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ORIGINAL ARTICLE

Regulation of Axl receptor tyrosine kinase expression by miR-34a and miR-199a/b in solid cancer

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Axl is a receptor that induces proliferation, migration and invasion in cancer. In this study, we show that specific microRNAs (miRNAs) target the 3′-UTR of Axl. Luciferase-reporter assays with wild-type and deleted miR-34 and miR-199a/b seed sequences of Axl 3′-UTR confirmed the specificity of targeting. An inverse correlation between Axl protein and miR-34a expression in a panel of non-small cell lung cancer (NSCLC), colorectal cancer (CRC) and breast cancer (BRC) cell lines was observed, while miR-199a/b expression was completely suppressed. Pre-miR transfection inhibited *in vitro* migration and invasion and, *in vivo*, reduced the number of distant lung- or liver-metastases in a chorion-allantoic-membrane (CAM) assay. Moreover, methylation-specific PCR on bisulfite-converted DNA obtained from the cell lines showed that the miR-34a promoter methylation status was inversely correlated with its expression, and that miR-199a/b promoter regions were methylated in all cells tested. In a panel of NSCLC tissues ($n = 44$), miR-34a and miR-199a/b were found to be downregulated and significantly co-expressed. A lower expression of all three miRs was significantly associated with squamous histotypes, and, in a preliminary series, NSCLC patients with miR-34a upregulation showed a positive association towards a longer survival. These results indicate that Axl receptor expression can be regulated by miR-34a and miR-199a/b, which are suppressed by promoter methylation in solid cancer cells.

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Keywords: non-small cell lung cancer; breast cancer; colorectal cancer; Axl; microRNA

Introduction

Cancer is a complex disease occurring as a result of progressive accumulation of genetic aberrations and epigenetic changes that enable escape from normal cellular and environmental controls (Weinberg, 1995). Ninety percent of patient deaths in solid tumors are due to metastasis, therefore, an understanding of the process and pathogenesis at the systemic, cellular and molecular levels is one of the most ambitious goals in cancer research (Gupta and Massague, 2006; Esteller, 2007). Epigenetic changes in the promoters of classical and small non-coding (miRNA) genes has a pivotal role in the acquisition of tumorigenic and metastatic properties (Feinberg and Tycko, 2004; Dumont *et al*., 2008; Lujambio *et al*., 2008). Methylation occurs in specific genomic areas called CpG islands, especially within the promoter region of a gene, thereby preventing gene expression (Weber *et al*., 2007).

Axl is a 140-kDa protein, activated either with growth-arrest-specific gene 6 (Gas6) or homophilic interactions and activates different signaling molecules like phosphatidylinositol 3-kinase, Akt, Src, extracellular signal-regulated kinase and nuclear factor kappaB (Goruppi *et al*., 1997; Lee *et al*., 2002; Vajkoczy *et al*., 2006). Overexpression of *Axl* can transform fibroblasts even in the absence of a ligand (Burchert *et al*., 1998). Axl is known to induce cell survival (Melaragno *et al*., 2004; van Ginkel *et al*., 2004), proliferation (Stenhoff *et al*., 2004; Sainaghi *et al*., 2005), stimulation of cell migration (Fridell *et al*., 1998) and cell–cell adhesion (McCloskey *et al*., 1997). Moreover, an increased expression of *Axl* is associated with invasion, metastasis, angiogenesis, and is found in metastatic colon, prostate carcinoma, gastric and endometrial cancers, breast cancers, lung cancers and sarcomas (Hafizi and Dahlback, 2006). Axl is transcriptionally regulated by Sp1/Sp3 transcription factors and further controlled by CpG island methylation (Mudduluru and Allgayer, 2008). Additionally, overexpression of MZF1 transactivates Axl gene expression and induces migration, invasion and *in vivo* metastasis formation (Mudduluru *et al*., 2010). However, little is known about the epigenetic regulation of Axl and especially its post-transcriptional regulation by microRNAs (miRNAs).

MiRNAs are a class of ~22 nt endogenous RNAs, which can be expressed in a cell and tissue-specific manner, influencing mRNA stability and translation. They control a wide range of biological functions such as cellular proliferation, differentiation and apoptosis (Zhao and Srivastava, 2007; Erson and Petty, 2008;

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Eulalio *et al*., 2008). Recent reports showed strong evidence that miRNAs can act as oncogenes or tumor suppressors, having key roles in cancer initiation and progression (Cho, 2007; Tili *et al*., 2007). In the attempt to understand which mechanisms underlie abnormal miRNA expression in cancer, an increasing number of studies have investigated how miRNAs are regulated, and it is now widely accepted that miRNAs undergo the same regulatory mechanisms as any other classical protein-coding genes, including epigenetic regulation (Valeri *et al*., 2009). Recent papers have demonstrated that the epigenetic regulation of miRNAs in cancer is a widespread phenomenon, for example, as shown for miR-9-1, miR-107, miR-127, miR-193a, miR-137, miR-342, miR-203, miR-34b/c and miR-1 (Lujambio and Esteller, 2009; Valeri *et al*., 2009).

All this emerging evidence prompted us to design a study on several non-small cell lung cancer (NSCLC), breast cancer (BRC) and colorectal cancer (CRC) cell lines, as well as in a case-series of resected tissues of NSCLC patients, to test the hypothesis and the possible impact of Axl regulation by miRNAs.

Results

An evolutionary conserved target sequence for miR-34a and miR-199a/b in the 3′-UTR of Axl

The 1868 nt 3′-UTR of Axl was screened for complementary seed sequences of known miRNAs via a bioinformatic search. A 100% match target sequence for miR-34a at nts 24–50, and for miR-199a/b at nts 25–56, was found (Figure 1a). The minimum free energy predicted for hybridization with the Axl 3′-UTR and miR-34a or miR-199a/b at their site is ΔG –31.8, ΔG –20.7 and ΔG –21.7 kcal/mol, respectively, determined by mFold analysis (Supplementary Figure S1), this being consistent with authentic miRNA targeting (Doench and Sharp, 2004). Comparing the human sequence for interspecies homology, we found that the miR-34a and miR-199a/b target sequences at nts 24–50 and at nts 26–54 of the Axl 3′-UTR are highly conserved among species (Figure 1b).

The Axl 3′-UTR is a target for miR-34a and miR-199a/b

Given the results from homology search across species, we asked whether the 3′-UTR of Axl is a functional target of miR-34a and miR-199a/b. We cloned a reporter plasmid driven by the cytomegalovirus basal promoter, harboring the 1834 nt 3′-UTR of Axl at the 3′-position of the luciferase-reporter gene (Axl 3′-UTR). We also cloned a shorter fragment (1741 nt 3′-UTR) by removing 93 nt at the 5′-end of the Axl 3′-UTR (Axl del-3′-UTR), which does not contain the seed sequences for miR-34a and miR-199a/b. As miR-199 expression was comparatively low in the screened cells (Supplementary Figure S4b), both of the constructs were transfected into high miR-34a-expressing cells (H460, MCF-7 and Colo 320). Axl del-3′-UTR significantly induced luciferase activity when compared with the complete Axl 3′-UTR construct (Figure 2a, left column panel) ($*P<0.05$). Furthermore, co-transfection of the Axl 3′-UTR along with AM-miR-34a significantly induced luciferase activity when compared with the corresponding control-miR (Figure 2a, middle column panel) ($*P<0.05$). Additionally, PM-miR-34a or PM-miR-199a/b were co-transfected along with the Axl 3′-UTR-luciferase construct into H1299, MDA-MB-231 and Rko cells, which lack endogenous expression of these three miRs, significantly reducing the luciferase activity when compared with control-miR (Figure 2a, right column panel) ($*P<0.05$). Taken together, these data suggest that the 3′-UTR of Axl is a functional target for miR-34a and miR-199a/b.

