

Predictive relevance of miR-34a, miR-224 and miR-342 in patients with advanced squamous cell carcinoma of the lung undergoing palliative chemotherapy

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Abstract. Attributing to their pathophysiological role and stability in biological samples, microRNAs (miRNAs) have the potential to become valuable predictive markers for non-small cell lung cancer (NSCLC). Samples of biopsy tissue constitute suitable material for miRNA profiling with the aim of predicting the effect of palliative chemotherapy. The present study group included 81 patients (74 males, 7 females, all smokers or former smokers) with the squamous cell carcinoma (SCC) histological subtype of NSCLC at a late stage (3B or 4). All patients received palliative chemotherapy based on platinum derivatives in combination with paclitaxel or gemcitabine. The expression of 17 selected miRNAs was measured by reverse transcription-quantitative polymerase chain reaction in tumor tissue macrodissected from formalin-fixed paraffin-embedded (FFPE) tissue samples. To predict the effect of palliative chemotherapy, the association between gene expression levels and overall survival (OS) time was analyzed. From the 17 miRNAs of interest, low expression levels of miR-342 and high expression levels of miR-34a and miR-224 were associated with a reduced OS time in subgroups of patients based on smoking status and treatment modality. Using cluster analysis, associations between combinations of miR-34a, -224 and -342 expression levels with patient survival were identified. The present study revealed that patients with the simultaneous high expression of miR-224 and -342 had a similar prognostic outcome to those with the low expression of miR-224 and -342, which was significantly reduced, compared with patients exhibiting high expression of either miR-224 or miR-342 with low expression of the other. We hypothesize that

the effect of a particular miRNA is dependent on the expression level of other members of the miRNA network. This finding appears to complicate survival analyses based on individual miRNAs as markers. In conclusion, the present study provides evidence that specific miRNAs were associated with OS time, which may be candidate predictors for the effectiveness of palliative treatment in SCC lung cancer patients. This objective can be better achieved by combining more markers together than by using individual miRNAs.

Introduction

Lung cancer is the most common type of cancer, with high mortality rates worldwide (1); the incidence in the Czech Republic was 86.9 cases in men and 38.0 in women per 100,000 people in 2011 (2). Approximately 85% of all lung cancer cases are non-small cell lung cancer (NSCLC), which includes two major histological subtypes: Squamous cell carcinoma (SCC) and adenocarcinoma. SCC represents ~25-30% of cases of NSCLC (3). The prognosis for patients with advanced SCC is poorer than that of those with adenocarcinoma (4).

Chemotherapy is an essential modality of palliative treatment for inoperable SCC at advanced stages. The response rate to chemotherapy varies widely from patient to patient; therefore, it is of interest to find biomarkers that predict the effect of cytostatic therapeutics. The resistance of cancer cells to chemotherapy can be caused by the increased export of anti-cancer drugs out of the cells, improved DNA repair ability or apoptosis resistance (5). The expression of genes participating in these processes is regulated by the microRNA (miRNA/miR) network. miRNAs are small non-coding RNA molecules of ~22 nucleotides that participate in the post-transcriptional regulation of gene expression (6). The human genome encodes >2,500 miRNAs (7), which target ~60% of mammalian genes and are abundant in a number of human cell types (8) (see miRNA database available online at www.mirbase.org).

The aim of the present study was to evaluate the association of the expression of miRNAs involved in the processes resulting in chemotherapy resistance with the overall survival (OS) time of patients with advanced SCC receiving palliative

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care. All patients in the cohort of the present study received palliative chemotherapy based on platinum derivatives (cisplatin or carboplatin) in combination with paclitaxel or gemcitabine.

On the basis of previously published literature, the present study focused on miRNAs whose effect on the processes involved in chemotherapy resistance may be expected (miR-15b, miR-21, miR-27a, miR-34a, miR-99a, miR-106a, miR-107, miR-143, miR-150, miR-192, miR-193, miR-211, miR-218, miR-221, miR-224, miR-342 and miR-375). A list of the main characteristics of miRNAs of interest, including references, is included in Table I.

