (±2.72)	70.69	
≥65 years	80	1.48 (±2.09)	70.36	ns
Sex				
Female	53	1.76 (±2.05)	76.36	
Male	87	1.54 (±2.56)	66.93	ns
Smoking history				
Never	15	1.07 (±1.39)	64.14	
Ever	123	1.65 (±2.41)	70.15	ns
Stage				
Stage I	96	1.35 (±2.21)	65.73	
Stage II	44	2.22 (±2.65)	80.91	0.033
Recurrence				
No	73	1.12 (±1.85)	62.97	
Yes	67	2.18 (±2.75)	78.71	0.017
Recurrence location				
Loco-regional	21	1.87 (±2.33)	34.43	
Distance	46	2.32 (±2.93)	33.80	ns
Life status				
Alive	63	1.25 (±2.16)	63.31	
Dead	77	2.06 (±2.55)	78.80	0.019
Event (death or recurrence)				
No	68	0.93 (±1.64)	60.17	
Yes	72	2.28 (±2.76)	80.26	0.002

AUC: area under the curve.

Table 2. Disease-free survival analysis (Multivariate Cox model) of miR-34b/c methylation in 140 early stage lung AC patients.

Covariates in the model		Hazard Ratio	95% confidence interval	*P-value*
Age, continuous		1.02	0.994 - 1.05	ns
Gender	Female	1.00	-	
	Male	1.17	0.72 - 1.92	ns
Stage	Stage I	1.00	-	
	Stage II	2.73	1.69 - 4.40	<0.001
miR-34b/c	Low to medium	1.00	-	
methylation	High (AUC > 1.34)	2.16	1.32 - 3.52	0.002

Main Figures legends.

Figure 1. Methylation status of miR-34b/c in lung AC cell lines and primary tumors. (**A** and **B**) Representative examples of MCA for miR-34b/c. Melting curves and derivative peaks from two cell lines (H2087 and H1838) are shown in the left panel (**A**) and from a lung AC and its corresponding nonmalignant lung tissue in the right panel (**B**). Differences in the GC content after bisulfite treatment results in variation of the melting temperature that make possible to discriminate the methylation status of samples assessed. Whereas homogeneously unmethylated or methylated samples yielded a sharply defined melting curve, heterogeneously methylated samples resulted in a double-peak pattern. This bimodal pattern may correspond to the presence of non-tumoral DNA from stromal cells within the tumoral samples or also may correspond to methylation that affects only one allele. (**C** and **D**) Representative chromatograms from bisulfite genomic sequencing of the miR-34b/c promoter. CpG sites are depicted as pink bars and the transcription start sites as blue bars. Five clones of each sample were sequenced and each row represents one sequenced allele. Black and white squares represent methylated and unmethylated cytosines at CpG sites, respectively.

Figure 2. MiR-34b/c expression in lung AC cell lines (**A**) and primary tumors (**B**). We observed a strong correlation between DNA methylation, *TP53* status and miR-34b/c expression. These boxplots show miR-34b/c expression (log2) in a set of 15 lung AC cell lines (**A**) and 49 lung AC tumors (**B**) according to DNA methylation (M, methylated; U, unmethylated) and *TP53* status (mut, mutated; WT, wild type). *P* values correspond to Kruskal-Wallis test among all 4 categories.

Figure 3. Kaplan-Meier plots of DFS according to miR-34b/c methylation level. Lung AC tumors were divided according to their source into training (Bellvitge Hospital, **A**) and test set (University of Michigan, **B**).

Figure 4. Impact of miR-34b/c on lung AC cell proliferation, migration and invasion. (**A**) Cell proliferation of miR-34b/c transfected cells was assessed using WST-1 assay. Values are expressed as the means ± SD of 3 experiments. Cell proliferation was significantly lower in cells expressing miR-34b/c as compared to cells transfected with the empty vector (ev). (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (**B**) Wound healing assay in SK-LU-1 cells. Quantitative values indicate the mean distance ± SD between the migration fronts measured at different time-points per 3 wells and are representative of 3 experiments. Cells expressing miR-34b/c closed the induced cell wound significantly slower than cells transfected with ev. (**C**) Invasion experiments using Boyden chamber in SK-LU-1 cells. Invasive cells stained by Diff-Quick were counted. The quantitative values represent the mean ± SD of 5 microscopic fields per 2 wells and are representative of 2 experiments. Cells expressing miR-34b/c were significantly less invasive as compared to control ev transfected cells.

