

miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer

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MicroRNAs (miRNAs) have been identified as promising prognostic markers in non-small-cell lung cancer (NSCLC) since they play an important role in oncogenesis. The miR-34 family is composed of three miRNAs (miR-34a, miR-34b and miR-34c) that are part of the p53 network and whose expression is directly induced by p53 in response to DNA damage or oncogenic stress. We have analyzed the impact of miR-34 expression on relapse and overall survival in surgically resected NSCLC patients. For this purpose, we used stem-loop reverse transcription-polymerase chain reaction to analyze the expression of the miR-34 family in paired tumor and normal tissue from 70 surgically resected NSCLC patients who received no postsurgical treatment until relapse. In addition, in patients with sufficient tumor tissue, we assessed p53 mutations and the methylation status of the *MIRN34A* gene promoter region and correlated these findings with miR-34a expression. Molecular findings were correlated with relapse and overall survival. The miR-34 family was downregulated in tumor compared with normal tissue, and low levels of miR-34a expression were correlated with a high probability of relapse ($P = 0.04$). A relation was also found between *MIRN34A* methylation and miR-34a expression ($P = 0.008$). Patients with both p53 mutations and low miR-34a levels had the highest probability of relapse ($P = 0.001$). In the multivariate analysis, miR-34a expression emerged as an independent prognostic marker for relapse. In summary, we have identified miR-34a as a novel prognostic marker in NSCLC patients, providing a potential mechanism for estimating a patient's risk of disease recurrence and a useful tool to help guide treatment decisions.

Introduction

Lung cancer is the most common cause of cancer death in Europe and worldwide (1). Despite years of research, the prognosis for patients with lung cancer remains dismal. The most frequent type, non-small-cell lung cancer (NSCLC) (85%), shows an overall 5 year survival of

Abbreviations: miRNA, microRNA; MSP, methylation-specific polymerase chain reaction; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction.

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10% (1,2). Although it is difficult to determine a clear prognosis for an individual NSCLC patient, in part because of the marked heterogeneity of patients with the disease (2), in surgical NSCLC patients, tumor stage is the most important prognostic factor (3). Even after complete resection, recurrence rates are substantial (20–85%, depending on tumor stage) (3). During recent years, various molecular prognostic factors have been analyzed, including markers of tumor proliferation, cellular adhesion and cellular growth (Ras, retinoblastoma and epidermal growth factor receptor) or apoptosis (p53 and Bcl-2) (3). One of the markers that has been extensively studied is the mutational status of the tumor suppressor gene p53 (4,5), which is frequently mutated or inactivated in human cancers (6). Although several studies have assessed p53 mutations as a prognostic factor for NSCLC, conflicting results have been reported (7–13). About 55% of NSCLCs are reported to contain mutations of the p53 gene, most of which are point mutations located in the region between exons 5 and 8 (14,15), which contains the DNA-binding site of the p53 protein (16).

Recently, the analysis of tumor biology has led to increased interest in microRNAs (miRNAs) (17–22). miRNAs are small non-coding RNAs (22–24 nucleotides in length) that negatively regulate messenger RNA translation to protein. miRNAs are encoded in the entire genome, including the exonic, intronic and intergenic regions, but 90% are found in intronic regions. Their expression levels are regulated by transcription factors (23,24)—for example, the miR-17-92 cluster that is activated by c-myc (24)—or by epigenetic mechanisms—such as methylation of the CpG islands in the promoter region of the gene (25). Moreover, all the mechanisms that alter the normal expression of a gene can affect the expression of an miRNA, including chromosomal translocations, amplifications and deletions or mutations (26,27). High-throughput analyses have shown that miRNA expression is commonly deregulated in several types of cancer including NSCLC (18,28–30). Some miRNAs, like the let-7a family, which appears downregulated in many tumors, have prognostic implications in postoperative survival in NSCLC through Ras regulation (31); patients with low levels of let-7a had shorter survival (20). Other miRNAs, like miR-155 (20,30) or miR-21 (19), also have a role as prognostic markers in NSCLC, where shorter survival has been associated with high levels of miR-155 or miR-21.

