Effect of miR-34b Overexpression on the Radiosensitivity of Non-small Cell Lung Cancer Cell Lines

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Abstract. *Background: The miR-34 family, under-expressed in-non small cell lung cancers (NSCLCs), are effectors of p53 activation upon irradiation of cells. We evaluated whether the miR-34b overexpression modulates the NSCLCs response to radiation. Materials and Methods: NSCLC cell lines A549 with V-KI-RAS2 Kirsten Rat Sarcoma viral oncogene (KRAS) codon 12 mutation and with wild type p53, and H1299, not expressing p53, were irradiated after transfection with pre-miR-34b. Cell survival was assessed by clonogenic survival assays. The apoptosis and the cell cycle were evaluated by flow cytometry. Results: In the A549 cell line, overexpression of miR-34b significantly reduced cell survival at lower than 4 Gy radiation doses. There was a significant reduction in B-cell CLL/lymphoma 2 (BCL2) expression but no significant differences were observed in the apoptotic cell population or the cycle profile. No significant effect was recorded in the H1299 irradiated cells. Conclusion: In the p53 wild type, KRAS mutated NSCLC cells, the overexpression of miR-34b increases radiosensitivity at low doses of radiation.*

Lung cancer is considered the primary leading cause of death from malignant neoplasms in males and the second cause of death in females. Radiotherapy, usually in combination with chemotherapy, is routinely used in lung cancer treatment, especially for non-small cell lung cancer (NSCLC), allowing for better local control of the disease and reduction of metastasis occurrence. As radiation resistance is common, contributing to the dismal prognosis, intense investigation is being carried out in the quest for radiosensitizers.

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The genetic effects of ionizing radiation primarily arise by ejected electrons (ionization events) that damage DNA directly or indirectly by the generation of reactive oxygen species (ROS), causing DNA double-strand breaks (DSB) or single-strand breaks (SSB) (1). In response to ionizing radiation-induced injury, a complex DNA damage response is activated, which determines cell fate: block of cell cycle at G_1 and G_2 checkpoints, damage repair, senescence or activation of death signals (2, 3). p53 suppressor protein is a key player in these responses.

A true guardian of the human genome, p53 is involved in DNA repair, apoptosis and cell cycle regulation, preventing the proliferation of mutated cells. The *p53* gene is deleted or mutated in about 50% of NSCLC and in 90% of small cell lung cancer (SCLC) cases (4, 5). The loss of p53 function abrogates activation of the G_1 cell cycle checkpoint and the induction of apoptosis which is reflected by the accumulation of mutations by increased aggressiveness of cancer and by the frequent resistance to cytotoxic therapies (6, 7). The roles of p53 as a tumor suppressor are predominantly mediated through transcription activation or repression of target genes, leading to increased expression of cyclin-dependent kinase inhibitor $P21^{WAF1}$ and pro-apoptotic proteins, such as PMA-induced protein 1 (NOXA), p53-upregulated modulator of apoptosis (PUMA) and BCL2-associated X protein (BAX) or to reduced levels of anti-apoptotic proteins such as B-cell CLL/lymphoma 2 (BCL2) (6, 8-9). Recently, microRNAs (miRNAs) have been also added to the list of p53 targets.

miRNAs are an abundant class of small noncoding RNAs with a well known role in the regulation of gene expression, through blocking the translation of targeted mRNAs (10). There is multiple evidence linking abnormal expression of

miRNAs to the development and progression of cancer in humans, where they act as promoting (oncomirs) or inhibiting (suppressors) factors (11). In lung cancer, typically there are reduced expression levels of LET7 and the miR-34 family, whereas miR-21 and miR-155 are overexpressed (12-14), while miRNAs expression profiles have been suggested as useful diagnostic and prognostic tools (15, 16).

The miR-34 family includes three members with high homology, originating from two loci: one located at 1p36, encodes the miR-34a, the other, located at 11q23, gives rise to miR-34b and miR-34c from a single primary transcript (17). Recently, several studies have implicated the miR-34 family as being important effectors of p53-mediated cellular response to stress (12, 17-22). Based on experimental data and computer analysis, several targets have been proposed for miR-34 family members, such as transcripts from cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase 6 (CDK6), cyclins E2 and D1, mesenchymal epithelial transition factor (MET) or BCL-2, among others (17-19), in accordance with the roles of p53 in cell cycle regulation and apoptosis. As for other tumor suppressors miRNAs (23-25), overexpression of miR-34a has been tried in an attempt to overcome resistance to cytotoxic therapy, with interesting results (19, 26).

Thus far, there are limited studies of miR34 family members other than miR34a. In this work, we sought to determine the effect of overexpression of pre-miR-34b in the radiosensitivity of p53-negative and p53-wild type ($p53^{wt}$) NSCLC cell lines.

