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# Genetic variants in *miR-146a, miR-149, miR-196a2, miR-499* and their influence on relative expression in lung cancers

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## Abstract

**Background:** The presence of sequence variants in miRNA genes may influence their processing, expression and binding to target mRNAs. Since single miRNA can have a large number of potential mRNA targets, even minor variations in its expression can have influences on hundreds of putative mRNAs.

**Methods:** Here, we evaluated 101 paired samples (cancer and normal tissues) from non-small cell lung carcinoma (NSCLC) patients to study the genotype distribution of single nucleotide polymorphisms (SNPs) in *miR-146a (rs2910164 C-G), miR-149 (rs2292832 C-T), miR-196a2 (rs11614913 C-T)* and *miR-499 (rs3746444 G-A)* and their influence on the expression of respective miRNAs.

**Results:** Relative expression of *miR-146a, miR-149* and *miR-499* were comparable in NSCLC and in paired control tissues. On the contrary, we clearly detected a significant increase ($p < 0.001$) of *miR-196a2* expression in NSCLC. In particular we found a significant association between *miR-196a2* CC genotype and high expression, whereas TT genotype showed a very low expression in comparison to both CT ($p < 0.005$) and CC patients ($p < 0.01$). We did not find any association between *miR-149, miR-196a2* and *miR-499* genotype and risk of NSCLC. Conversely, CG genotype of *miR-146a* appeared associated to an increased risk for NSCLC ($p = 0.042$ and 1.77 OR).

**Conclusions:** Our results seem to demonstrate that sequence variants of *miR-196a2* can have an influence on its expression, while *miR-146a* can have a role in increasing the risk of NSCLC.

**Keywords:** high resolution melting analysis; miRNA expression; miRNA genotypes.

## Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post transcriptional level. It is estimated that there may be 1000 miRNA genes in the human genome (1). miRNAs are expressed in a tissue-specific manner and changes of their expression can correlate to disease status (2, 3). Identification of putative miRNA targets is difficult since only the seed sequence (about 6–8 bases) aligns perfectly with the target mRNA 3′ untranslated region (4, 5). The residue miRNA sequence may bind perfectly to the target mRNA, but more often it does not. Target mRNAs can be identified by the bioinformatics approaches, but miRNA effects need to be assayed in vitro or in vivo to determine if they truly affect the proposed mRNA. 'In silico' data indicated that a single miRNA could bind hundreds mRNA targets and therefore each miRNA would be involved in several biological processes.

Several lines of evidences indicate that miRNAs are involved in malignant transformation of human cells (6) acting either as a tumor suppressor or oncogene (7). miRNAs profiling revealed that most of them are significantly deregulated in human cancers (2, 8). The molecular mechanism of that is still not fully clarified, but transcriptional inhibition, epigenetic mechanisms, hystone deacetylation, mutations or regulation of miRNA stability could be involved (9).

An additional mechanism connected to abnormal miRNA expression is the presence of sequence variants in miRNA (10). A mutation or a SNP in a miRNA gene region might affect the transcription of primary miRNA, the processing of a precursor to mature miRNA or miRNA-target interactions (11). Whereas most of the identified SNPs in miRNA genes do not affect their expression and function (9, 12, 13), in a few cases it has been reported that there was a loss or reduction of miRNA expression as a consequence of sequence variants (14). Because small variations in the quantity of miRNAs may have an effect on hundreds of target mRNAs, one SNP in a miRNA sequence could represent an important functional alteration and may be a putative cancer biomarker (15).

A genetic variant in *miR-196a2* was associated to a major susceptibility to hepatocellular carcinoma (16). SNPs in *miR-146a, miR-196a2* and *miR-499* were described as low penetrance alleles in familial breast cancer in the German and Italian population (17). A functional genetic variant in *miR-196a2* was found to be associated with increased susceptibility to lung cancer (18) and decreased risk of glioma (19). However, in these association studies the influence of single SNPs on the expression of corresponding miRNA is not always clarified. A SNP in pre-*miR-146a* decreases mature miRNA expression and predisposes to papillary thyroid carcinoma (20).