The miR-34 conserved family is composed of three miRNAs: miR-34a, miR-34b and miR-34c. There are two miR-34 loci, one encoding miR-34a and the other encoding both miR-34b and miR-34c. The promoter regions of both loci contain a p53-binding site (32). The miR-34 family has been shown to form part of the p53 network (32–35), and the expression is directly induced by p53 in response to DNA damage or oncogenic stress. Moreover, the promoter region of the *MIRN34A* gene contains CpG islands, and aberrant CpG methylation that reduces miR-34a expression levels has been reported in multiple types of cancer (36). The high frequency of miR-34 silencing in tumors suggests that the miR-34 family acts as a tumor suppressor gene, indicating a potential role as a prognostic marker, although this has not yet been investigated.

In the present study, we have analyzed the prognostic value of miR-34 expression and its relationship with p53 mutations in 70 surgically resected NSCLC patients who received no treatment from curative surgery to relapse.

Materials and methods

Tissue samples

Samples of tumor and paired normal tissue were retrospectively selected from 70 NSCLC patients who underwent potentially curative resection as their first and only treatment between February 1996 and September 2002 at a single institution, Hospital Clinic (Barcelona, Spain). Median age was 64 years (range, 36–86), and 94% were males (Table I). At a median follow-up of

Table I. Main clinical characteristics and correlation with relapse and survival according to the univariate analysis

Characteristic	N = 70 (%)	P-value relapse	P-value survival
Gender		0.82	0.9
Male	66 (94)		
Female	4 (6)		
Median age, years (range)	64 (35–85)	0.24	0.04
Stage			
I	39 (55)	0.09	0.9
II	17 (25)		
III	14 (20)		
IA versus others		0.033	0.23
PS			
0	17 (25)	0.81	0.5
1	49 (71)		
2	3 (4)		
Histologic subtypes			
Adenocarcinoma	31 (44.3)	0.5	0.8
Squamous cell carcinoma	36 (51.4)		
Others	3 (4.3)		
Smoking history			
Current	38 (54.3)	0.47	0.5
Never	1 (1.4)		
Ex	31 (44.3)		
p53 mutation			
No	39 (55.7)	0.042	0.4
Yes	18 (25.7)		

38 months (range, 1–127), overall 5 year survival was 53.3% and 33 patients (47%) had relapsed. Approval for this study was obtained from the Institutional Review Board of Hospital Clinic, Barcelona, Spain. Signed informed consent was obtained in accordance with the Declaration of Helsinki.

RNA extraction and miRNA quantification

Total RNA was extracted from formalin-fixed paraffin-embedded tumor tissues using RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's protocol. Taqman quantitative real time-polymerase chain reaction (PCR) miRNA assay of miR-34a, miR-34b and miR-34c was used to quantify expression levels, as described previously (37), using an Applied Biosystems 7500 Sequence Detection System. Normalization was performed with miR-191, based on preliminary analyses comparing the stability of miR-24, RNU6B and miR-191; miR-191 had the lowest expression variability in the miRNA expression patient dataset (38) and was therefore used in this study.

MIRN34A gene methylation analysis

The methylation status of the promoter region of the *MIRN34A* gene was examined in DNA extracted from tumor tissue. The DNA methylation status of the CpG islands was determined by methylation-specific polymerase chain reaction (MSP) after genomic DNA (1 µg) was subjected to sodium bisulfite treatment using the EZ DNA Methylation kit as recommended by the manufacturer (Zymo Research, Orange, CA). For MSP, the modified DNA was amplified with primers capable of annealing either methylated or unmethylated cytosine residues. Oligonucleotide sequences used for the MSP were as follows: mir34U 5'-IIGGTTTGGGTAGGTGTGTTT-3'; mir34U.r 5'-AATCCTCATCCCCTTACCACCA-3'; mir34M 5'-GGTTTTGGGTAGGCGGTTTC-3' and mir34M.r 5'-TCCTCATCCCCTTACC-GCCG-3'. Primer sequences, polymerase chain reaction (PCR) conditions and product sizes for each methylation marker were analyzed, and the specificity of the MSP assays has been described previously (36). PCR products were visualized after gel electrophoresis in 2% agarose wt/vol containing ethidium bromide. Commercially modified DNA (Chemicon International, Temecula, CA) and normal blood DNA were used as positive controls for methylated and unmethylated alleles, respectively.

p53 mutation analysis

PCR was performed on 100 ng DNA samples to identify p53 mutations. The mutation analysis included p53 exons 5–8. PCR primers and conditions for mutation analysis have been described previously (39). The PCR products were analyzed by agarose gel electrophoresis (2% wt/vol), purified and used in two sequencing reactions per exon (forward and reverse), using the Big Dye

Terminator Cycle Sequencing kit (version 3.1, Applied Biosystems). Reactions were loaded into an ABI-3100 DNA sequencer (Applied Biosystems). All sequences were analyzed with the SeqScape software (Applied Biosystems).