MiR-34a and miR-199a regulate Axl gene expression and competes for binding sequence

To corroborate the reporter assay results on miR-34- and miR-199-inhibited Axl regulation at the mRNA and protein level, H1299, MDA-MB-231, HCT116 and Rko cells were transfected with control-miR, miR-34a or miR-199a. As miR-199a and miR-199b have similar seed sequences and binds with more or less equal free energy to the Axl 3′-UTR, miR-199a was used for further transfection experiments. qRT–PCR results showed that both the miRs (miR-34a and miR-199a) significantly reduced Axl-transcript levels (Figure 2b). Furthermore, western blot analysis confirmed the downregulation of Axl protein amounts (Figure 2c). Similarly, other known targets of these miRs (miR-34a: c-Met and Notch 1; miR-199a: HIF1a) were screened through western blot analyses and observed the downregulation of the respective molecules by the respective miRs (Supplementary Figure S2) (Li *et al*., 2009; Yeligar *et al*., 2009). However, transfection efficiency of miR-34a/miR-199a and expression were measured with qRT–PCR (Supplementary Figure S3a). Taken together, our results suggest that both the miRs are regulating Axl at the transcript level, which downregulates protein amounts.

Additionally, experiments were carried by transfecting AM-miR-34a, which binds to endogenous miR-34a and thereby antagonizes its activity. As expected, in A549 cells Axl protein amounts were increased, but no Axl protein band was detected in MCF-7 and Colo 320 cell lines (Figure 2d). Transfection efficiency of the AM-miR-34a and expression levels were measured with qRT–PCR (Supplementary Figure S3b). This result is not surprising with MCF-7 and Colo 320 cells, as we know that Axl is also epigenetically regulated by hypermethylation of its promoter (Mudduluru and Allgayer, 2008).

As miR-34a and miR-199a seed sequences are partially overlapping, additional experiments were performed transfecting Rko and H1299 cells with PM-miRs, or with the combination of PM-miRs and AM-miRs, and by the analysis of Axl protein expression by western blot. The results showed that silencing of either miR-199a or miR-34a through the respective AM-miR mutually enhances the miR-34a/miR-199a efficiency in downregulating Axl, respectively (Figure 3). Compared with miR-34a, miR-199a showed to downregulate Axl

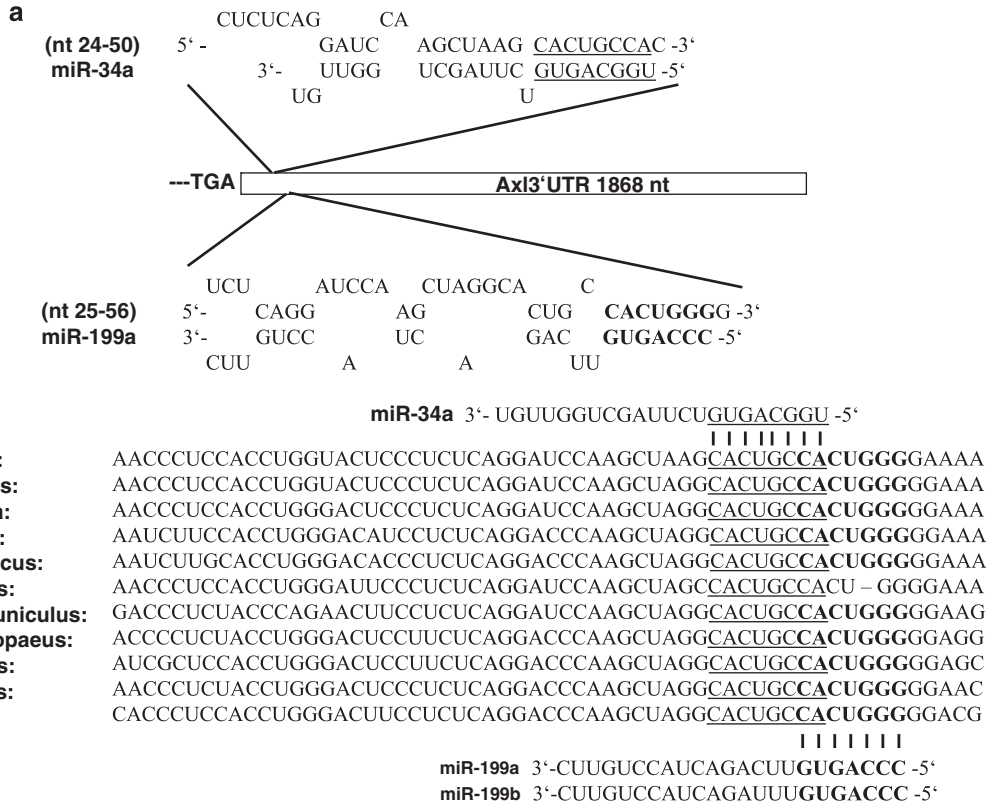


Figure 1 miR-34a and miR-199a/b target sites reside in close proximity with the Axl 3′-UTR, and are highly conserved across 11 species. (**a**) The location of the putative miR-34 and miR-199a/b target sites are shown with underlined and bold letters, respectively, unpaired bases are shown either above or below the duplexes. (**b**) Comparison of nucleotides between the miR-34 and miR-199a/b seed sequence and the target Axl site within the 3′-UTR across 11 species.

expression less efficiently (Figures 2c and 3). Moreover, an evident downregulation of Axl protein was observed after transfecting cells with AM-miR-199a and PM-miR-34a, while AM-miR-34a and PM-miR-199a co-transfection had poorly significant effects. These results indicate that miR-34a and miR-199a are competing for their seed sequence within the Axl 3′-UTR, miR-34a being more efficient than miR-199a in controlling Axl expression.

Axl-mRNA and Axl protein correlates inversely with miR-34a expression and invasion of NSCLC, BRC and CRC cell lines

The expression of Axl-mRNA, Axl protein, miR-34a and miR-199a/b were screened in a panel of NSCLC, BRC and CRC cell lines and correlated with cell invasive ability. MiR-34a, miR-199a/b and Axl endogenous expression was measured by qRT–PCR (Supplementary Figures S4a, b and S5a). MiR-199a/b endogenous expression was not detected in most of the screened cancer cells (data not shown). Axl protein amounts were estimated through western blot analysis and densitometry as the ratio Axl/β-actin (Supplementary Figure S5b, c). The invasive ability of NSCLC, BRC and CRC cells was measured by Matrigel assay and plotted as the percentage of invading cells after 15 h (Figure 4a). A statistically significant positive correlation between Axl-mRNA, or Axl protein expression and the invasion of NSCLC, BRC and CRC cells, respectively, was found ($R_S = 0.64$, $P = 0.001$; $R_S = 0.74$, $P < 0.001$). Moreover, an inverse correlation between miR34a expression and the invasiveness of NSCLC, BRC and CRC cells was found ($R_S = -0.45$, $P = 0.03$). Axl protein and miR-34a expression correlated inversely with a statistically positive association, when compared with all cell lines ($R_S = -0.38$, $P = 0.07$) (Figures 4b–e). However, specifically NSCLC cells showed statistically significant correlations (Supplementary Figure S6a–c). Correlations were evaluated by the Spearman's rank correlation method. Taken together, from these results we can hypothesis that Axl is positively regulating invasion, and miR-34a is inhibiting Axl expression and invasion.