Patients and methods

Ethics statement. This study was approved by the Ethics Committee of the University Hospital in Pilsen (Pilsen, Czech Republic). Written informed consent was obtained from all the subjects. Anonymized data were used to conduct the present study.

Patients. The present study was retrospective. The study group consisted of 81 patients with late-stage (3B or 4) and the SCC histological subtype with an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0-2 of NSCLC treated between January 2000 and June 2014 at the Department of Pneumology and Phthisiology of the University Hospital in Pilsen. Stage of disease was determined using the TNM (Tumor Nodus Metastasis) system of the International Union Against Cancer (IUCC; 7th edition) (9). The median patient age was 62.4 years (range, 32.7-79.3 years), and there were 74 males and 7 females. All patients underwent palliative chemotherapy using platinum derivatives in combination with paclitaxel or gemcitabine. The use of sequential radiotherapy was permitted for patients with stage 3B disease; patients with concurrent radiotherapy were excluded from the present study. In certain patients with stage 3B disease, radiotherapy was not indicated due to poor PS. The exclusion criteria for entering the study were >80 years of age, other malignancy and high cardiopulmonary risk. Clinicopathological data, including age at the time of diagnosis, smoking status, clinical disease stage, radiotherapy status and chemotherapy regimen, are listed in Table II.

Tissue samples and RNA isolation. Biopsy tissue samples were obtained using bronchoscopy for diagnostic purposes prior to chemotherapy and were processed by standard laboratory techniques at the Department of Pathology of the University Hospital in Pilsen. Formalin-fixed paraffin-embedded (FFPE) tissue samples were stored at room temperature until use. Paraffinized sections were stained with hematoxylin and eosin, microscopically verified by pathologists and examined to identify sites with cancer cells for macrodissection. Total RNA (including miRNA) was extracted from 15- μ m thick FFPE sections following the macrodissection of tumor tissue using the miRNeasy FFPE kit (Qiagen, Hilden, Germany) as described previously (10).

Quantitative estimation of microRNA expression. A quantitative estimation of 17 selected miRNAs (Table I) was performed

by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method using TaqMan[®] MicroRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in technical duplicates on the Stratagene Mx3005P apparatus (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The two-step protocol included reverse transcription with a miRNA-specific primer, followed by qPCR with TaqMan[®] probes. Briefly, 5 ng of RNA was reverse transcribed in a 20- μ l reaction containing 2.5 μ l of primers specific to particular miRNA (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 20- μ l PCR reactions included 2.5 μ l of RT product. The reactions were incubated in 96-well plates at 95°C for 15 min and then followed by 48 cycles of 95°C for 15 sec and 60°C for 60 sec. The $2^{-\Delta\Delta C_q}$ method was used for the quantification of qPCR data, as described previously (11); the expression was normalized to RNU6B (U6snRNA). Details of all kits for estimation of all miRNAs and RNU6B are included in Table I.

Statistical analysis. SAS version 9.3 statistical software (SAS Institute, Inc., Cary, NC, USA) was used to perform all statistical calculations. Although the group of patients in the present study was as homogenous as possible (SCC subtype of NSCLC, stages 3B and 4), certain patients underwent radiotherapy, which may have caused confounders. The potential effect of treatment inconsistency was mitigated by evaluating miRNA expression in the subgroups of patients.

The evaluation of prognostic significance (the association between markers and time to recurrence) was performed as a univariate analysis of maximum likelihood estimates using the Cox regression hazard model. For markers significant in the Cox model, an optimal cut off was identified. There is no standard method for biomarker cut-off determination to split continuous variables into two groups; the simplest approach used in exploratory studies is to set the median as a cut-off value. However, in the case of unequal distribution of events in the studied group, this approach is far from optimal. The present study used continuous search for the cut-off value by searching for the lowest P-value of the log-rank test, as described previously (12,13).

miRNAs identified as significant by univariate analysis were incorporated into a multivariate analysis and the Kaplan-Meier survival distribution functions were generated for combinations of miRNA expression levels (clustering) with the cut-off values from univariate analysis and later, the median. $P < 0.05$ was considered to indicate a statistically significant difference. DIANA-TarBase v7.0 and DIANA-miRPath v3.0 bioinformatic tools were used to identify overlapped target genes of the miRNAs of interest (14,15).