Statistical methods

All statistical analyses were performed with SPSS 14.0 (SPSS Inc., Chicago, IL) and R v. 2.6.0 software. The primary end points of the study were relapse and overall survival. miR-34a expression was dichotomized using the maxstat package of R in order to determine the cutoff that best discriminated between different groups of patients for relapse and overall survival (40). The univariate analysis of relapse was performed using the Gray test (41). The cumulative incidence was computed with the cmprsk package for R. All variables that were significant in the univariate analysis were included in a multivariate analysis of relapse performed using the subdistribution regression model of Fine *et al.* (42) with the cmprsk package. Overall survival was calculated with the Kaplan–Meier method and compared using the log-rank test.

Group comparisons of categorical variables were performed with Fisher's exact test. All *P*-values were two sided and represent raw values. Statistical significance was set at *P* < 0.05.

Results

miR-34a, miR-34b and miR-34c expression and clinical outcome

The normalized real-time PCR results from 70 primary NSCLC tumor samples showed that miR-34a (*P* < 0.001) and miR-34b (*P* = 0.02) were downregulated in tumor tissue compared with paired normal tissue, but no significant difference in miR-34c expression levels was observed (Figure 1).

In order to examine the prognostic implications of the expression levels of miR-34a, miR-34b and miR-34c, we used the cutoff selected by the maxstat package of R. A significant association with relapse was observed only for miR-34a. Maxstat identified two significant cutoffs: one below (cutoff 1) and one above (cutoff 2) the median expression of normal tissue (Figure 2). When patients were divided according to cutoff 1, 16 of 24 patients (67%) with low levels of miR-34a relapsed compared with 17 of 46 patients (37%) with high levels (*P* = 0.04). Cutoff 2 identified seven patients (10%) with the highest levels of miR-34a, none of whom relapsed (*P* = 0.023). Using both cutoffs enabled us to identify three groups of patients with different rates of relapse: 67% for those with low levels; 43% for those with high levels and 0% for those with the highest levels (*P* = 0.039) (Figure 2). No significant correlation was observed between either miR-34b or miR-34c and relapse. In the univariate analysis for relapse, in addition to miR-34a, only stage IA versus other stages and the presence of p53 mutations significantly correlated with probability of relapse.

We performed two independent multivariate analyses for relapse: one without p53 mutational status (all 70 patients) (Table II) and another including p53 mutational status (57 patients) (Table II). In the first analysis, miR-34a (relative risk = 2.17; *P* = 0.004) and disease stage (stage IA versus others) (relative risk = 2.9; *P* = 0.04) were independent factors for relapse. In the second analysis, only miR-34a expression emerged as an independent factor for relapse (relative risk = 1.9; *P* = 0.024).

No correlation was observed between survival and miRNA expression or clinical characteristics, with the exception of age (*P* = 0.04).

Regulation of miR-34a expression: p53 mutations and methylation status

In order to further investigate the reasons for differences in miR-34a expression levels between patients, we analyzed p53 mutations and the methylation status of the promoter region of the *MIRN34A* gene in patients for whom sufficient tumor tissue was available.

p53 mutations

p53 mutations were assessed in 60 samples, 18 of whom (30%) had p53 mutations (Table III). Although no significant correlation was observed between mutation status and miR-34a expression levels (*P* = 0.1) (Figure 3A), the median miR-34a expression in those with mutations (median = 0.11 ± 0.57) was lower than in those without mutations (median = 0.26 ± 0.58). Furthermore, when patients were