MiR-34a and miR-199a inhibits in vitro *migration and invasion and* in vivo *distant metastasis*

To further investigate the ability of miR-34 and miR-199 to regulate diverse phenomena of the metastatic cascade, a wound-healing assay was performed with H1299 cells by transfecting control-miR, pre-miR-34a or pre-miR-199a (Figure 5a). Furthermore, a migration assay was performed with trans-well chambers by transfecting control-miR, pre-miR-34a or pre-miR-199a, into H1299 and Rko cells (Supplementary Figure S7). Moreover, Matrigel invasion assays were performed with highly invasive H1299, MDA-MB-231 and Rko

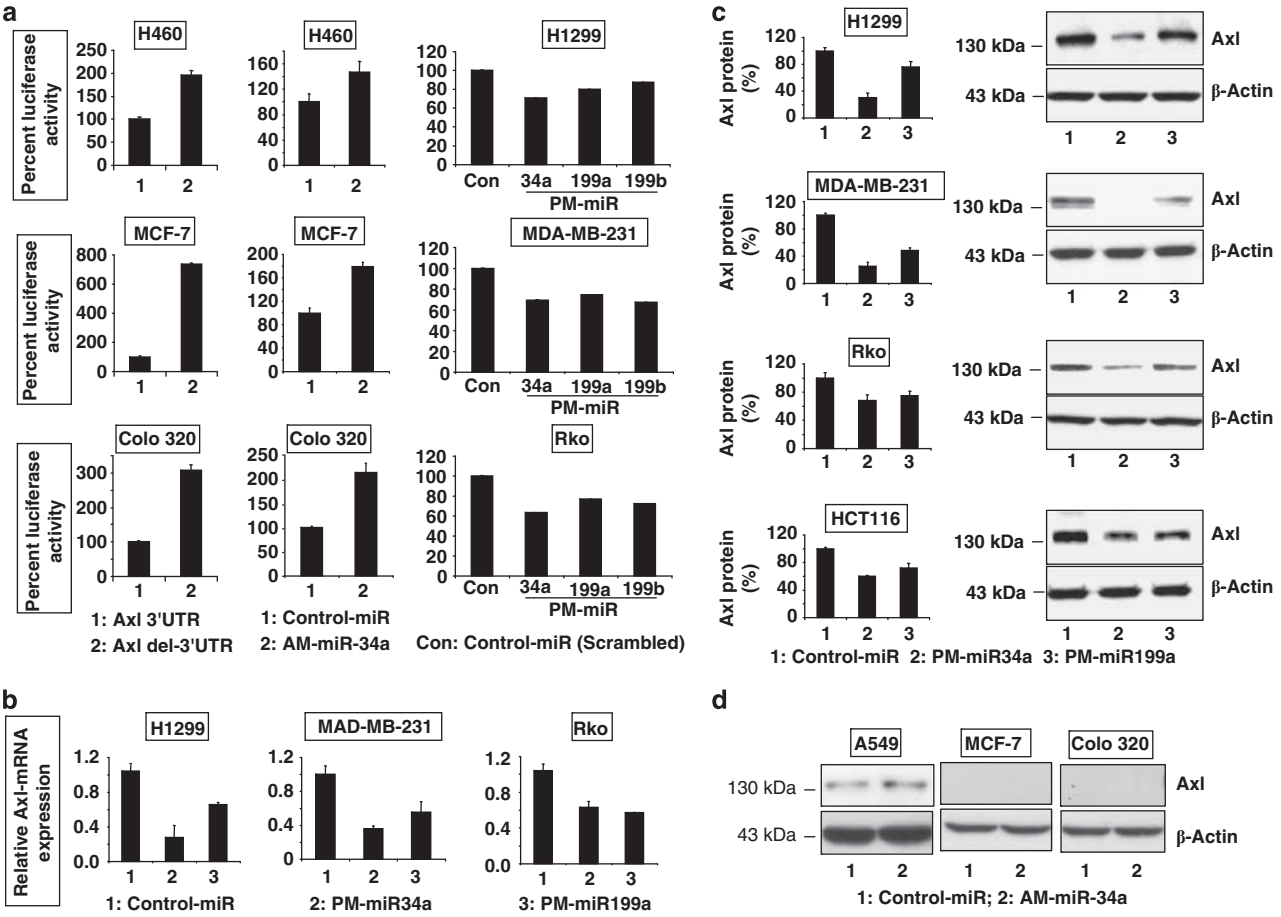


Figure 2 miR-34a and miR-199a/b target the Axl 3′-UTR and regulate Axl expression. (**a**) Luciferase-reporter assays of Axl 3′-UTR (complete 3′-UTR) and Axl del-3′-UTR (seed sequence of miR-34a and -199a/b, 5′ region of Axl-3′-UTR removed) in H460, MCF-7 and Colo 320 (left column) or Axl 3′-UTR with co-transfection of either with control-miR or AM-miR-34a in H460, MCF-7 and Colo 320 (middle column) or Axl 3′-UTR with co-transfection of either with control-miR or PM-miR-34a, PM-miR-199a/b in H1299, MDA-MB-231 and Rko (right column) as indicated. Percent luciferase activity was calculated either with Axl 3′-UTR or control-miR samples set as 100%. Each bar represents values of quadruplicates ($P \leqslant 0.05$) (s.d. is small). H1299, MDA-MB-231, HCT116 and Rko cells were transfected either with miR-34a or miR-199a. After 48 h, total RNA and protein was isolated, and (**b**) Axl expression levels were evaluated by RT–PCR ($P < 0.05$), (**c**) Axl protein amounts were estimated by western blot analysis and quantified by densitometry, density ratio of Axl/β-actin is represented as a bar graph. In control-miR transfected samples, Axl protein amounts were set as 100% and relative Axl protein amounts were calculated in PM-miR transfected samples and represented as indicated. Western blots were represented adjacent to the bar graphs, respectively. (**d**) A549, MCF-7 and Colo 320 were transfected either with control-miR or AM-miR-34a and after 48 h protein was isolated and western blot analysis for Axl and β-actin was performed.

cells (Figure 4a) transfected with control-miR, pre-miR-34a or pre-miR-199a (Figure 5b). In all experiments, miR-34a and miR-199a significantly reduced migration and invasion when compared with control-miR transfected cells (*$P < 0.05$).

To determine the ability of miR-34a and miR-199a in suppressing the ability of cancer cells to metastasize *in vivo,* a chorion-allantoic-membrane (CAM) assay was performed with H1299 and Rko cells transfected with control-miR, pre-miR-34a, or pre-miR-199a. The number of metastatic H1299 and Rko cells into embryonic chicken liver and lungs was dramatically reduced when compared with control-miR transfected cells (Figures 6c and d) (*$P < 0.05$). Additionally miR-34a and miR-199a inhibited the primary tumor growth in the upper CAM, in a separate experiment (Figures 6a and b) (*$P < 0.05$). To check Axl and miR-expression in parallel to the migration and invasion process, RT–PCR and western blot quantifications were additionally performed 8 days after transfection. The results showed a reduction in miR-expression after day 8 (<30-fold) as compared with the second day of transfection, but no significant changes in Axl protein amounts (data not shown). Taken together, these results suggest that miR-34a and miR-199a are inhibiting several different steps of metastasis, migration, invasion and the formation of *in vivo* distant metastasis.