Results

Effect of treatment modalities on OS time. Prior to the analysis of the association between gene expression and OS, the outcomes for subgroups of patients undergoing different treatment were compared. A significantly longer OS time was identified in the subgroup of patients who underwent chemotherapy combined with radiotherapy in comparison with patients who underwent chemotherapy alone ($P = 0.0498$; Fig. 1A). There were no significant differences in OS between

Table I. Analyzed miRNAs and their involvement in pathogenesis and treatment of NSCLC.

Symbol	miRBase accession no.	Cat. no. 4427975 assay ID	Relation to NSCLC	(Refs.)
miR-15b	MIMAT0000417	000390	Regulates cisplatin resistance and metastasis by targeting PEBP4 in lung adenocarcinoma cells	(35)
miR-21	MIMAT0000076	000397	Regulates NSCLC cell invasion and chemo-sensitivity through SMAD7	(36)
miR-27a	MIMAT0000084	000408	Higher expression levels in advanced NSCLC patients resistant to EGFR-TKI	(37)
miR-34a	MIMAT0000255	000426	Sensitizes lung cancer cells to cisplatin via p53/miR-34a/MYC axis	(38)
miR-99a-3p	MIMAT0004511	002141	Promotes proliferation, migration and invasion of NSCLC cell lines	(39)
miR-106a	MIMAT0000103	000578	Confers cisplatin resistance in non-small cell lung cancer A549 cells	(40)
miR-107	MIMAT0000104	000443	Regulates cisplatin chemosensitivity of A549 non small cell lung cancer cell lines by targeting cyclin dependent kinase 8	(41)
miR-143	MIMAT0000435	002249	Regulates cell apoptosis in lung cancer by targeting PKC ϵ	(42)
miR-150	MIMAT0000451	000473	Downregulation induces cell proliferation inhibition and apoptosis in NSCLC by targeting BAK1	(43)
miR-192	MIMAT0000222	000491	Regulates chemo-resistance of lung adenocarcinoma for gemcitabine and cisplatin combined therapy by targeting Bcl-2	(44)
miR-193a-3p	MIMAT0000459	002250	Suppresses the metastasis of NSCLC by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway	(45)
miR-211	MIMAT0000268	000514	Promotes NSCLC proliferation by targeting SRCIN1	(46)
miR-218	MIMAT0000275	000521	Regulates cisplatin chemosensitivity in NSCLC by targeting RUNX2	(47)
miR-221	MIMAT0000278	000524	Overexpressed in aggressive NSCLC and regulates TRAIL resistance through PTEN and TIMP3	(48)
miR-224	MIMAT0000281	002099	Is implicated in lung cancer pathogenesis through targeting caspase-3 and caspase-7	(19)
miR-342-3p	MIMAT0000753	002260	Suppresses proliferation and invasion of NSCLC by targeting RAPB2	(21)
miR-375	MIMAT0000728	000564	Predictive for response for non-small cell lung cancer treated with cisplatin-vinorelbine A	(49)
RNU6B	-	001093	Reference gene	(50)

miR/miRNA, microRNA; NSCLC, non-small cell lung cancer.

subgroups of patients with different chemotherapy regimens (Fig. 1B).

Association of miRNA expression with OS time. The Cox regression hazard model was used to determine the association between the levels of miRNA expression with OS time. From the 17 miRNAs of interest, in the subgroup of smokers, the low expression of miR-342 ($P=0.0500$) and high expression level of miR-34a and miR-224 ($P=0.0338$ and $P=0.0400$, respectively) were associated with a shorter OS time. High expression levels of miR-34a were associated with shorter OS time in the subgroup of patients treated with platinum derivate-based chemotherapy in combination with gemcitabine ($P=0.0364$). High expression levels of miR-224 were associated with shorter OS time in the subgroup of patients who underwent chemotherapy combined with radiotherapy ($P=0.0250$).