Recently, the distribution of SNPs in four miRNA genes (*miR-146a, miR-149, miR-196a2,* and *miR-499*) was associated to differences in overall survival of NSCLC in the Chi-

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nese population (15). In our study, we decided to evaluate the distribution of the above-mentioned polymorphic variants in 101 Caucasian patients affected by NSCLC. The study aimed to evaluate possible relationships between miRNA genotypes and expression, but also to explore their association with increased risk of NSCLC.

## Materials and methods

### Tissues and blood samples

We collected tissues from 101 consecutive patients (age range from 37 to 82 years; mean 67.2 years) who underwent surgical resection for NSCLC. A sample of apparently non-affected tissue was also collected from each patient and used as paired control. Cancer tissues were taken at 3–5 cm from cancer during lobectomy and 5–7 cm in pneumonectomy. In all patients, comparable tissues were processed for histological examination. The study protocol was approved by the local Ethical Committee. Informed consent was obtained from all patients.

The ex-smoking group were patients that had stopped smoking for at least about 12 years.

NSCLC samples and corresponding non-affected tissues were snap frozen and stored in liquid nitrogen until analysis. Tissues were disrupted by TissueLyser with Stainless Steel Beads 5 mm (Qiagen Gmbh, Hilden, Germany). DNA extraction was performed by BIOROBOT EZ1 (Qiagen) and DNA Tissue kit (Qiagen). For miRNA extraction we used mirVana™ miRNA Isolation kit (Ambion, Applied Biosystems, Monza, Italy) according to the manufacturer's protocol. The concentration of DNA and total RNA was determined with Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Tumor histological type and grade of differentiation were assessed according to the World Health Organization criteria (21). TNM staging was evaluated according to UICC system (22).

To compare allele distribution, we collected a blood sample from 129 subjects without any evidence of diseases, matched for sex and age with NSCLC patients. DNA was extracted from nucleated cells with QIAamp DNA Blood Mini kit (Qiagen). All volunteers provided an informed consent.

### Real-time RT-PCR measurement of miRNAs

To measure miRNA expression levels, 10 ng total RNA were reverse transcribed with TaqMan Reverse Transcription Kit and assayed by the respective TaqMan microRNA kit (Applied Biosystems, Forster City, CA, USA) according to manufacturer's instructions. Using this procedure, expression of *miR-146a*, *miR-149* and *miR-196a2* was easily detectable in real time amplification for all samples, with their Ct values constantly lower than 35. Conversely, *miR-499* resulted at very low expression levels. In this latter case we decided to consider as real amplification kinetics all duplicates showing a clear fluorescent signal over a threshold value within 38 cycles. All samples that did not generate significant improvement of fluorescence within this range were considered as negative. According to this procedure *miR-499* was detectable in 70/101 NSCLCs but only in 10 paired non-affected tissues.

The *miR-U6B* was used as endogenous control, as already reported (15). Relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ formula (23).

### Genotype identification with high-resolution melting analysis (HRMA)

For HRMA, 100 ng DNA were amplified in a 25 μL final volume, containing 10× Buffer, 1.5 mM $MgCl_2$, 800 μM dNTPs, 300 nM of each primer, 1.5 μM of SYTO9 Dye (Invitrogen, Carlsbad, CA, USA) and 1U of Taq Gold Polymerase (Applied Biosystems). PCR was performed with an initial step at 95°C for 10 min, followed by 35 cycles for 30 s at 95°C, 30 s at the annealing temperature (see Table 1) and final extension at 72°C for 20 min.