Figure 1.

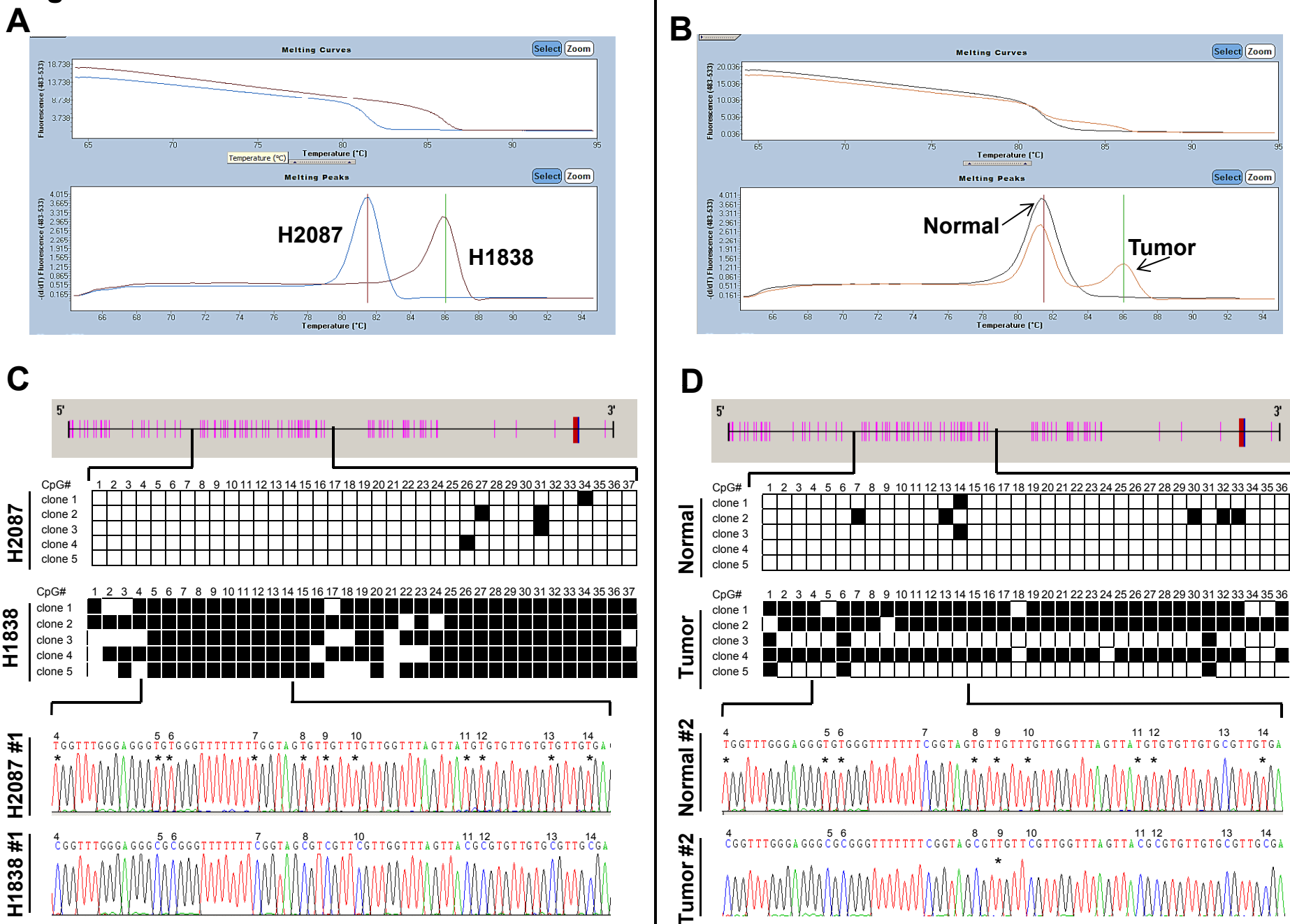


Figure 2.