For the statistically significant miRNA markers, optimal cut-off values were identified and Kaplan-Meier survival distribution functions for OS were generated. Statistically significant differences in OS time between subgroups with marker expression levels below and above the cut-off value were obtained for miR-342 in the subgroup of smokers ($P=0.0243$; Fig. 2A), miR-34a in a subgroup of patients that were treated with gemcitabine in chemotherapy regimen ($P=0.0239$; Fig. 2B) and miR-224 in the subgroup of patients that underwent chemotherapy combined with radiotherapy ($P=0.0093$; Fig. 2C). Statistical values obtained from the Kaplan-Meier analyses are summarized in Table III.

miRNAs associated with OS (miR-34a, -224 and -342) were the subject of the subsequent cluster analysis. Pairs of these miRNAs (miR-34a and -224, miR-34a and -342, and miR-224 and -342) were analyzed for their association with

Table II. Clinicopathological characteristics of patients with squamous cell carcinoma of the lung (n=81).

Characteristic	Patients, n (%)
Sex	
Male	74 (91.4)
Female	7 (8.6)
Age, years	
<55	11 (13.6)
55-65	41 (50.6)
>65	29 (35.8)
Smoking status	
Non-smoker	0 (0)
Ex-smoker	42 (51.9)
Smoker	39 (48.1)
Clinical stage	
3B	42 (51.9)
4	39 (48.1)
Eastern Cooperative Oncology Group performance status	
0	2 (2.5)
1	58 (71.6)
2	18 (22.2)
3	3 (3.7)
Radiotherapy	
Yes	25 (30.9)
No	56 (69.1)
Chemotherapy	
Paclitaxel and carboplatin	35 (43.2)
Gemcitabine and cisplatin	46 (56.8)

OS time. For each pair of miRNAs, patients were stratified into groups according to the miRNA expression being above (high) or below (low) the cut-off value: Group A (high miR-224 and high miR-342); group B (high miR-224 and low miR-342); group C (low miR-224 and high miR-342); and group D (low miR-224 and low miR-342). Initially, the cut-off value obtained from univariate analysis was used also for cluster analysis; however, this led to a highly disproportional distribution of patients among subgroups. Therefore, a median was used as a cut off value for cluster analysis.

Fig. 3 demonstrates the Kaplan-Meier survival distribution functions of patients stratified into groups according to the expression of miR-224 and miR-342. There are two pairs of groups with similar OS distributions; Fig. 4 includes a comparison of the OS of two groups of patients created by combining the groups from Fig. 3 with similar survival outcomes (group A/D vs. group B/C). There was a significant difference in survival between these groups ($P=0.0018$), as detailed in Table IV. The same approach was used to analyze the other pairs of miRNAs; however, no significance was identified. All three miRNAs were analyzed together in the same manner (miR-34a, -224 and -342). Patterns of expression

associating patients with significantly shorter survival times were identified (Fig. 5; Table IV).

Identification of potential target genes for miR-34a, -224 and -342. Using DIANA-TarBase v7.0 and DIANA-miRPath v3.0 bioinformatic tools (11,12), 6 overlapping target genes with $P<0.05$ were identified between miR-34a, -224 and -342. These genes, including GNAS complex locus (GNAS), insulin like growth factor 1 receptor (IGF1R), cyclin D1 (CCND1), cyclin G2 (CCNG2), serpin family E member 1 (SERPINE1) and ribonucleotide reductase regulatory subunit M2 (RRM2), are associated with cell cycle regulation, p53 signaling and DNA repair.