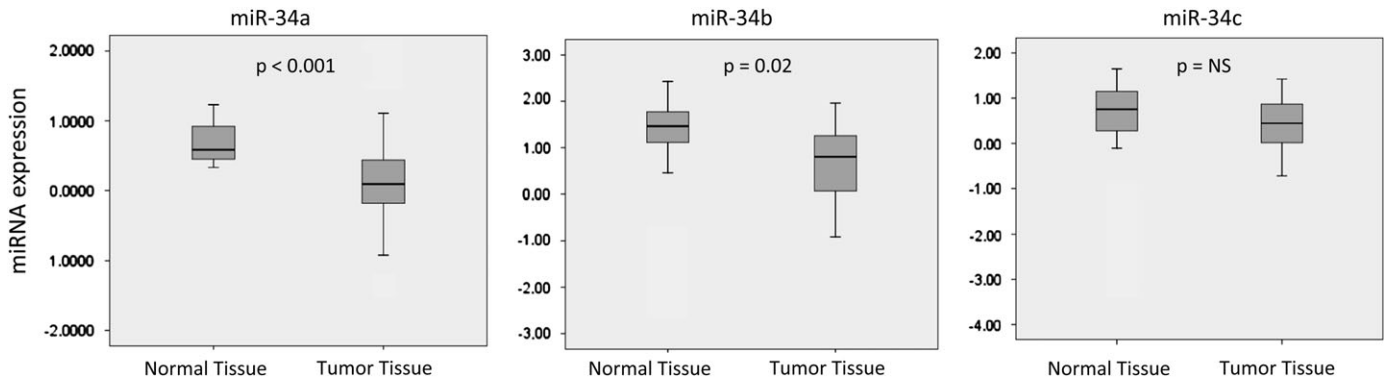


Fig. 1. miR-34a, miR-34b and miR-34c expression. The data are shown as Log10 of relative quantification ($2^{-\Delta\Delta C_t}$). miR-191 was used as endogenous normalization control. miR-34a ($P < 0.001$) and miR-34b ($P = 0.02$) were downregulated in tumor tissue compared with paired normal tissue, but no significant (NS) difference in miR-34c expression levels was observed.