5-Aza treatment reactivates miR-34a, miR-199a and b expression and inhibits Axl protein

To further differentiate the epigenetic mechanism of regulation of miR-34, miR-199 and Axl, highly invasive (H1299, MDA-MB-231 and Rko), and less invasive cells (H520, Colo 206f and Geo) were treated with a selective

inhibitor of DNA methyltransferases (5-aza). Cells were treated for 5 days with 2 μM 5-aza by changing the fresh medium with drug and DMSO control. Interesting results were observed regarding Axl gene expression after 5-aza treatment. Axl-mRNA levels were significantly downregulated in highly invasive cells, and the opposite effect was observed in less invasive cells (Figure 7b, top row panel). The fact that 5-aza treatment induced Axl gene expression in less invasive cells is consistent with our previous results (Mudduluru and Allgayer, 2008). However, Axl protein amounts were drastically reduced after 5-aza treatment (Figure 7a). This suggests that Axl regulation is controlled at transcriptional level by CpG hypermethylation and also post-transcriptionally by miRs. 5-Aza treatment induced the constitutive expression of miR-34a (Figure 7b, second row panel), miR-199a (Figure 7b, third row panel) and miR-199b (Figure 7b, last row panel). In some cell lines (MDA-MB-231, H520, Geo and Colo 206f), mixed results were observed, regarding miR-expression: either no significant change in expression of any one/two miRs, or a significant induction of one particular miR was observed (Figure 7b). This suggests that, apart from CpG methylation, other mechanisms could control the expression of these miRs, possibly in a cell-line specific manner.

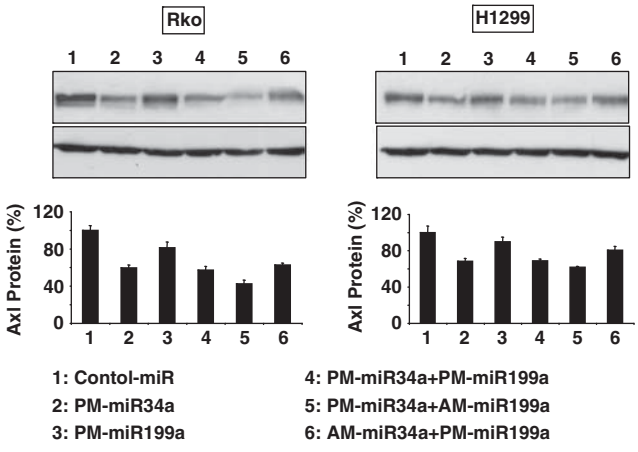


Figure 3 MiR-34a and miR-199a compete for seed sequences. Axl protein amounts were estimated by western blot analysis and quantified by densitometry, density ratio of Axl/β-actin is represented as a bar graph. In control-miR-transfected samples, Axl protein amounts were set as 100%, and relative Axl protein amounts were calculated in PM-miR or in combination (PM-miR and AM-miR) transfected samples.

Hyper-methylation of miR-34a, miR-199a/b and Axl promoters

To understand the mechanisms behind the downregulation of miRs (miR-34a (MI0000268), miR-199a1 (MI0000242), miR-199a2 (MI0000281) and miR-199b (MI0000282) in solid cancer cells (NSCLC, BRC, and CRC), DNA was isolated and subjected to bisulfite conversion and to a subsequent methylation-specific PCR with 'methylated' and 'unmethylated' pairs of primers designed in the CpG island promoter region of miR-34a, miR-199a and b, and also Axl (Supplementary Figure S8). As expected, miR-199a and b promoters were hyper-methylated in most of the screened cells, correlating with their expression. miR-34a expression levels were high in A549 and H460; particularly in these two cell lines, miR-34a promoter hypo-methylation was observed. Axl promoter hyper-methylation was described by us previously (Mudduluru and Allgayer, 2008)

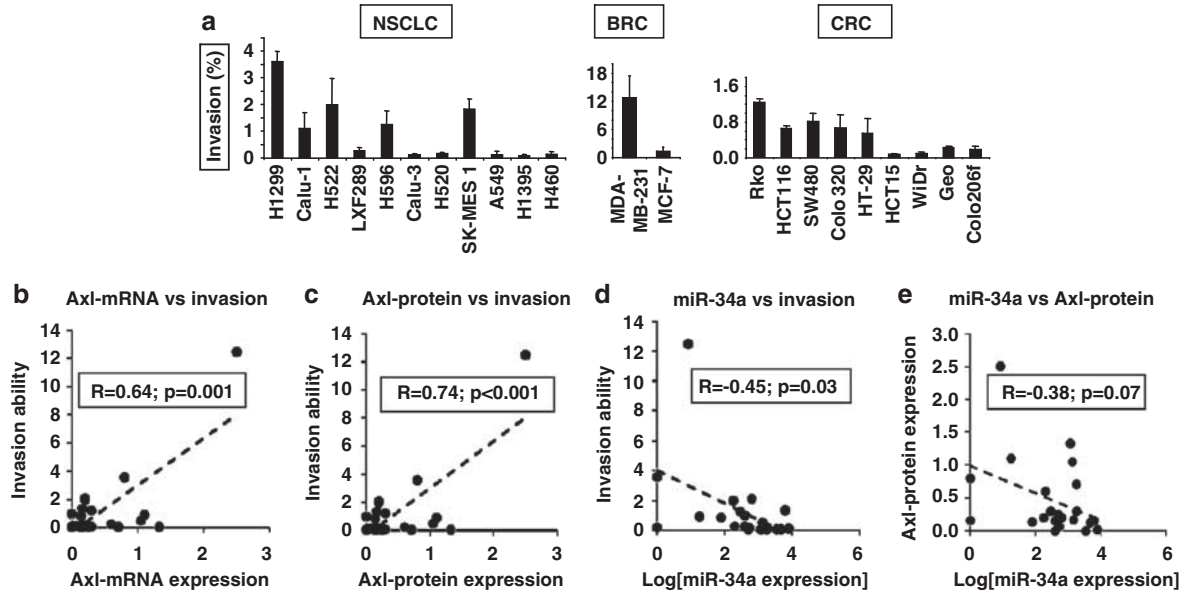


Figure 4 Invasive ability of NSCLC, BRC and CRC cell lines and correlations between invasion, miR34a and Axl endogenous expression. (**a**) Invasive ability of the cells evaluated by Matrigel assays, data are expressed as percentage of invaded cells compared with non-invading cells. Correlations between (**b**) Axl-mRNA expression vs invasion, (**c**) Axl protein vs invasion, (**d**) miR34a-expression vs invasion, (**e**) miR-34a expression vs Axl protein in **a** of panel of NSCLC, BRC and CRC cell lines (*P*-value is from a Spearman's correlation test).

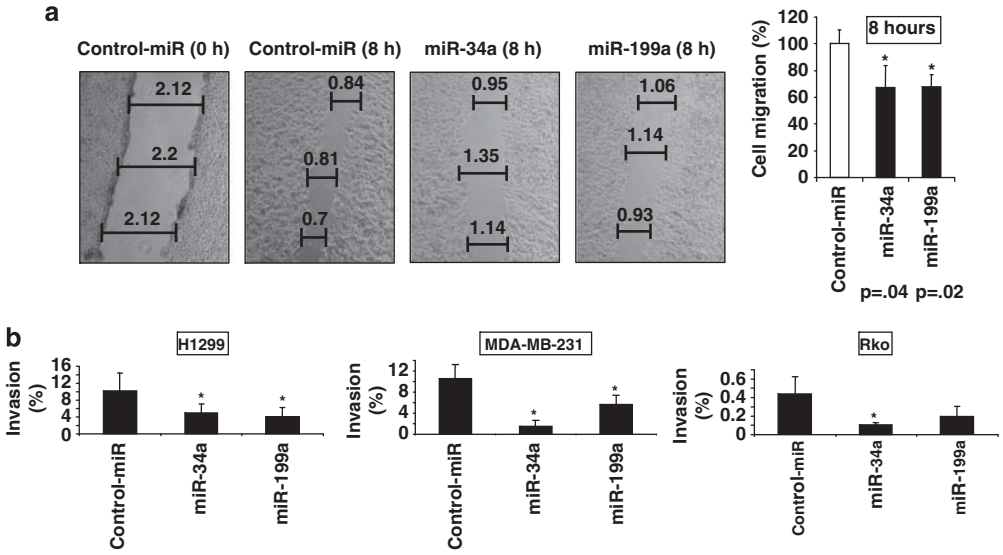


Figure 5 MiR-34a and miR-199a inhibits the migration and invasion *in vitro*. (**a**) H1299 cells were transfected with PM-miR-34a and PM-miR-199a. After 48 h wounds were created with a yellow tip, pictures were taken and again after 8 h. Wounds were measured in three different places and mean wound distance of control-miR transfected set as 100%. PM-miR-34a and PM-miR-199a transfected samples were calculated and represented as a graph ($*P<0.05$). (**b**) H1299, MDA-MB-231 and Rko cells were transfected with control-miR or PM-miR-34a or PM-miR-199a. After 48 h cells were plated on top of the Matrigel-coated Boyden chambers, invaded cells were measured as described in Materials and methods. Data are represented as the percentage of invading cells, as mean ± s.d. of four replicates ($*P<0.05$).

correlated with Axl expression in some of the screened cells (H520, Colo 206f, Geo), but not in all of them, suggesting the biological relevance of the Axl-regulating mechanisms besides epigenetics (Figure 7b, top panel; Figure 8).