Discussion

miRNAs may have the potential to become accurate, easily measurable biomarkers, with features convenient for diagnostic testing methods, including stability in FFPE tissue blocks, blood, and potentially, other bodily fluids (16). The present study focused on patients with the NSCLC SCC subtype with an advanced-stage SCC. The included patients were unable to undergo surgical resection and received palliative treatment only. For these patients, there were multiple treatment modalities. The main clinical concern in such cases is deciding which therapeutic regimen is indicated. However, in the group of patients in the present study, it was only possible to analyze the potential predictors for the treatment response to platinum base derivatives in combination with either paclitaxel or gemcitabine, with or without the application of radiotherapy.

Initially, the present study focused on a univariate analysis of the association between miRNA expression and OS time. Subsequently, a multivariate analysis was performed that included the miRNAs that had been identified to exhibit associations with OS. On the basis of the results of the present study, we hypothesize that the effect of a single miRNA may depend on the level of expression of other members of the miRNA network, to be further discussed.

Higher levels of miR-224 indicated shorter OS times for patients with chemotherapy combined with radiotherapy in the present study. Cui *et al* (17) reported that miR-224 expression was significantly upregulated in NSCLC tissues and suggested it performed its oncogenic role in lung cancer pathogenesis through targeting caspase-3 and -7. Wang *et al* (18) identified through microarray analysis that miR-224 expression was upregulated in cisplatin-resistant cell lines, and demonstrated that miR-224 could promote cisplatin resistance via regulating the G₁/S cell cycle transition and apoptosis by targeting p21. These findings indicated the association of miR-224 with the effect of chemotherapy based on DNA damage, and its potential as a predictor for the response to treatment. However, Zhu *et al* (19) reported that miR-224 expression levels were downregulated in NSCLC compared with non-cancerous lung tissue. These authors also observed that decreased miR-224 expression was significantly associated with lymph node metastasis, an advanced tumor-node-metastasis stage and a reduced OS time (19). Furthermore, Wang *et al* (20) recently identified that miR-224 was significantly upregulated in NSCLC tissues and hypothesized that miR-224 expression promotes NSCLC cell proliferation by downregulating Ras association domain

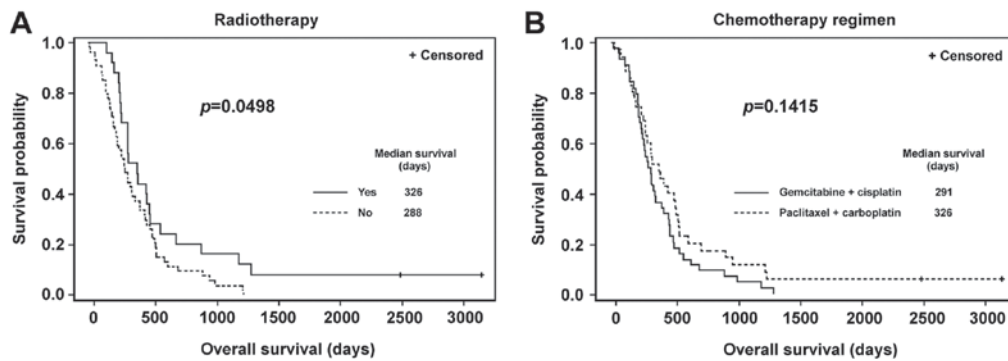


Figure 1. Association between the treatment modality and OS time for non-small cell lung cancer patients, as determined with the Kaplan-Meier method. All patients were treated with chemotherapy. (A) There were significantly longer OS times in the subgroup of patients who underwent chemotherapy combined with radiotherapy, compared with the patients who underwent chemotherapy without radiotherapy. (B) OS was independent of the chemotherapy regimen received. OS, overall survival.