For the HRMA, performed on a RotorGene 6000 (Corbett Research, Sydney, Australia), samples were initially maintained for 5 min at 95°C, for 1 min at 40°C and then submitted to specific melting profiles (see Table 1). Primers for HRMA analysis were selected using Primer 3 software (24). To confirm HRMA results, sequencing analysis was performed in 10% randomly selected samples. In these cases, after HRMA samples were purified with a PCR Purification Kit (Qiagen) and submitted to cycle sequencing with 2 μL of BigDye Terminator Ready Reaction Mix (Applied Biosystems), 2 μL of sequencing buffer and the same primers used in HRMA but 0.8 μM in a final volume of 10 μL. After purification with a DyeEx 2.0 Spin Kit (Qiagen), samples were analyzed with the ABI Prism 310 Genetic Analyzer (Applied Biosystems). All sequenced samples perfectly matched HRMA genotyping.

### Statistical analysis

Statistical analysis was carried out using the SPSS software package. Hardy-Weinberg equilibrium was tested by the $\chi^2$-test for goodness-of-fit using a web-based program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The associations between miRNA polymorphisms and NSCLC risk were estimated by odds ratios (OR) and their 95% confident intervals (95% CI).

**Table 1** Primers and experimental procedures for miRNA genotyping.

| SNP | Primers forward | Primers reverse | Annealing temperature PCR, °C | Range of temperature HRM, °C |
|---|---|---|---|---|
| *miR-146a* (rs2910164 C-G) | GGGTCTTTGCACCATCTCTG | TCCAGTCTTCCAAGCTCTTCA | 57 | 77–87 |
| *miR-149* (rs2292832 C-T) | GTGTCTTCACTCCCGTGCTT | ACCTCTCACACCCCCTCAC | 58 | 88–98 |
| *miR-196a2* (rs11614913 C-T) | TCCTCCAGATAGATGCAAAGC | GAGGGTAGGAGTGGGAGAGG | 60 | 80–90 |
| *miR-499* (rs3746444 G-A) | CGGCTGTTAAGACTTGCAGTG | CACCCCTTCCCCACAAAC | 59 | 80–90 |

## Results

### Genotype distribution in NSCLC and paired non-affected tissues

In a recent study, it was demonstrated that in about 6% of papillary thyroid carcinomas, variants of *miR-146a* were the result of somatic mutations (20). Therefore, our initial goal was to test whether NSCLC transformation may induce selective modifications of allele variants in the four miRNAs investigated in this study. To this purpose, DNA was extracted from all paired NSCLC and unaffected tissues and analyzed for their genotype distribution. We found that in all samples miRNA genotype in normal tissues exactly matched that found in cancer biopsies (data not shown). This finding seems to exclude that, at least in NSCLC, cell transformation can induce sequence modifications in these four miRNAs.

### Expression of miRNAs in NSCLC and paired non-affected tissues

As previously reported, we had the opportunity to determine simultaneously the expression of the four miRNAs in paired NSCLC and non-affected tissues. We found that *miR-146a, miR-149* and *miR-196a2* were constantly expressed in all paired samples. Conversely, *miR-499* was expressed at the very low levels and was detectable in 70/101 NSCLCs but only in 10 paired non-affected tissues. For the best graphic viewing, the value of *miR-499* was multiplied by 1000.

Relative expression of *miR-146a* (mean ± SE) was comparable in NSCLC (1769 ± 482) and in paired control tissues (1548 ± 274). Equally, for *miR-149* it was impossible to demonstrate any difference between NSCLC (47 ± 22) and non-affected tissues (22.3 ± 5.4). Due to the limited numbers of non cancer samples in which *miR-499* was detectable, the comparison between paired NSCLC (2.3 ± 1.0) and normal tissues (23.9 ± 11.9) had a limited statistical value. On the contrary, we clearly detected a significant increase ($p < 0.001$) of *miR-196a2* expression in NSCLC (33.7 ± 8.9) in comparison to paired non-affected tissues (1.9 ± 0.5) (see Table 2).