MiR-34a, miR-199a and miR-199b are frequently downregulated and co-expressed in NSCLC patients

To investigate an *in vivo* relevance of the mechanisms found for cancer patients and their tumor diseases, miR-34a, miR-199a and miR-199b expression was tested in NSCLC samples and in the corresponding normal lung tissues. Results were analyzed adopting a cutoff value of 0.75 (that is, 1.5-fold) for expression. Concerning miR-34a, the results showed a significant downregulation in 24 out of 44 patients (54.5%; $\Delta\Delta Ct < -0.75$), while 12 (27.3%) patients had a ratio >0.75, that is, miR-34a significantly upregulated. Concerning miR-199a, the results showed a significant downregulation in 19 out of 44 patients (43.2%), while 13 (29.5%) patients, miR-199a was upregulated. Finally, miR-199b was found to be downregulated in 23 out of 44 patients (52.3%), while in 9 cases (20.5%) this miR was upregulated. Rest of the patients for these three miRs did not show significant changes in their expression when compared with their respective normal tissues.

The three miRs were found to be significantly co-regulated (see Figures 9a–c) and a lower expression of all three was significantly associated with a squamous NSCLC histotype (Figure 9d). No other significant correlations between miR levels and patients' characteristics such as gender, age, tumor size, lymph nodal status and clinical stage were found (not shown). Survival analysis, dividing the patients between these with up-regulated and those with down-regulated or equal, showed a positive association towards a longer survival for the patients with an upregulation of miR-34a ($P=0.07$), while miR-199a and 199b were not correlated with patients' prognosis (Figure 9e). Furthermore, an immunohistochemical quantification of Axl protein was performed in those tumor specimens with miR-34a normal/tumor ratio <-2 or >2 (that is, strongly downregulated and upregulated). As a result, we found no differences of Axl expression in terms of both intensity and percentage of positive cells, but interestingly a more frequent cytoplasmic staining was observed in those tumors with strong miR-34 downregulation as compared with upregulated tumors (62 vs 14%, see Figure 9f).

Discussion

The major finding of this study is that three microRNAs, miR-34a, miR-199a, and miR-199b can inhibit the expression and functions of Axl tyrosine kinase, thereby impairing migration, invasion and formation of distant metastasis of cancer cells (Vajkoczy *et al*., 2006; Mudduluru *et al*., 2010). As 5-aza treatment was shown to induce the constitutive expression of miR-34a, miR-199a, and miR-199b and to inhibit Axl protein expression, we found that miR-34a and miR-199a/b were frequently methylated and that their expression levels significantly inversely correlated with invasive capacity and Axl expression. Our study extends the results of others showing the miR-34a and miR-199a2 regulation is controlled by methylation (Lodygin *et al*.,

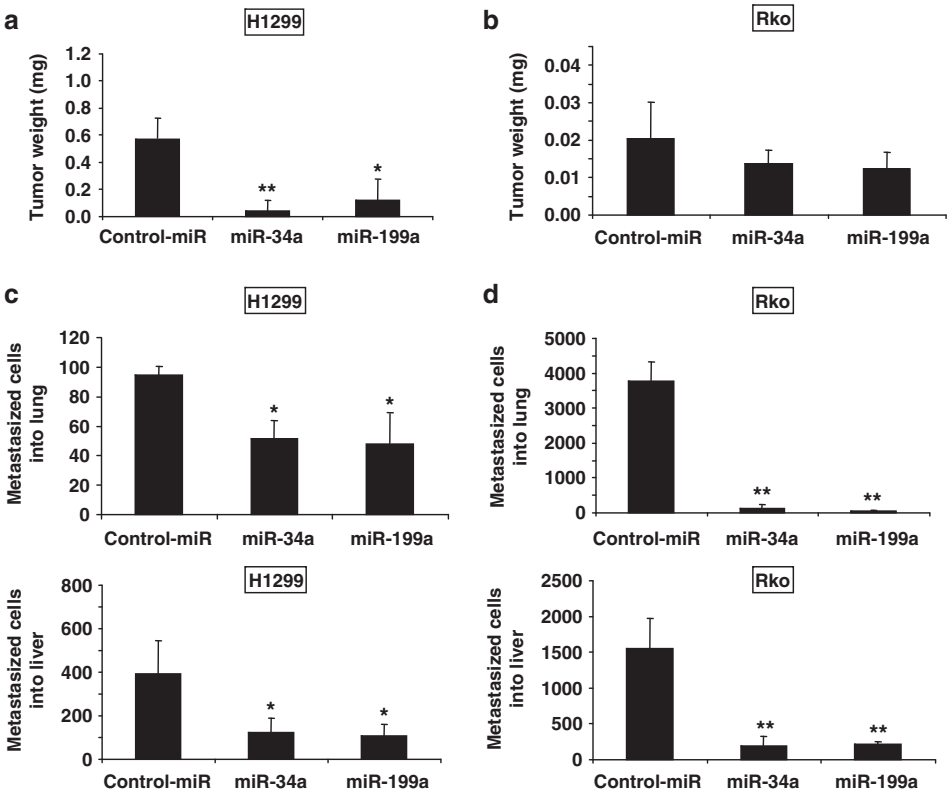


Figure 6 MiR-34a and miR-199a reduces tumor growth and inhibits metastasis *in vivo*. Chicken embryo metastasis (CAM) assay was performed with H1299 and Rko cells transfected with control-miR or PM-miR-34a or PM-miR-199a. Ten eggs were included for each group. (**a**, **b**) Upper CAM tumors were removed and weights were measured and represented as indicated. (**c**, **d**) Genomic DNA was analyzed by real-time Alu-PCR to determine the number of metastasized cells into liver and lung (right), ±s.d. of eight replicates (*$P<0.05$ and **$P<0.01$).

2008; Toyota *et al*., 2008; Cheung *et al*., 2010). Moreover, to the best of our knowledge this is the first report to show the methylation of miR-199a1 and miR-199b promoters.

In our work, by *in silico* analysis, we have identified miR-34a and miR-199a/b as potential candidates capable of targeting the Axl oncogene. By co-transfecting miRNAs and Axl 3′-UTR either with or without seed sequences for the predicted miRNAs, we have shown that Axl is a true target for the miR-34a and miR-199a/b. Moreover, exogenous expression of each miR-34a or miR-199a resulted in a strong decrease in Axl protein, whereas blockade of endogenous miRNAs (by anti-miRs) led to a twofold increase of Axl expression in A549. The lack of a specific Axl band observed for MCF-7 and Colo 320 cells was not surprising, as Axl transcriptional regulation is epigenetically controlled by CpG methylation in these particular cell lines (Mudduluru and Allgayer, 2008).