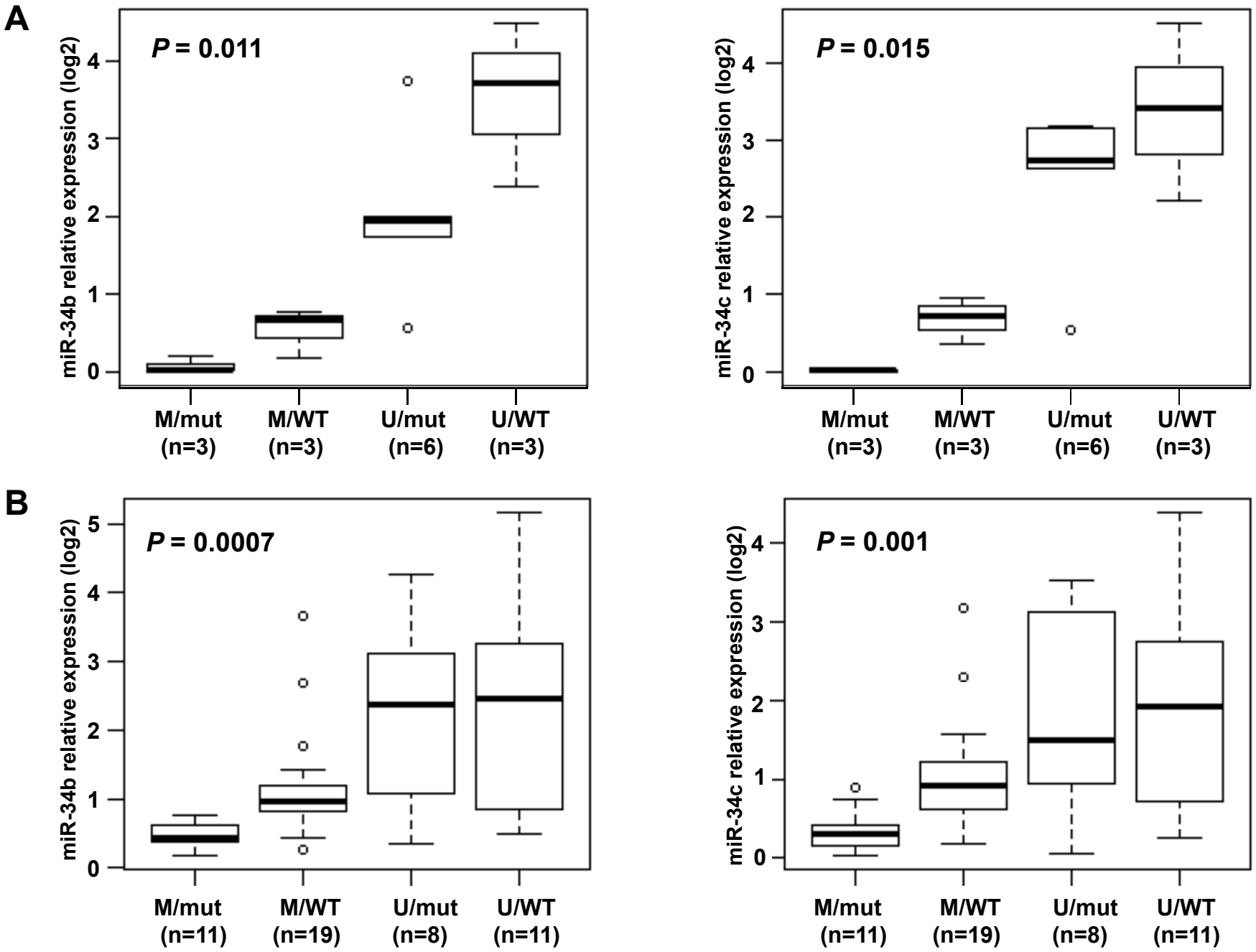
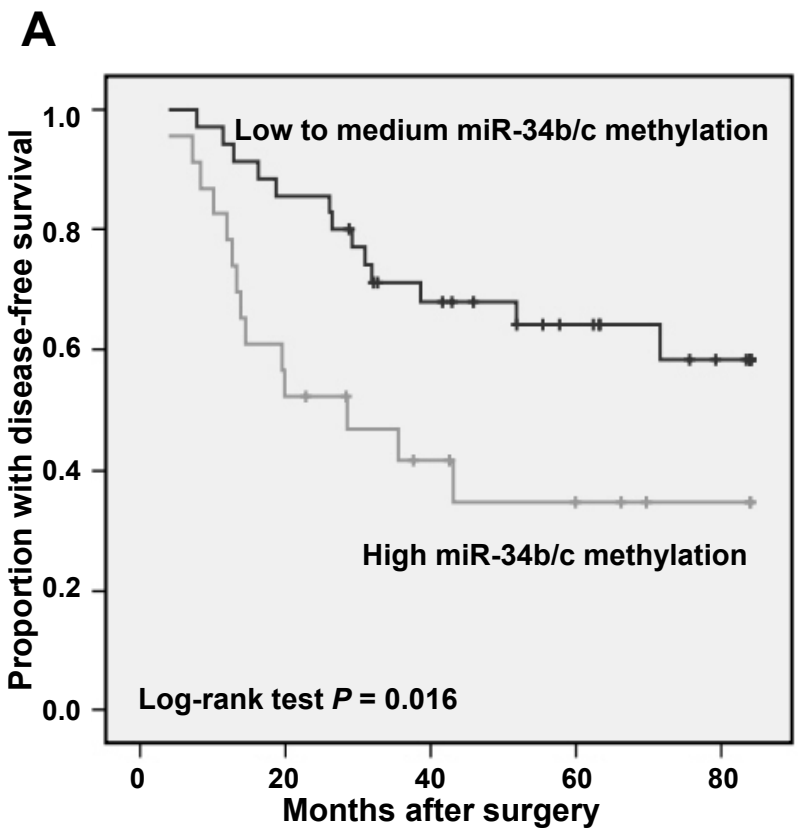