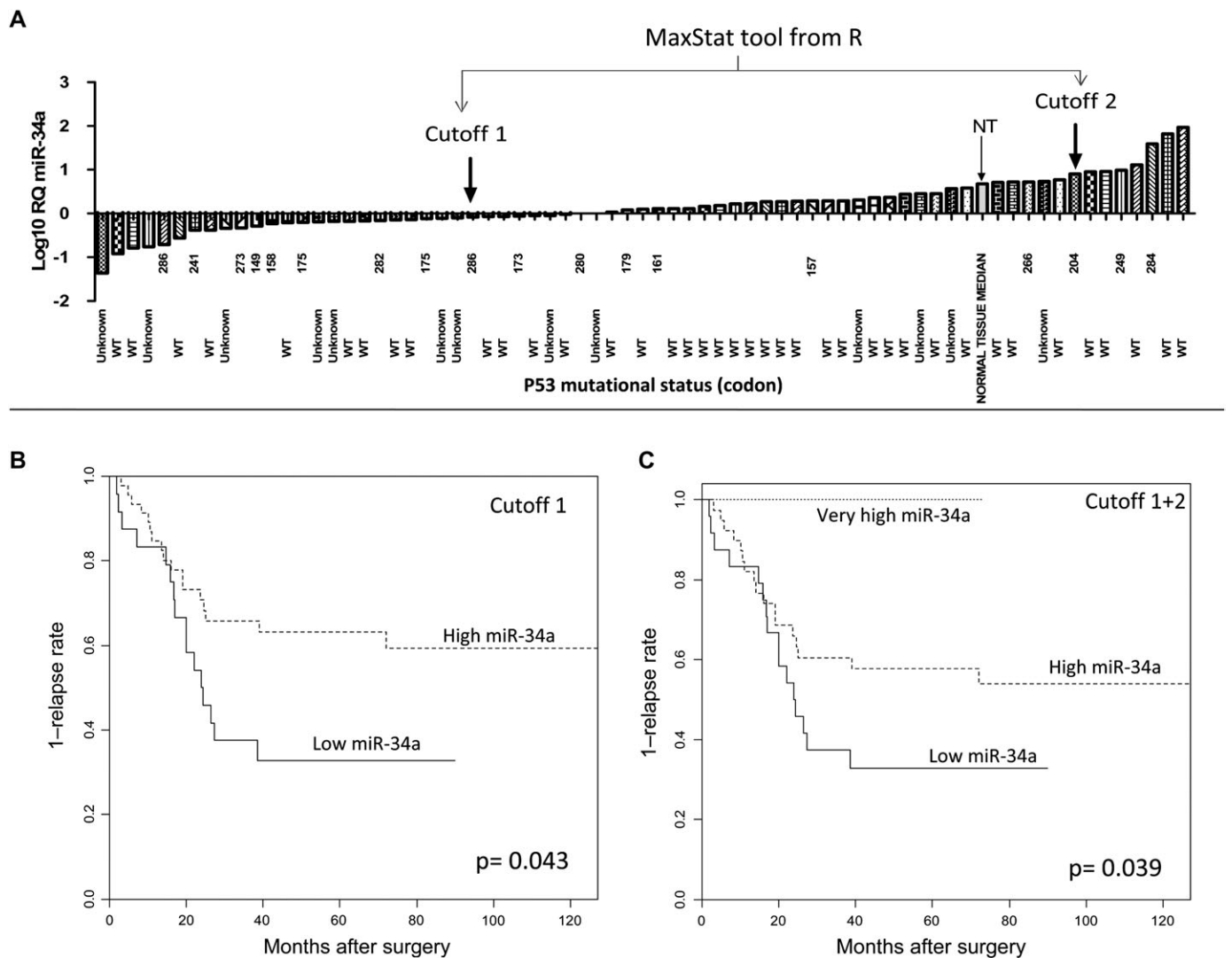


Fig. 2. miR-34a expression and relapse. miR-191 was used as normalization control. (A) miR-34a expression and cutoffs, as selected by maxStat. The data are shown as Log10 of relative quantification (RQ) ($2^{-\Delta\Delta C_t}$). miR-191 was used as endogenous normalization control. Patients are classified according to their miR-34a expression, and numbers indicate patients harboring p53 mutations. Unknown refers to patients in whom p53 status was not clear; WT indicates wild-type (non-mutated) p53 and the number indicates the codon where the mutation is located. NT indicates the median of expression in the normal tissues analyzed. miR-34a expression was dichotomized using the maxstat package of R in order to determine the cutoff that best discriminated between different groups of patients for relapse. Maxstat identified two significant cutoffs: one below (cutoff 1) and one above (cutoff 2) the median expression of normal tissue. (B) Probability of relapse using cutoff 1. (C) Probability of relapse using cutoffs 1 and 2.

divided into those with high versus low miR-34a expression, p53 mutations were found more frequently in those below (13/29, 45%) than in those above (5/28, 18%) the median expression level ($P = 0.04$).

No significant correlation was found between the location of the p53 mutation (exon 5, 6, 7 or 8) and miR-34a expression levels. However, patients with mutations in exon 5 showed a more marked

Table II. Multivariate analysis of relapse including all factors with univariate P -value <0.1

Factor	RR	P -value
A) Multivariate analysis of relapse without p53 mutational status (all 70 patients)		
Stage IA versus others	2.9	0.04
miR-34a expression	2.17	0.004
B) Multivariate analysis of relapse including p53 mutational status (57 patients)		
Stage IA versus others		0.1
P53 mutations		0.2
miR-34a expression	1.9	0.024

RR, relative risk.

Table III. Patients carrying p53 mutations and their miR-34a expression by cutoff 1

Patient	Exon	Codon	Nucleotide/change	AA change	Type of mutation	miR-34a levels (cutoff 1)
P1	5	161	GCC/ACC	Ala/Thr	Transition	High
P2	5	157	GTC/TTC	Val/Phe	Transversion	High
P3	5	175	CGC/CAC	Arg/His	Transition	Low
P4	5	149	TCC/TTC	Ser/Phe	Transversion	Low
P5	5	179	CAT/CGT	His/Arg	Transversion	High
P6	5	158	CGC/CTC	Arg/Leu	Transversion	Low
P7	5	175	CGC/CAC	Arg/His	Transition	Low
P8	6	204	GAG/TAG	Glu/STOP	Transversion	High
P9	7	249	AGG/AGT	Arg/Ser	Transversion	High
P10	7	241	TCC/TAC	Ser/Phe	Transversion	Low
P11	8	280	AGA/ATA	Arg/Ile	Transversion	High
P12	8	273	CGT/CAT	Arg/His	Transition	Low
P13	8	282	CGG/TGG	Arg/Trp	Transition	Low
P14	8	273	CGT/CTT	Arg/His	Transition	High
P15	8	286	GAA/AAA	Glu/Lys	Transition	Low
P16	8	286	GAA/AAA	Glu/Lys	Transition	Low
P17	8	284	ACA/ATA	Thr/Ile	Transition	High
P18	8	266	GGA/AGA	Gly/Arg	Transition	High

downregulation of miR-34a than those with mutations in the other exons ($P = 0.06$) (Figure 3B).