### Expression of miRNAs and miRNA genotype in control tissues

We explored the possible relationship between miRNA genotypes and their expression. The initial quantification analysis was performed on control lung samples, obtained by surgical biopsies in non cancer areas (see Materials and methods). We postulated that, even these samples cannot be considered as real normal tissues, they represented the specimens which better identify the steady state of miRNA expression in the lung. Only in the case of *miR-146a* we found a significant difference of expression among genotypes, this being the expression in the GG group, which was significantly higher ($p < 0.05$). For *miR-149* we did not find any significant correlation between genotype and expression, whereas *miR-196a2* tended to have maximal expression in CC patients if compared to TT patients, without reaching the significance. In the case of *miR-499*, since the analysis was possible in only 10 patients in which its expression was detectable, no definitive indication was deducible (see Figures 1 and 2).

**Table 2** Expression of miRNAs in paired NSCLC and non-affected tissues. For a better graphic viewing, the values of miR-499 were multiplied by 1000.

| | *miR-146a* | *miR-149* | *miR-196a2* | *miR-499* × 1000 |
|---|---|---|---|---|
| NSCLC | 1769 ± 482 | 47 ± 22 | 33.7 ± 8.9 | 2.3 ± 1.0 |
| Controls | 1548 ± 274 | 22.3 ± 5.4 | 1.96 ± 0.5 | 23.9 ± 11.9 |
| p-Value | 0.672 | 0.225 | < 0.001 | 0.123 |

### Expression of miRNAs and miRNA genotype in NSCLC samples

Subsequently, the expression of each miRNA was stratified according to respective genotype in the group of 101 NSCLC and compared to results obtained in paired control tissues. Analysis of results seems to indicate that in cancer tissues only *miR-196a2* maintained an expression profile comparable to normal lung tissues. In detail, we confirmed a significant association between CC genotype and high expression, whereas TT genotype showed a very low expression in comparison to both CT ($p = 0.005$) and CC patients ($p < 0.01$) (Figure 2). A statistical difference was demonstrable also between CC and CT patients ($p = 0.027$). For the other three miRNAs we detected evident discrepancies of their expression profiles between normal and pathological tissues. In the case of *miR-146a*, the CG genotype appeared clearly associated to a relevantly high expression of respective miRNA. For *miR-149* maximal expression was associated to CC genotype. Once again we considered of limited value, the comparison of results of *miR-499* due to the limited number of comparable normal samples (see Figures 1 and 2).

### Expression of miRNAs in NSCLC and clinical features

As reported in Table 3 we did not find any significant relationship between the expression of single miRNAs and pathological features of NSCLC patients. The only exception was represented by the association between the increased expression of *miR-499* and high tumor grade.

### Allelic frequencies and genotype distributions in NSCLC patients and controls

Frequency of the four SNPs was tested in 101 NSCLC patients and compared with 129 control subjects, matched for sex and age. Genotype distribution of *miR-149*, *miR-196a2* and *miR-499* did not reveal any difference between cases and controls (see Table 4). Conversely, the analysis of *miR-146a* revealed a significant difference in genotype distribution between cases and controls. In particular, CG frequency was significantly higher in NSCLC patients (47.9%) in comparison to controls (34.9%) (CG vs. GG: OR = 1.77, 95% CI 1.02–3.09, $p = 0.042$). The genotype distributions of the four miRNAs