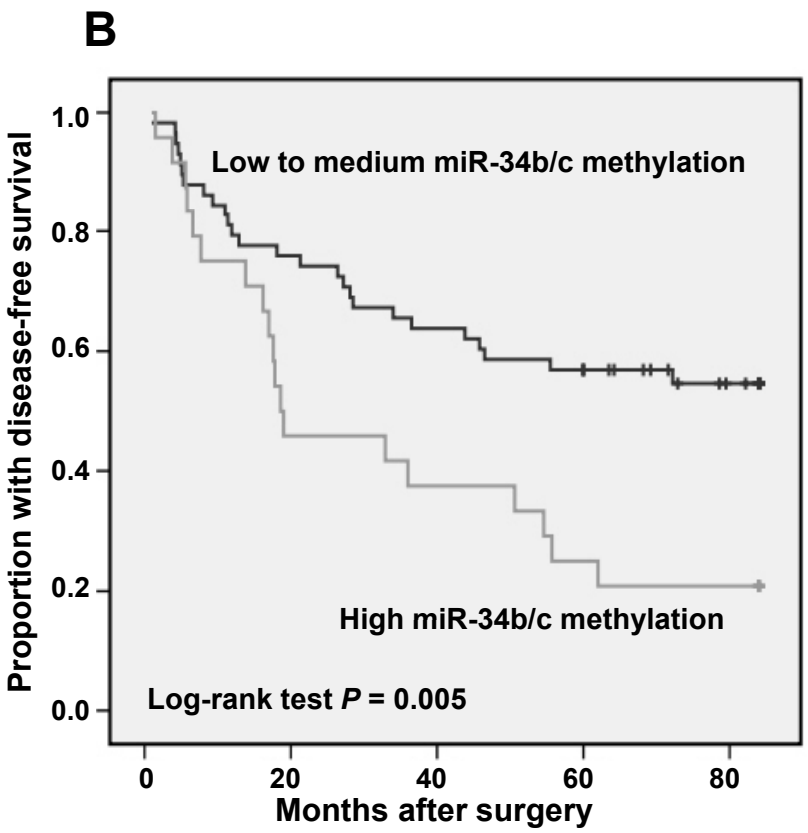