The relapse rate for patients with p53 mutations was 61.1% compared with 35.9% for those without p53 mutations ($P = 0.04$). Eight patients had both p53 mutations and miR-34a expression below the cutoff 1 level. A total of 87.5% of these patients relapsed compared with 36.7% of the remaining patients ($P = 0.001$) (Figure 4B).

Methylation status

Methylation status in the promoter region of the *MIRN34A* gene was assessed in 31 patients. Eight (26%) were unmethylated, 20 (64%) were methylated/unmethylated (patients that showed amplification in the MSP assay with both pairs of primers) and 3 (10%) were methylated (supplementary Figure 1 is available at *Carcinogenesis* Online). Unmethylated patients had significantly higher levels of miR-34a than the other two groups ($P = 0.008$) (Figure 5). In addition, the group of unmethylated patients showed a wide range of miR-34a expression levels [median = 0.51; range (-0.18 to 0.98); variance = 0.159].

Finally, when we analyzed the relation between *MIRN34A* methylation status and miR-34a expression in the 23 patients without p53 mutations, 6 (26%) were unmethylated, 14 (61%) were methylated/unmethylated and 3 (13%) were methylated. In this subgroup of patients, the variance decreased [median = 0.56; range (0.18 to 0.73); variance = 0.058], and the difference in median expression levels was more significant between methylated and unmethylated patients ($P = 0.004$) (Figure 5).

Discussion

In the present study, we have examined the role of miR-34 as a prognostic factor in NSCLC patients treated only with curative surgery. In our series of patients, miR-34a and miR-34b were significantly downregulated, and low levels of miR-34a in tumor samples correlated with a high rate of relapse. Moreover, miR-34a levels were modulated by methylation of the promoter region of the *MIRN34A* gene. The frequency of p53 mutations was significantly higher in patients with low miR-34a expression, and the group of patients with both p53 mutations and low miR-34a expression had a very poor prognosis, indicating a potential synergism for these two factors.

miR-34 transcription is directly induced by p53 in response to genotoxic stress and acts downstream to promote cell-cycle arrest or apoptosis (32,43,44). Importantly, miR-34 enables p53 to regulate the expression of a large number of proteins, even after their transcripts have already been synthesized, without the need for translation of additional effector proteins in situations of cellular stress. *In vitro* miR-34a overexpression leads to decreased proliferation and activation of apoptosis in multiple tumoral cells (43,45–49), indicating a role for miR-34a as a tumor suppressor gene, and in fact, miR-34a is lost or downregulated in many tumors (43,45–52). In the present study,

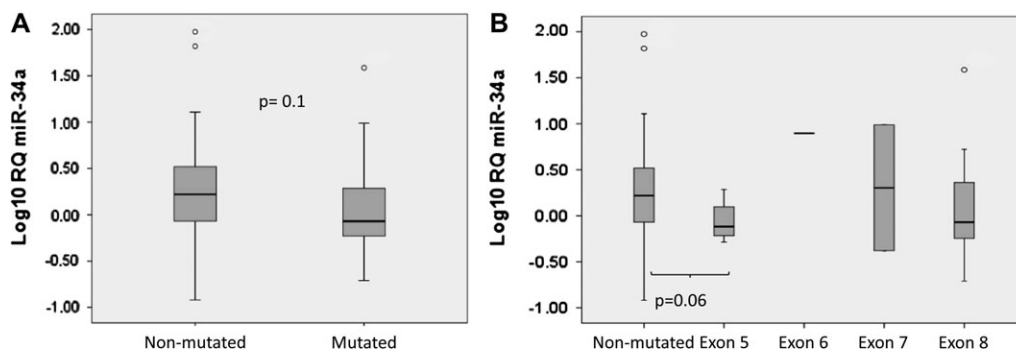


Fig. 3. Association between miR-34a expression (with miR-191 as normalization control) and p53 mutational status. (A) miR-34a expression in patients with and without p53 mutations. (B) miR-34a expression according to location of p53 mutation. Patients with mutations in exon 5 showed a non-significant trend toward greater downregulation of miR-34a.