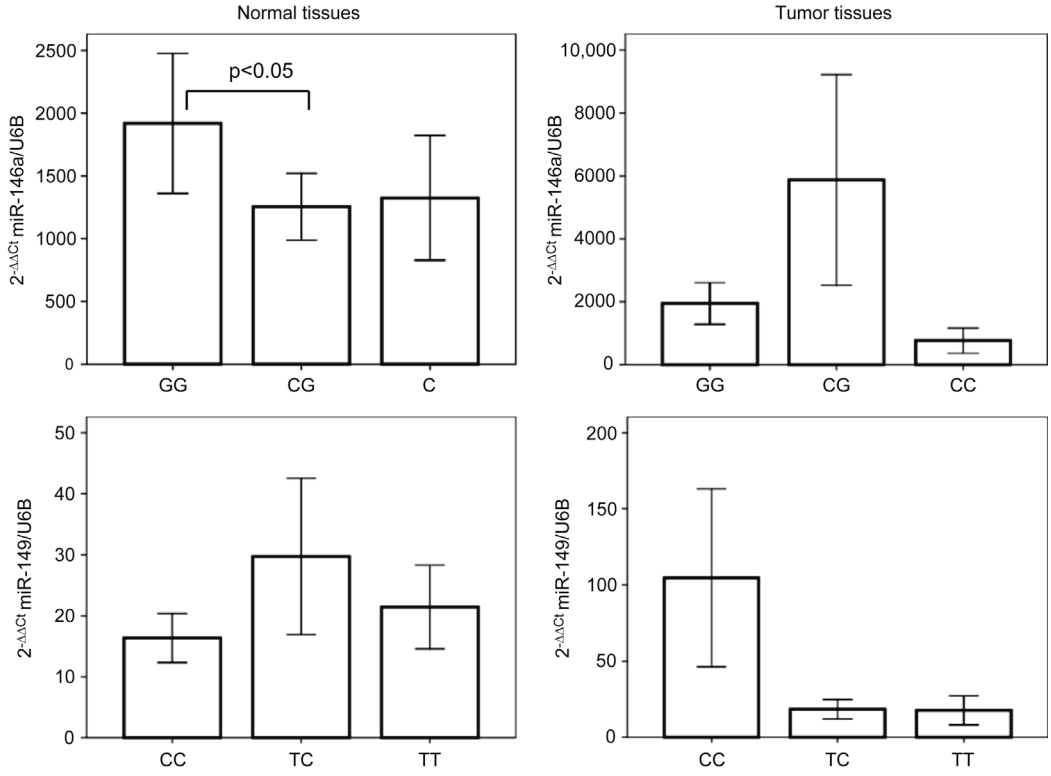


**Figure 1** Comparison of expression of *miR-146a* and *miR-149* in benign lung and NSCLC tissues stratified according to respective genotype.

in NSCLC and healthy controls were all consistent with Hardy-Weinberg equilibrium (data not shown).

### Prognostic significance of genotypes and miRNA expression

We analyzed the prognostic significance of genotype distribution for each miRNA genotype. According to the Kaplan-Meier curves, we did not find any statistical association between *miR-146a, miR-149, miR-196, miR-499*, and patient overall survival. We repeated Kaplan-Meier analyses after patient stratification according to arbitrary cut-offs corresponding to the median level of expression of each miRNA in NSCLC. Survival analysis was obtained by comparing patients with high miRNA expression ($>$median value) vs. patients with low expression ($\leq$median value). According to this division, we did not find a significant relationship between the four miRNA expressions and unfavorable outcome (data not shown).

## Discussion

Previous studies on miRNA profiling have shown that miRNAs are up- or down-regulated in human cancers (2). Given that most of the known miRNAs have a large number of potential mRNA targets, minor variations in miRNA expression can influence the expression of a large number of proteins. The mechanism of miRNA deregulation in cancers is very complex and only partially clarified and the influence of genetic and epigenetic mechanisms has been proposed (25–29).

More recently it was demonstrated that modifications of miRNA expression in cancers could be due to a single nucleotide replacement resulting from a mutation or an SNP. An 'in silico' study revealed the presence of 323 known SNPs located within 227 human pre-miRNA sequences (30). In another investigation on 474 human miRNA genes, sequence variants were demonstrated in about 10% of human miRNA precursors, while $<1\%$ of miRNAs had SNPs in their functional seed region (31). Recently, a study on four genetic variants of miRNA sequences in NSCLC indicated that a SNP in the *miR-196a2* pre-miRNA *(rs11614913)* was associated to patient survival in stage I and II NSCLC (15). The same variant was associated to susceptibility of lung cancer (18), breast cancer (32), HBV-related hepatocarcinoma (16), as well as to congenital heart disease (33). These possible relationships were postulated to be connected to the altered expression of mature *miR-196a2* and its differential binding capacity to the target *mRNAs*. All these studies were performed on the Chinese population, favoring the hypothesis that additional studies on the Caucasian population should be performed to verify these findings.