Figure 3.

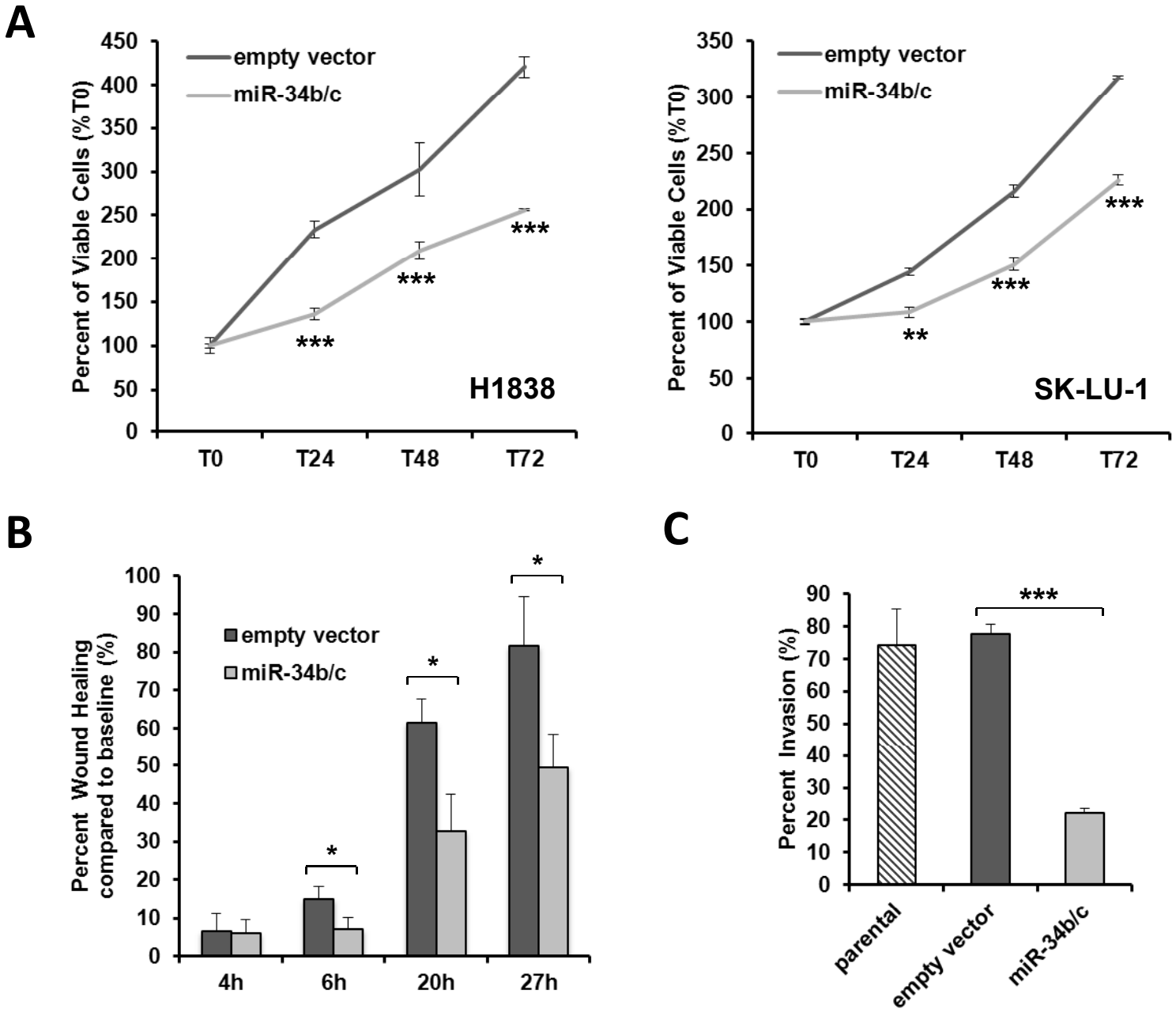




miR-34b/c methylation	*n*	Events (%)	Median DFS (95% CI)
Low to medium	35	13 (37%)	Not reached
High (AUC > 1.34)	23	14 (61%)	28.5 (6.01-50.9)

miR-34b/c methylation	*n*	Events (%)	Median DFS (95% CI)
Low to medium	58	26 (45%)	Not reached
High (AUC > 1.34)	24	19 (79%)	18.6 (0.24-36.9)

Figure 4.



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Epigenetic inactivation of microRNA-34b/c predicts poor disease-free survival in early stage lung adenocarcinoma

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