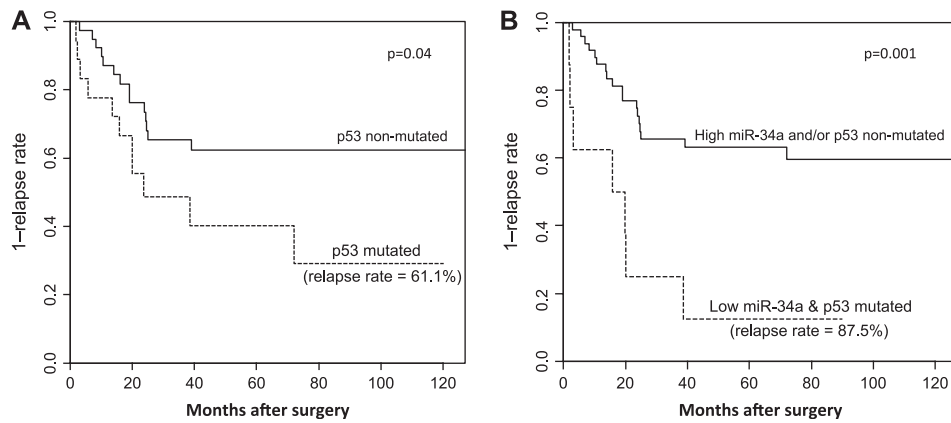


Fig. 4. Probability of relapse for patients according to p53 mutation status and miR-34a levels. **(A)** Relapse rate for patients according to p53 mutation status. Those with mutations had a higher relapse rate. **(B)** Relapse rate for patients with both low miR-34a levels and p53 mutations versus all other patients. Those with low miR-34a levels and p53 mutations had a higher relapse rate, indicating that miR-34a and p53 mutations synergize to stimulate relapse.