5-Aza treatment increased Axl-mRNA expression in H520, Geo and Colo 206f (Mudduluru and Allgayer, 2008), but not in H1299, MDA-MB-231 and Rko cells. H1299, MDA-MB-231 and Rko cells are more invasive and have high endogenous Axl expression. In particular, these cells might be bypassed the epigenetical regulation, where as H520, Geo and Colo 206f cell lines are less invasion and no Axl expression. Axl-mRNA levels were induced in H520, Geo and Colo 206f cells, since it is controlled by CpG methylation. However, protein amounts were significantly reduced after 5-aza treatment in all of these cells (Vajkoczy *et al*., 2006; Mudduluru and Allgayer, 2008; Mudduluru *et al*., 2010). In general, it implies different stages of controlling mechanisms of a gene, especially of an oncogene. Moreover, miR-34a and miR-199a2 are reported that they are epigenetically controlled by CpG methylation (Lodygin *et al*., 2008; Cheung *et al*., 2010). 5-Aza treatment significantly induced the expression of either one or all of the miRs (miR-34a, miR-199a and b) in the screened cell lines. Epigenetic regulation by CpG methylation of these miRs could be cell line specific or other epigenetic mechanisms and specific transcriptional factors might interfere with the expression. Bisulfite conversion and methylation-specific PCR supported the existing data and also revealed that miR-34a, miR-199a1, miR-199a2 and miR-199b promoters were hyper-methylated and inversely correlated with their expression in a panel of cancer cells (Lodygin *et al*., 2008; Garzia *et al*., 2009; Cheung *et al*., 2010). Our data shows in particular that miR-199a1 and miR-199b promoters were hyper-methylated in NSCLC, BRC and CRC cell lines. CpG methylation might account for the loss of these three miRs in a substantial portion of carcinomas (Bommer *et al*., 2007; Chang *et al*., 2007; Tazawa *et al*., 2007;

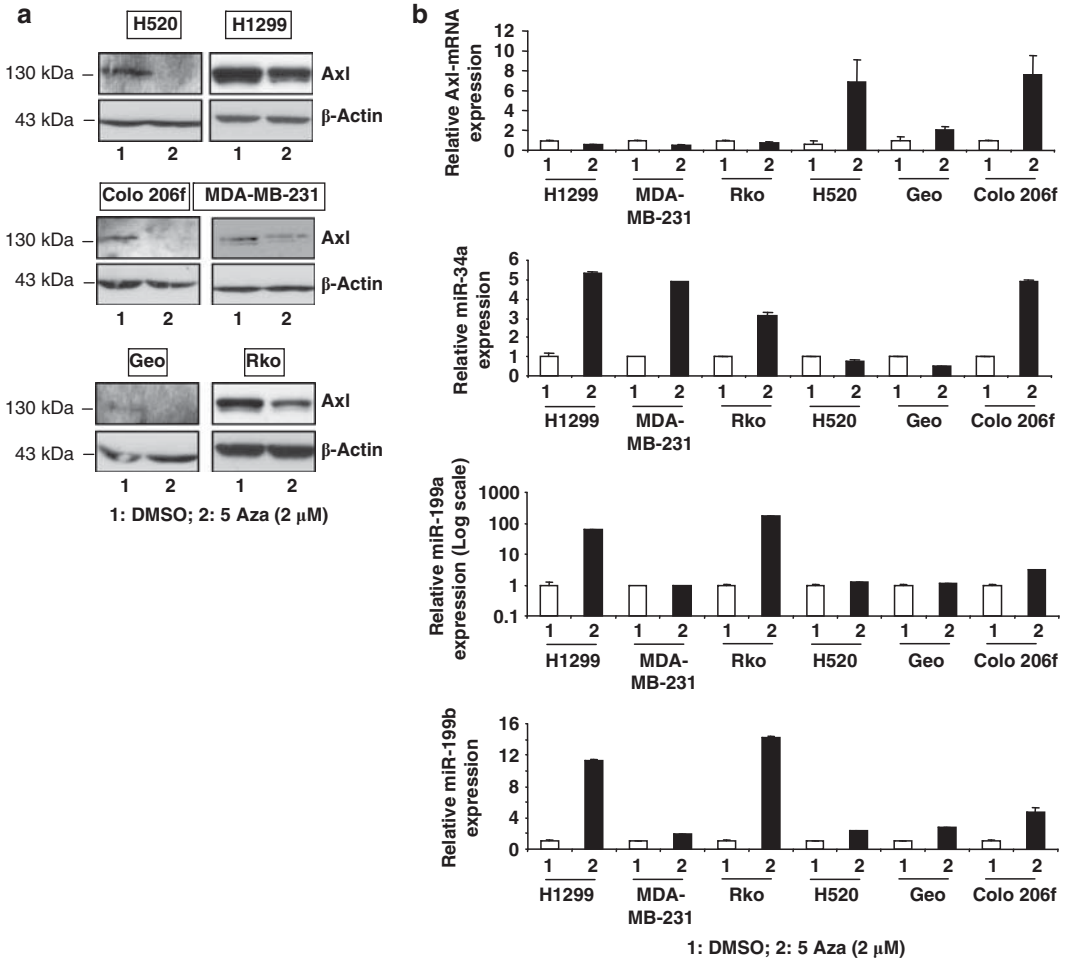


Figure 7 5-Aza-dC (5-aza)-treatment for 5 days induced the expression miR-34a, miR-199a, miR-199b and deregulated Axl gene expression. H520, H1299, MDA-MB-231, Colo 206f, Geo and Rko cells were treated with 5-aza (2 μM) for 5 days and total RNA and protein was isolated. (**a**) Western blot analysis was performed for Axl and β-actin as indicated, (**b**) Axl-, miR-34a-, 199a/b-expression levels were evaluated by RT–PCR (s.d. is small, thus hardly visible).

Lodygin *et al*., 2008; Cheung *et al*., 2010). Our data show that Axl-targeting miRs are epigenetically silenced in cancer cell lines/tumors, and that miR-34a and miR-199a/b are regulating Axl expression at mRNA levels by degrading it and also inhibiting translation, which is corroborating the Axl overexpression in different types of cancers (Hafizi and Dahlback, 2006). As Axl and miR-34a have opposite functions; their endogenous expressions were quantified and correlated with NSCLC, BRC and CRC invading capacity. Axl-mRNA and Axl protein expression significantly inversely correlated with the miR-34a expression, and cell invading capacity positively correlated with Axl protein amounts and inversely with miR-34a expression. In general, our findings strongly support the existing literature that Axl is an oncogene and miR-34a is a tumor suppressor (Hafizi and Dahlback, 2006; Vajkoczy *et al*., 2006; Bommer *et al*., 2007; Welch *et al*., 2007; Mudduluru *et al*., 2010).