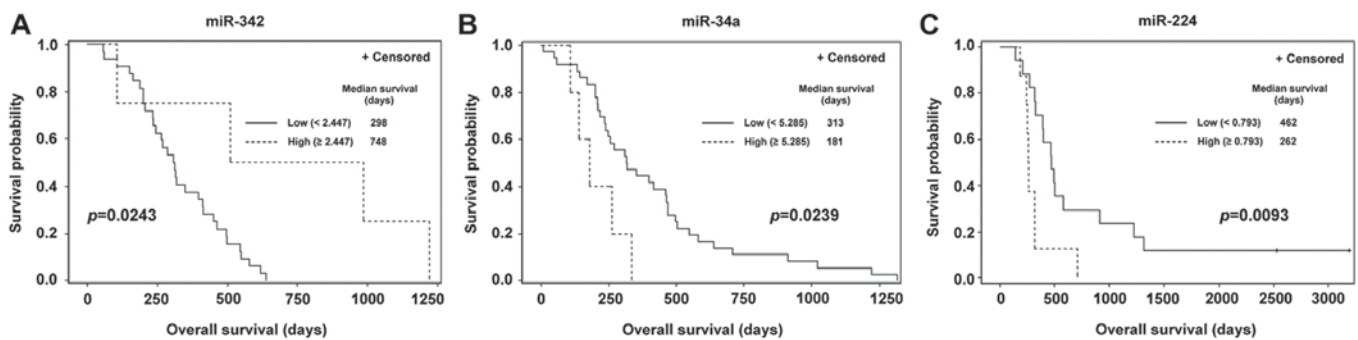


Figure 2. Association of miRNA expression with OS in the subgroups of non-small cell lung cancer patients (Kaplan-Meier curves). (A) Low expression of miR-342 was associated with shorter OS time in the subgroup of smokers. (B) High expression of miR-34a was associated with shorter OS time in the subgroup of patients treated with chemotherapy based on platinum derivatives in combination with gemcitabine. (C) High expression of miR-224 was associated with shorter OS time in a subgroup of patients who underwent chemotherapy combined with radiotherapy. OS, overall survival; miR, microRNA.

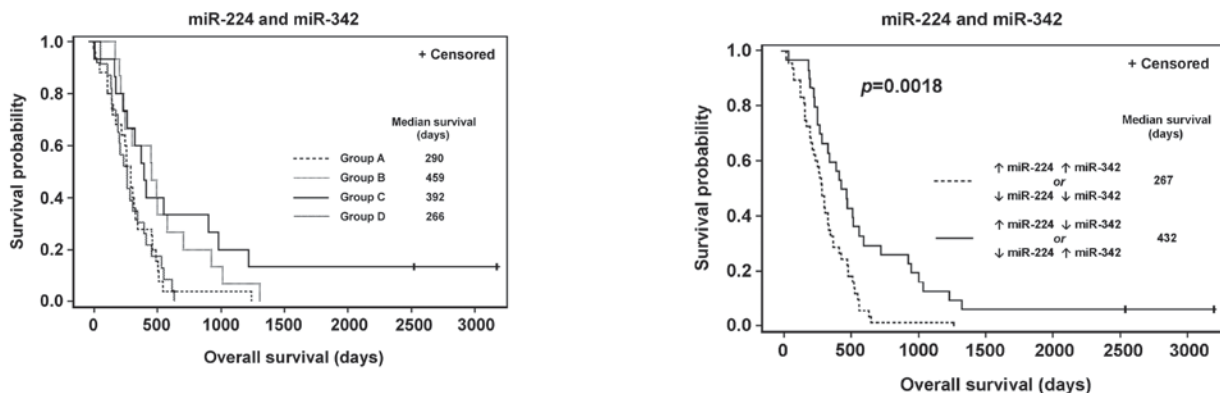


Figure 3. Kaplan-Meier curves for the overall survival of patients stratified into four groups according to the expression of miR-224 and -342: Group A, high miR-224 and -342; group B, high miR-224 and low miR-342; group C, low miR-224 and high miR-342; and group D, low miR-224 and -342. miR, microRNA.

Figure 4. Comparison of survival of two groups of patients created by putting groups with similar survival from Fig. 3 together (group A and D vs. group B and C). Patients with the high expression of both miR-224 and -342 or low expression of both miR-224 and -342 have significantly shorter OS times than those with the high expression of either miR-224 and -342 and the low expression of the other. miR, microRNA.

family member 8 expression; the inconsistency of these studies will be discussed in the following paragraph.