In the present study we evaluated genotype distribution and expression of *miR-146a (rs2910164 C-G), miR-196a2 (rs11614913 C-T), miR-149 (rs2292832 C-T)* and *miR-499*

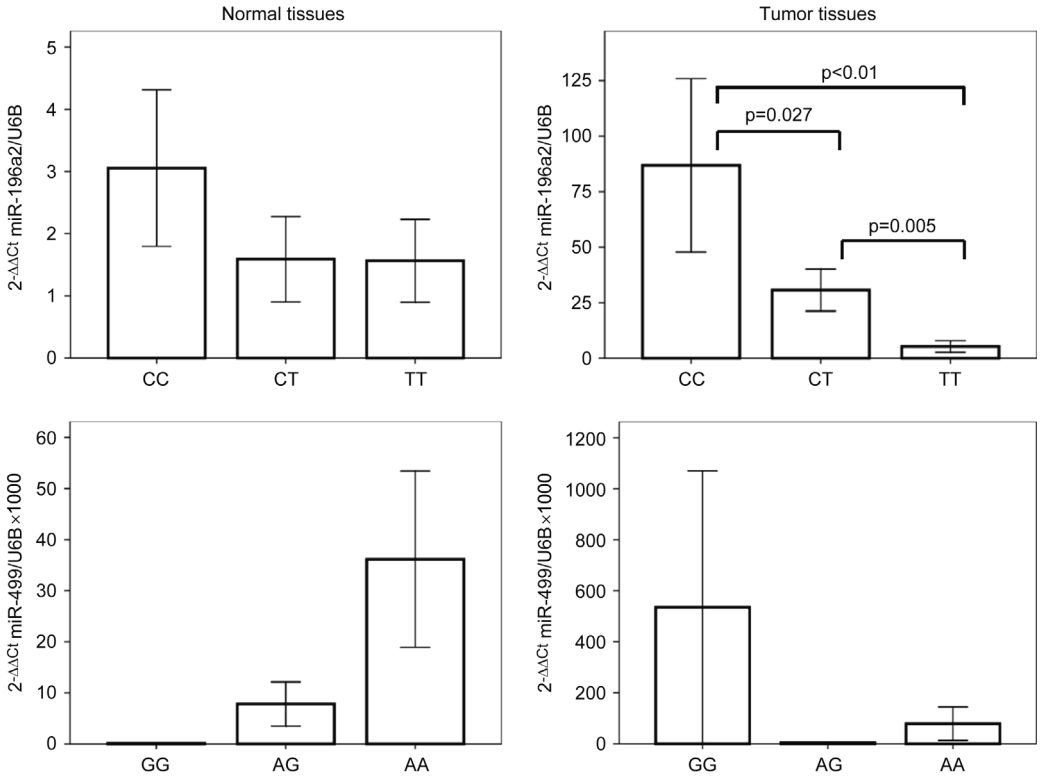


**Figure 2** Comparison of expression of *miR-196a2* and *miR-499* in benign lung and NSCLC tissues stratified according to respective genotype.
For better graphic viewing, the value of *miR-499* was multiplied by 1000.

*(rs3746444 G-A)* in 101 patients affected by NSCLC. Our study was mainly focused on the evaluation of their expression profile in paired normal and cancer tissues, to better evaluate the separate influence of genotypes on miRNA expression.

Our results tend to exclude that SNPs in these miRNAs may undergo somatic mutations during cell transformation, in contrast to what was recently demonstrated in papillary thyroid carcinomas (20).

In normal lung tissues the expression of *miR-146a* was apparently connected to its genotype, being significantly higher in GG patients ($p < 0.05$) in comparison to GC and CC. On the contrary, the expression profile of the same miRNA in paired NSCLC showed a modified trend with maximal expression in CG genotype (about a six-fold increase of the mean), the same connected to a major risk of NSCLC. Apparently, therefore this increased tendency to develop NSCLC is strictly connected to an increment of related miRNA expression, but limited to the specific CG genotype.