Previous studies reported that miR-34a can inhibit cell cycle (CCNE2, CDK4, CDK6, Cyclin E2 and E2F5), anti-apoptotic protein (BCL2) and invasion (MET) inducing genes (Bommer *et al*., 2007; Chang *et al*., 2007; He *et al*., 2007; Raver-Shapira *et al*., 2007). Additionally, 199a and 199b can inhibit cell proliferation by targeting cell proliferation inducers like IKKβ, HES1, Cyclin D1 and C-Myc (Chen *et al*., 2008; Garzia *et al*., 2009). miR-34a is a p53 target gene that presumably mediates induction of apoptosis, cell cycle arrest and senescence by p53. Subsequently, Axl is known to induce migration, invasion and distant metastasis (Vajkoczy *et al*., 2006; Mudduluru *et al*., 2010). We now demonstrate that miR-34a and miR-199a are potent inhibitors of migration, invasion (H1299, MDA-MB-231 and Rko) and tumor growth and, *in vivo* distant metastasis by CAM assay (H1299 and Rko). Thus, the inhibition of migration and invasion by these miRs might in part be mediated via negative regulation of Axl. However, the present work on miR-34a and miR-199a does not exclude other important mechanisms in terms of known and/or unknown additional targets of these miRs, especially in the context of tumor progression, invasion and metastasis. Towards this end, in the present work we

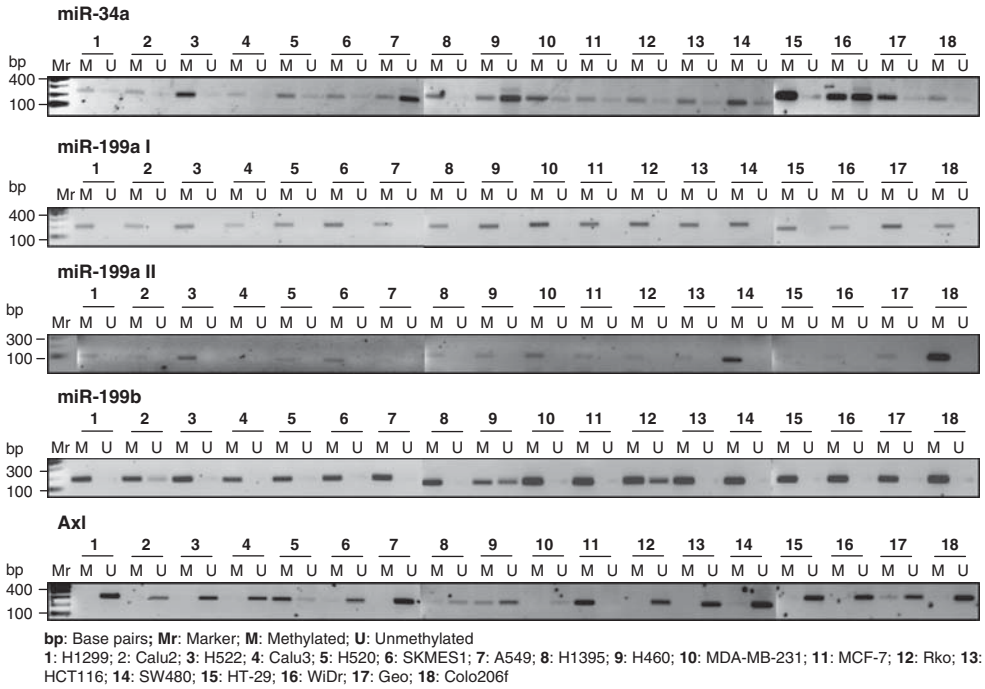


Figure 8 Methylation status of Axl, miR-34a and 199a/b promoters in cancer cells. Gel electrophoresis of PCR products obtained from methylation-specific PCR for miR-34a, miR-199a, miR-199b and Axl in a panel of cancer cell lines.

have confirmed that c-Met and Notch-1, and also HIF1a, are targets of miR-34a and miR-199a, respectively, molecules that are known to be highly relevant for tumor formation and progression, and also metastasis. The targeting of these mRNAs by miR-34a/199a has been shown by Li *et al*. (2009) and Yeligar *et al*. (2009), in glioblastoma and liver sinusoidal endothelial cells, respectively. These and our results, together with the very likely notion that further miR-34a/199a targets will be discovered soon, we consider it very likely that these two miRs are capable of regulating a whole program of concerted regulation of progression- and metastasis-related mRNAs.

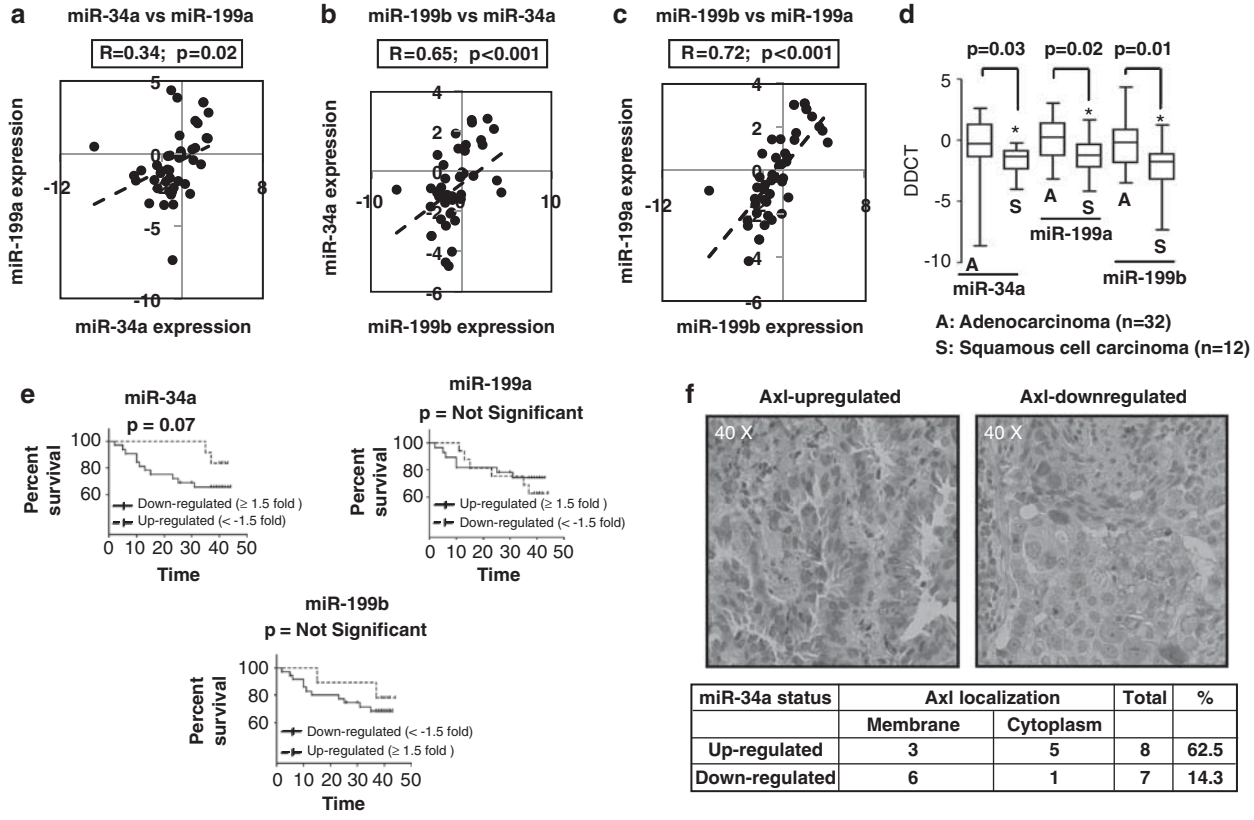
To confirm some of the observations obtained in cell line experiments, an expression analysis was performed in primary tumor specimens from a case-series of consecutively resected NSCLC patients. As a result of qPCR experiments, miR-34a, miR-199a and miR-199b were found frequently downregulated in the tumors when compared with the corresponding normal tissues, and a significant co-expression in the tumor tissues among the three miRs was observed. While no significant correlation between miR expression levels and patients' clinico-pathological characteristics (gender, age, tumor size, lymph nodal status and clinical stage) was found, survival analysis indicated a positive association towards a longer survival for those patients with miR-34 upregulation. This is in line with recent results indicating miR-34 as a prognostic factor in NSCLC (Gallardo *et al*., 2009), and further confirm its putative role as a tumor suppressor miR in lung cancer. Interestingly, a comparably lower level of regulation (that is, expression) in squamous, as compared with non-squamous tumors, has been observed for all the three miRs. Additionally, the quantification of Axl protein by immunohistochemistry confirmed a differential pattern of expression depending on the status of miR-34a regulation, being Axl more frequently localized in the membrane (that is, active) in NSCLC patients with miR-34a downregulation. Hypothetically, the presence of Axl in the cytoplasm (more frequent in tumors with high miR-34a) could be due to proteosomal degradation or other proteolytic degradation (for example by ADAM10 or maybe other functional changes caused by specific miRs in tumors). We did not extend specific data on this aspect at this point of time in our present study (O'Bryan *et al*., 1995; Budagian *et al*., 2005), but consider it as an important aspect that those patients with high miR-34a expression appear to have less functional (membrane-bound) Axl protein as suggested by IHC, the molecular mechanism leading to this still being in need to be investigated.