In the present study, low levels of miR-342 indicated a poorer outcome in patients with a history of smoking, independent of treatment modality. Xie *et al* (21) demonstrated that miR-342 was downregulated in NSCLC and acted as a tumor suppressor through the repression of RAP2B, member of RAS oncogene family. Similarly, Tai *et al* (22) identified

that miR-342 was capable of indirectly regulating MYC activity via the direct repression of E2F transcription factor 1. Takahashi *et al* (23) investigated how cigarette smoking altered plasma miRNA profiles; they identified that there was a decrease in plasma miR-342 in subjects who quit smoking, compared with smokers.

Table III. Association between the level of miRs and overall survival time as determined by Kaplan-Meier estimation.

Patient group	Treatment	Marker	Patients, n	Cut-off	Below cut-off		Above cut-off		P-value
					n	Median, days	n	Median, days	
Smokers only	Chemotherapy	miR-342	36	2.447	32	298	4	748	0.0243
Smokers and ex-smokers	Gemcitabine and cisplatin	miR-34a	41	5.285	36	313	5	181	0.0239
Smokers and ex-smokers	Chemotherapy and radiotherapy	miR-224	25	0.793	17	462	8	262	0.0093

miR, microRNA.

Table IV. Association between combinations of miRs and OS (Kaplan-Meier estimation).

Expression pattern	Patients, n	Median OS, days
miR-342 and -224		
High miR-224 and -342, or low miR-224 and -342	48	267
High miR-224 and low miR-342, or low miR-224 and high miR-342	30	432
miR-342, -224 and -34a		
High miR-224, -342 and -34a, or low miR-224, -342 and -34a	39	250
Other combinations	35	451

OS, overall survival; miR, microRNA.

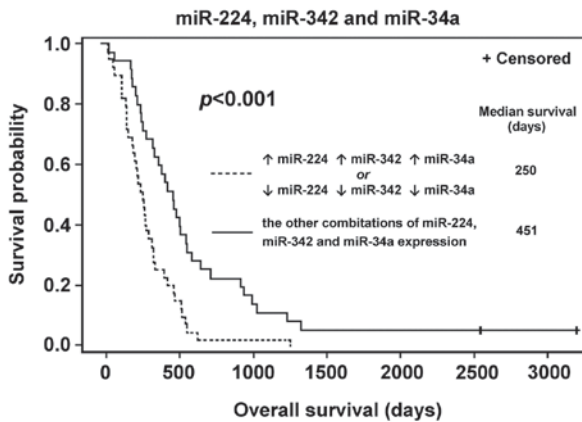


Figure 5. Comparison of the OS of two groups of patients based on patterns of miR-224, -342 and -34a expression. Patients with the high or low expression of all three miRs exhibit a significantly shorter OS time than those with other combinations of miR-224, -342 and -34a expression. OS, overall survival; miR, microRNA.

miR-34a is a member of the miR-34 family that is associated with the p53 pathway, and is implicated in cell death/survival signaling (24). The miR-34 family is transcriptionally activated by p53; in turn, p53 is a direct miR-34a target. However, the effect of miR-34a on p53 depends on the cellular context (25). miR-34a can have also a positive effect on p53 transcriptional activity and protein stability by targeting multiple p53 inhibitor genes (including MDM4, p53 regulator, sirtuin 1, metastasis associated 1 family member 2, histone deacetylase 1 and YY1 transcription factor) (26). A previous

study identified that miR-34a inhibits cell proliferation (27). Expression of the miR-34 family was downregulated in tumor tissue compared with normal tissue, and low levels of miR-34a expression were associated with a higher probability of relapse in surgically resected NSCLC (28). However, higher levels of circulating miR-34a were observed in patients with NSCLC compared with healthy controls (29). Higher levels of miR-34a indicated a shorter OS time in patients receiving palliative platinum derivate-based chemotherapy in combination with gemcitabine in the present study.