Concerning the prognostic significance of *miR-146a*, we did not detect any correlation with patient survival either for the miRNA expression or specific genotype.

Also in the case of *miR-149 (rs2292832 C-T)* paired normal and cancer tissues tended to have a different trend of expression according to their genotype. In normal tissues, the miRNA expression does not seem influenced by respective genotype. In corresponding NSCLC samples CC genotype seems associated to an increased miRNA expression (about a six-fold increase of the mean). In this case the presence of higher levels of *miR-149* expression seems to exploit a protective effect, as demonstrated by the significant improvement of overall survival in the CC or CT groups in comparison to TT patients ($p < 0.05$).

For the two remaining miRNAs, the trend of expression was similar between normal and cancer tissues. In particular, for *miR-196a2* the maximal expression was associated to the CC genotype and the minimal to TT. Therefore, the significant upregulation of *miR-196a2* expression in NSCLC in comparison to paired control tissues ($p < 0.001$), as reported also by others cancers (34, 35), appeared independent from genotype in our NSCLC patients. According to our results, other mechanisms are responsible for the deregulation of *miR-196a2* detected in NSCLC as well as for other cancers like gene amplification, deregulation of a transcription factor, epigenetic mechanisms (i.e., DNA methylation) (36).

Finally, the clinical significance of *miR-499* in NSCLC appeared of limited interest. We did not find any relevant association between genotype and expression neither in normal nor in affected tissues, with the highest expression detectable in patients carrying AA genotype. No association was demonstrable with patient survival. However, we found an association between the increased expression and high tumor grade only for *miR-499*.

**Table 3** Expression of miRNAs and clinical features in 101 NSCLC patients.

| | *miR-146a* | *miR-499* | *miR-196a2* | *miR-149* |
|---|---|---|---|---|
| Gender | | | | |
| Males (n=87) | 2105±615.9 | 10.6±5.2 | 38.9±11.2 | 64.5±32.5 |
| Females (n=14) | 3053±1734 | 2.6±0.8 | 32.5±17.0 | 31.9±16.8 |
| | n.s. | n.s. | n.s. | n.s. |
| Age | | | | |
| <55 (n=12) | 2237±1240 | 44.9±37.6 | 93.9±46.0 | 42.7±23.2 |
| ≥55 (n=89) | 1810±359 | 4.7±1.2 | 33±11.0 | 64.5±33.9 |
| | n.s. | n.s. | n.s. | n.s. |
| Smoking status (≥12 years) | | | | |
| Current smokers (n=46) | 2295±1050 | 4.6±2.8 | 61.5±23.2 | 78.9±63.5 |
| Non smokers or former smokers (>12 years) (n=55) | 2714±874 | 15.4±8.8 | 30.2±10.2 | 51.6±29.1 |
| | n.s. | n.s. | n.s. | n.s. |
| Grade | | | | |
| Low (n=21) | 2150±978 | 3.6±1.4 | 42.2±25.4 | 97.8±72.8 |
| Medium (n=70) | 1726±643 | 4.7±1.4 | 30.3±13.3 | 62.4±40.2 |
| High (n=10) | 5949±4537 | 57.6±52.6 | 48.4±18.7 | 41.2±38.2 |
| | n.s. | p=0.008 | n.s. | n.s. |
| Size of tumor | | | | |
| T1 (n=22) | 3306±1932 | 5.2±3.3 | 11.9±5.5 | 22.8±10.5 |
| T2 (n=68) | 1442±398 | 4.3±0.9 | 43.0±15.1 | 85.0±44.6 |
| T3 (n=11) | 3518±2801 | 1.3±0.6 | 20.5±11.6 | 22.9±16.1 |
| | n.s. | n.s. | n.s. | n.s. |
| Lymph node involvement | | | | |
| N– (n=54) | 2492±896 | 3.3±0.7 | 44.9±18.0 | 71.6±50.5 |
| N+ (n=47) | 1458±537 | 5.8±2.2 | 21.5±8.9 | 61.1±34.5 |
| | n.s. | n.s. | n.s. | n.s. |
| Tumor differentiation | | | | |
| T1 (n=47) | 2168±880 | 3.5±0.8 | 48.5±20.6 | 81.0±57.9 |
| T2 (n=20) | 2248±1.35 | 3.0±1.1 | 40.4±18.8 | 82.59±72.7 |
| T3-4 (n=34) | 1725±769 | 7.2±3.5 | 10.1±3.8 | 35.8±12.0 |
| | n.s. | n.s. | n.s. | n.s. |
| Histology | | | | |
| Adenocarcinomas (n=40) | 1871±650 | 21.0±13.1 | 43.2±23.5 | 96.5±78.7 |
| Squamous (n=47) | 3220±1290 | 3.1±1.1 | 43.3±14.6 | 63.2±36.5 |
| Adenosquamous (n=14) | 736±366 | 1.5±0.9 | 4.7±2.4 | 7.3±3.1 |
| | n.s. | n.s. | n.s. | n.s. |