In conclusion, this evidence highlights a pivotal role for miR-34a and miR-199a/b in various aspects of tumorigenesis like tumor growth, migration, invasion, and *in vivo* distant metastasis in cancer cell lines especially through Axl regulation.

Materials and methods

Cell lines, cultures and drugs

Eleven human NSCLC cell lines (Calu-1, H520, SK-MES-1, H596, Calu-3, H522, H1395, H1299 H460, LXF289, A549 and H520), two BRC cell lines (MCF-7, MDA-MB-231) and



miR-34a status	Axl localization		Total	%
	Membrane	Cytoplasm		
Up-regulated	3	5	8	62.5
Down-regulated	6	1	7	14.3

Figure 9 Axl and miRs quantification in NSCLC tumors. MiR-34a, miR-199a and miR-199b expression in NSCLC tumor tissues. RUNB6 served as a normalization control. (**a**–**c**) Expression levels are given as DDCT (tumor/normal ratio). *R* and *P*-values are calculated by Spearman's rank correlation method. (**d**) Box plot of miRs (miR-34a, miR-199a and miR-199b) expression in the patients' tumor tissues according to histotypes. (**e**) Kaplan–Meier survival curves for the NSCLC patients divided by the status of miRs regulation. (**f**) Immunohistochemical quantification of Axl in the patients with miR-34a upregulated and downregulated tumors ($n = 15$). The box represents the number of cases with membrane or cytoplasmic staining. The percentage represents the proportion of cases with cytoplasmic staining in each group. A full colour version of this figure is available at the *Oncogene* journal online.

eight CRC cell lines (Rko, HCT116, SW480, Colo 320, HT-29, HCT15, WiDr and Colo 206f) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and grown at 37 °C with ATCC recommended media supplemented with 10% fetal calf serum. Geo (CRC cell line) was a gift from D Boyd (MD Anderson Cancer Center, Houston, TX, USA), and grown as similar to the other cell lines with DMEM. Original stock solutions of 5-aza-2′-deoxycytidine (5-aza-dC, Sigma Chemical Co., St Louis, MD, USA) at a concentration of 4 mM was stored at −20 °C and freshly dissolved in culture medium before use.

Patients and samples

Fresh snap-frozen surgical specimens of tumor tissues and of the corresponding normal specimens from 44 NSCLC patients completely resected between 2005 and 2006 at the San Luigi Hospital (Orbassano, Italy) were consecutively collected. The main patients' characteristics are reported in Supplementary Table S1. None of the patients received pre-operatory chemo/radiation therapy. All cases were reviewed and classified according to the WHO classification by one of the investigators (MP), using anonymous 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.

Construction of 3′-UTR-luciferase plasmids and reporter assays

The full-length 3′-UTR of Axl (1834 nt) was amplified using cDNA from H1299 and cloned into the *Hin*dIII-site of pMIR (Ambion, Austin, TX, USA), checked for orientation, sequenced and named Axl 3′-UTR. Axl del-3′-UTR (with deleted seed sequences of miR-34a and miR-199a/b) was amplified using the Axl 3′-UTR luciferase construct as a template. Cloning primers are provided in Supplementary Table S2. For reporter assays, cells were co-transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) either with 1 μg of luciferase construct and pRL-TK (50 ng, Renilla Luciferase; Promega, Madison, WI, USA) or along with 50 nM of control-miR or PM/AM miR. pRL-TK was co-transfected and luminescence was measured to normalize transfection efficiency. Reporter assays were performed 48 h post-transfection using the Dual-luciferase assay-system (Promega), normalized for transfection efficiency by co-transfected Renilla luciferase.

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

Protein isolation and western blot analysis were performed as described by Mudduluru *et al*. (2010) using specific antibodies of Axl (#sc-1096) or β-actin (#sc-1616-R) (sc: Santa Cruz Biotechnology, Santa Cruz, CA, USA). DNA isolation and purification from cells were performed with DNeasy Blood

and Tissue Kit (Qiagen). Total RNA was isolated from cell lines and from lung specimens with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Expression of mature miR-34a and miR-199a/b were determined by the TaqMan miRNA-assay (Applied Biosystems, Foster City, CA, USA), and normalized using the $2^{-\Delta\Delta Ct}$ method relative to U6-snRNA (RNU6B). Axl gene expression was determined as described previously by Mudduluru *et al*. (2010). Primers are provided in Supplementary Table S3.

Migration, invasion and CAM assay
Assays were performed as described before by Mudduluru *et al*. (2010). In brief, Control-miR- or miR-34a- or miR-199a-transfected cells were placed either for CAM assay on the upper CAM of 10-day-old chicken embryos for tumor growth and *in vivo* metastasis analysis or for transwell chambers (Costar, Corning, NY, USA) and rest of the assays were performed as described by Mudduluru *et al*. (2010), respectively. Light microscopy pictures of transfected cells were taken at $\times 40$ magnification for wound healing assays.

5-aza-dC treatment of cells, bisulfite conversion of DNA and methylation analysis
5′-Aza-dC treatment, bisulfite conversion and methylation analysis (PCR was performed using HotStarTaq Plus DNA Polymerase (#203605) from Qiagen, Hilden, Germany) were performed as described by Mudduluru and Allgayer (2008). Axl and miRs expression were quantified in comparison with DMSO-treated samples. CpG islands upstream of the transcription start site or pri-miR start site were determined with the CpG island searcher (http://www.uscnorris.com/cpgislands2/cpg.aspx), and PCR primers were designed using the Methprimer software (http://www.urogene.org/methprimer) ~ 1000 bp upstream to the miRs or Axl transcription start site. Primer sequences are provided in Supplementary Table S4.

Immunohistochemistry
Expression levels of Axl protein were detected by using goat anti-Axl antibody (sc-1096; Santa Cruz Biotechnology) at 4 °C overnight followed by rabbit anti-goat (biotinylated immunoglobulin G (IgG)) secondary antibody at room temperature for 30 min. Immunoreactions were revealed by a biotin-free dextran-chain detection system (Envision, DakoCytomation, Glostrup, Denmark), and developed using diaminobenzidine as the chromogen. One section with isotype-non-specific IgG served as negative controls for normal and tumor tissues. H1299 cell line served as positive controls. Semiquantitative scaling was used with slight modification as described previously by Wu *et al*. (2002). Results were categorized into three groups according to the percentage of positively stained cells: score 1 (no staining); score 2, $<10\%$ of positive tumor cells; score 3, $>10\%$ of positive tumor cells. Additionally, tumors were divided according to the localization of Axl staining (membrane or cytoplasm).

Statistical analysis
To test differential miR-34a, miR-199a and miR-199b expression between tumors and corresponding normal lung tissues, the $\Delta\Delta$Ct method was used, and miRs were considered significantly overexpressed when $\Delta\Delta$Ct values were $> \pm 0.75$. To test significant associations between expression levels and clinical-pathological variables, the Mann–Whitney U test and the Fisher's exact tests were applied. Univariate analysis of survival was done with the method of Kaplan and Meier. In all tests, the statistical significance was set at $P = 0.05$.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)