Multivariate analysis was performed with the miRNAs (miR-34a, -224 and -342) that were identified as associated with OS. The most notable finding was that patients with the high expression of miR-224 and -342 exhibited similar outcomes to those with low expression of miR-224 and -342, which was significantly shorter than that of patients with high expression of either miR-224 or miR-342 and the low expression of the other (Figs. 3 and 4).

We hypothesize that the effect of a single miRNA is dependent on the level of expression of the other members of the miRNA network. It has been established that an miRNA can have a predominantly oncogenic role in one type of cancer and a tumor suppressive role in another; for instance, miR-224 was identified to be a tumor suppressor in prostate cancer (30), whereas in other types of malignancy, including gastric (11,31) and colorectal cancer (32), an oncogenic role for miR-224 was described. The ambiguous role of miR-224 was also observed within the SCC histological subtype of NSCLC in the present study. Tumor progression occurs as a result of the dysregulation of a number of protein-coding genes and epigenetic processes,

including the deregulation of a number of miRNAs. Therefore, to understand the role of one particular miRNA, it is necessary to determine the levels of the other 'co-players'. In the present study, OS time was influenced by the mutual association of miR-224 and -342. The high level of miR-224 can be associated with adverse or favorable outcomes, depending on the simultaneous level of miR-342. These findings could explain the inconsistent results of previously published studies on miR-224 expression in NSCLC. In 2014, Zhu *et al* (19) reported that miR-224 was significantly downregulated in NSCLC and that a decrease in miR-224 expression was significantly associated with shorter OS time (19). Also in NSCLC, Cui *et al* (33) identified that miR-224 was significantly upregulated, with the increased expression of miR-224 promoting cell migration, invasion, and proliferation. As aforementioned, the present study also identified that the high expression levels of miR-224 were associated with shorter OS time in one subgroup of patients, specifically those who underwent chemotherapy combined with radiotherapy.

Using bioinformatic tools, the present study identified overlapping experimentally validated target genes for miR-34a, -224 and -342. Notably, all overlapping target genes identified in the present study (GNAS, IGF1R, CCND1, CCNG2, SERPINE1, and RRM2) are involved in processes associated with carcinogenesis, including cell cycle regulation, p53 signaling and DNA repair. This may explain the complicated mutual dependency of those miRNAs in relation to tumor progression and the effectiveness of treatment. We hypothesize that these molecules could be involved in competing endogenous RNA crosstalk, where RNA transcripts co-regulate each other by competing for shared miRNAs, thereby titrating miRNA availability (34). However, one limitation of the present study is the absence of immunoprecipitation data and reporter assays, which are methods that may confirm the interactions among the set of 3 miRNAs and 6 target genes. Nevertheless, the results of the present study may provide a stimulus for further research in this area.

With cluster analysis, novel associations between miR-34a, -224 and -342 that affected patient survival time were identified in the present study. The result may demonstrate that the effort to find a particular miRNA as a perfect marker for a particular event may be fruitless due to the complex interactions between RNA transcripts. In order to understand all aspects of the effect of miRNAs on the regulation of gene expression in cancer and their associations with phenotype and treatment outcome, miRNA profiling and deep bioinformatic analysis will be necessary. Only this approach can facilitate the future application of miRNAs in clinical practice. miRNAs can generally be assessed with more precision and ease than the mRNAs of coding genes, as miRNA analysis is less demanding in terms of the quality and quantity of isolated RNA, features that may be problematic in RNA samples extracted from FFPE tissue (16). FFPE tissue samples are routinely taken and analyzed during standard lung cancer management, which is why miRNAs may become clinically applicable predictors of the effectiveness of palliative treatment in patients with lung cancer. Nevertheless, the findings of the present study demonstrated that, due to the complex network of interactions, this objective could be achieved by combining more markers together rather than by using individual miRNAs. On the basis of the results of the

current study, miR-224, -342 and -34a could be members of this panel of predictors of treatment efficacy.

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