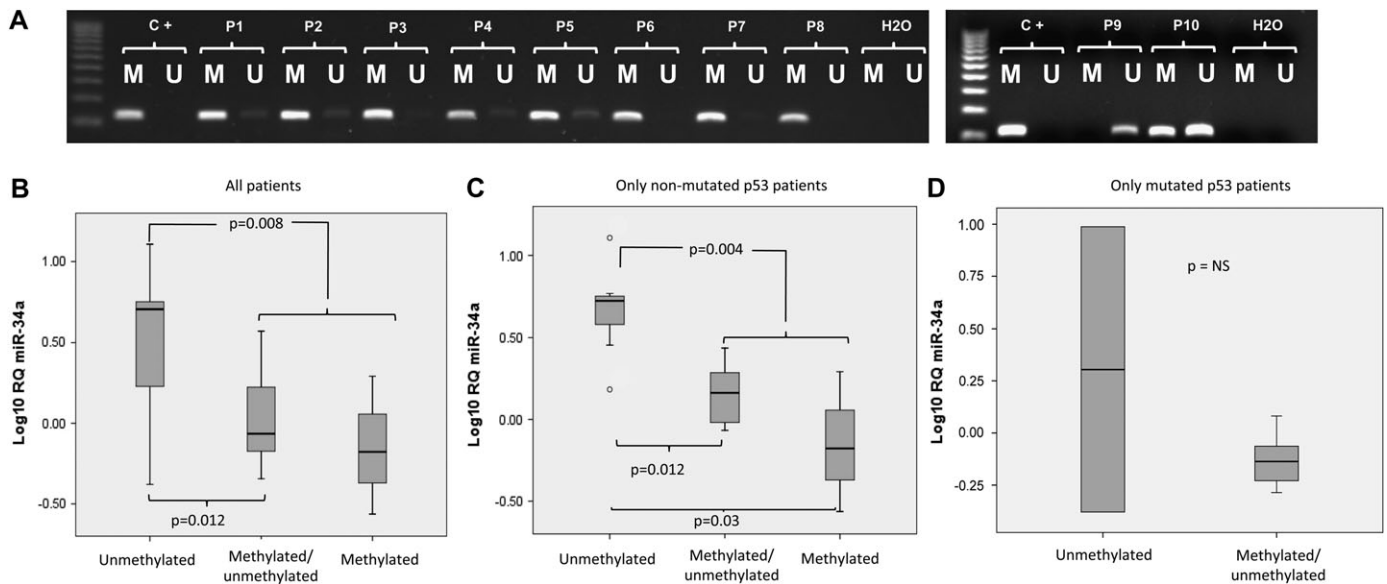


Fig. 5. miR-34a expression (miR-191 as normalization control) according to the methylation status of *MIRN34A*. The methylated/unmethylated group includes the patients that showed amplification in the MSP assay with both pairs of primers. **(A)** Examples of methylation analysis results (methylated, methylated/unmethylated and unmethylated). PCR products of 10 patients are shown in two gels (2% agarose). P1, P2, P3, P4, P5, P7 and P10 are methylated/unmethylated, P6 and P8 are methylated, and P9 is unmethylated. Commercially modified DNA was used as positive control (C+) for methylated alleles. The MSP analyses for all patients are shown in supplementary Figure 1 (available at *Carcinogenesis* Online). **(B)** miR-34a expression in all 31 patients in whom methylation status was assessed. **(C)** miR-34a expression in 23 patients without p53 mutations. **(D)** miR-34a expression in seven patients with p53 mutations.

patients with low miR-34a expression had a higher risk of relapse than those with high expression, indicating that interindividual differences in miR-34a expression may have an impact on rate of relapse.

In vitro inactivation of endogenous miR-34a effectively inhibits p53-dependent apoptosis. However, increasing miR-34a levels elicits only a very mild increase in apoptosis, suggesting that miR-34a may be a necessary but not sufficient requisite (44). Patients with both p53 mutations and low miR-34a expression have alterations in two important points of the p53 pathway. In the present study, this group of patients had the highest risk of relapse, leading us to hypothesize that the impact of miR-34a on cell death may be greatest when acting synergistically with additional modulators.

In chronic lymphocytic leukemia deleted and/or mutated p53 correlates with low levels of miR-34a (53), although a significant correlation was not found in the present study. However, there is some evidence in NSCLC that p53 mutations can have different repercussions depending on their location (54), and this may have had a bear-

ing on the results in the present study, where patients harboring exon 5 mutations tended to have lower miR-34a expression levels.

Moreover, in addition to p53 mutations, other mechanisms can decrease miR-34 expression; for example, the miR-34-encoding genes themselves may be targets for mutational or epigenetic inactivation in cancer (55). Loss of miR-34a expression in neuroblastoma was associated with the deletion of a region on chromosome 1p36, a common event in neuroblastoma (46,55,56). Aberrant CpG methylation that reduces the expression levels of miR-34a has also been reported in multiple types of cancer (36), and in the present study, a correlation was found between methylation status and miR-34a expression.

p53 induces cell-cycle arrest, cellular senescence and apoptosis through various targets. miR-34a is a direct transcriptional target of p53 and is upregulated in the presence of a functional p53 pathway. miR-34a itself induces apoptosis, cellular senescence and cell-cycle arrest through silencing of other potential targets, such as

cyclin-dependent kinases 4 and 6, CCND1, MYCN and Sirtuin1 (33,57–59). miR-34a-based therapeutic approaches could thus be useful in patients with a non-functional p53 pathway, where increasing miR-34a levels may provide an alternative to p53-induced apoptosis. Though miRNA-based therapies are still in the preliminary stages, some promising results have been reported. For example, aerosol application of let-7a has been shown to increase expression levels in mouse lungs, leading to a decrease in tumor size (60). The present study shows a link between *MIRN34A* methylation status and miR-34a expression levels, raising the possibility that miR-34a expression could be induced by demethylation therapies such as 5-azacytidine (61).

In summary, we have identified miR-34a as a novel prognostic marker in NSCLC patients, providing a potential mechanism for estimating a patient's risk of disease recurrence and an additional useful tool to guide treatment decisions regarding adjuvant therapy. Further investigation in a prospective study is warranted to validate these findings and to examine potential miR-34a-based therapeutic approaches.

Supplementary material

Supplementary Figure 1 can be found at <http://carcin.oxfordjournals.org/>

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Conflict of Interest Statement: None declared.

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