n.s., Non significant.

**Table 4** Allelic and genotype frequencies of miRNAs in NSCLC patients (n=101) and control subjects (n=129).

| SNP | Genotypes | NSCLC, % | Controls, % | OR | 95% CI | p-Value |
|---|---|---|---|---|---|---|
| *miR-146a* | GG | 44 (43.6) | 73 (56.6) | 1.00 | | |
| (rs2910164 C-G) | CG | 48 (47.5) | 45 (34.9) | 1.77 | 1.02–3.09 | 0.042 |
| | CC | 9 (8.9) | 11 (8.5) | 1.23 | 0.46–3.31 | 0.674 |
| | [G] vs. [C] | | | | | 0.149 |
| *miR-149* | CC | 44 (43.6) | 65 (50.4) | 1.00 | | |
| (rs2292832 C-T) | TC | 41 (40.6) | 53 (41.1) | 0.77 | 0.43–1.36 | 0.359 |
| | TT | 16 (15.8) | 11 (8.5) | 1.20 | 0.50–2.92 | 0.679 |
| | [C] vs. [T] | | | | | 0.103 |
| *miR-196a2* | CC | 35 (34.6) | 58 (45.0) | 1.00 | | |
| (rs11614913 C-T) | CT | 54 (53.5) | 61 (47.2) | 1.48 | 0.85–2.60 | 0.108 |
| | TT | 12 (11.9) | 10 (7.8) | 1.88 | 0.72–4.88 | 0.192 |
| | [C] vs. [T] | | | | | 0.126 |
| *miR-499* | AA | 53 (52.5) | 70 (54.2) | 1.00 | | |
| (rs3746444 G-A) | AG | 41 (40.6) | 48 (37.2) | 1.15 | 0.66–2.01 | 0.613 |
| | GG | 7 (6.9) | 11 (8.6) | 0.72 | 0.25–2.08 | 0.548 |
| | [A] vs. [G] | | | | | 0.914 |

In the case-control analysis of genotype distribution of *miR-149, miR-196a2* and *miR-499* we did not find any association with increased risk of NSCLC incidence. Conversely, the analysis of *miR-146a* revealed a significant difference in genotype distribution, being the CG frequency significantly associated to NSCLC ($p = 0.042$ and 1.77 OR). Unfortunately, due to the limited number of patients, this population study cannot provide definitive results. These data should be confirmed in a wider population of NSCLC patients.

In conclusion, our results seem to demonstrate that, the CG genotype of *miR-146* could be connected to an increased risk for NSCLC, presumably through an association with increased miRNA expression; however, we found no correlation with survival for any of the four miRNA investigated. Finally, we confirmed that miRNA-*196a2* is upregulated in NSCLC patients but, at least in our patients, in a manner independent from the genotype.

## Conflict of interest statement

**Authors' conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding the publication of this article.
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