Materials and Methods

Cell culture. A549 cell line was purchased from the American type Culture Collection (ATCC, Manassas, VA). This cell line originated from an NSCLC, expresses $p53^{WT}$ protein and has an activating mutation in codon 12 of the *KRAS* gene. H1299 cell line, also acquired from the ECACC, is originated from a lymph node metastasis of an NSCLC and has a homozygous deletion of the *p53* gene ($p53^{-/-}$). Both cell lines are radioresistant (27). They were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, in a humidified chamber at 37˚C under 5% CO_2.

Transfection. Cells were plated in antibiotic-free DMEM at a density of 2×10^5/5 ml in 25 cm^2 flasks. After 20 h, cells were trypsinized and transfected with miR-34b mimics Pre-miR™ miRNA precursor: hsa-miR-34b (corresponding to MIMAT0004676) or with an oligonucleotide transfection control, Pre-miR miRNA precursor molecules-negative control-1, using the siPORT *NeoFX* Transfection Agent (Ambion, Austin, TX, USA). A final concentration of 75 nM of miRNA and a 6 h incubation period were selected for both transfection molecules.

To optimize transfection conditions, cell viability was evaluated with the Alamar blue assay and transfection efficiency was assessed by real time quantitative reverse transcription PCR (qRT-PCR) for miR-34b as described below.

Quantification of miR-34 expression. Total RNA of irradiated and non-irradiated cells was extracted with an miRNeasy Mini Kit (Qiagen, Hilden, Germany) with a DNAse supplementary step. Expression of miR-34 a, b and c (MIMAT0000255, MIMAT0004676 and MIMA0000686) were evaluated using the TaqMan MicroRNA assays (Ambion, Austin, TX, USA; ID: 000426, APAB 4395168 for miR-34a; ID: 002102, APAB 4395213 for miR-34b; ID: 000428, APAB 4373036 for miR-34c). The small nucleolar RNA, C/DR box 48 (RNU48) was used as an internal expression control in order to normalize the miRNAs expression levels (Ambion, Austin, TX, USA; ID:001006, APAB 4373383). All PCR reactions were carried out in quadruplicate. Basal levels of expression of miR-34 members were calculated in relation to the ones of RNU48 using the $2^{-\Delta Ct}$ method; variation of expression levels between treated and non-treated cells were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell irradiation. Cell culture flasks of 25 cm^2 with 1×10^6 cells were exposed to different γ (140 keV) radiation doses (4, 8 or 12 Gy) using ^{99m}Tc. Irradiation was performed for the non-transfected cells and 24 h after transfection, for the transfected cells. Before irradiation, cells were checked for growth and viability.

Clonogenic cell survival assay. The clonogenic assay was performed for non-transfected non-irradiated cells, 28 h after transfection for the non-irradiated cells and 4 h after irradiation of the non-transfected and the transfected cells. Briefly, cells were tryspinized, counted, and the appropriate numbers of cells was seeded in six-well plates and incubated for 12 days. At day 7, 2 ml of DMEM with 10% FBS were carefully added to each well. For each irradiation dose, a different number of cells was used: 125, 400, 600 and 600 cells for 0, 4, 8 and 12 Gy, respectively. After 12 days of incubation, media were removed; plates were then gently washed with PBS, fixed with methanol and stained with 0.5% of crystal violet. Colonies with >50 cells were counted. Plate efficiency (PE) was calculated by dividing the number of colonies formed by the number of cells plated. The survival factor (SF) was calculated by dividing the PE of irradiated samples by the PE of the respective non-irradiated samples. SF values were plotted as a function of radiation dose. Radiation-survival data were fitted to a linear model, where $SF=e^{\frac{D}{D_e}}$ for each cell line and condition, using the software OriginPro v8.0 (OriginLab Corporation, Northampton, Massachusetts, USA). The sensitizer enhancement ratio (SER) was defined as the ratio between the isoeffective dose at SF 0.5 in the negative control and that of the overexpression of miR-34b. The assays were carried out in triplicate for each condition in three independent experiments.

Cell death analysis. Cell death analysis (apoptosis/necrosis) was performed by flow cytometry using the Annexin V/propidium iodide (PI) assay. A volume containing 1×10^5 cells (both adherent and detached) was centrifuged at 200 ×*g* for 10 minutes and 100 μl of binding buffer was added to the pellet. Annexin V-fluorescein isothiocyanate (FITC) (5 μl) and PI (5 μl) were added and the suspension was incubated for 15 minutes in the dark at 25˚C. The cells were immediately analyzed by flow cytometry. The percentage of stained cells was quantified using the Paint-a-gate software (Becton Dickinson, San Jose, CA). Cell death analysis was performed in non-transfected non-irradiated cells and at 24 h after irradiation. For each condition, samples were analyzed in triplicate in three independent experiments.

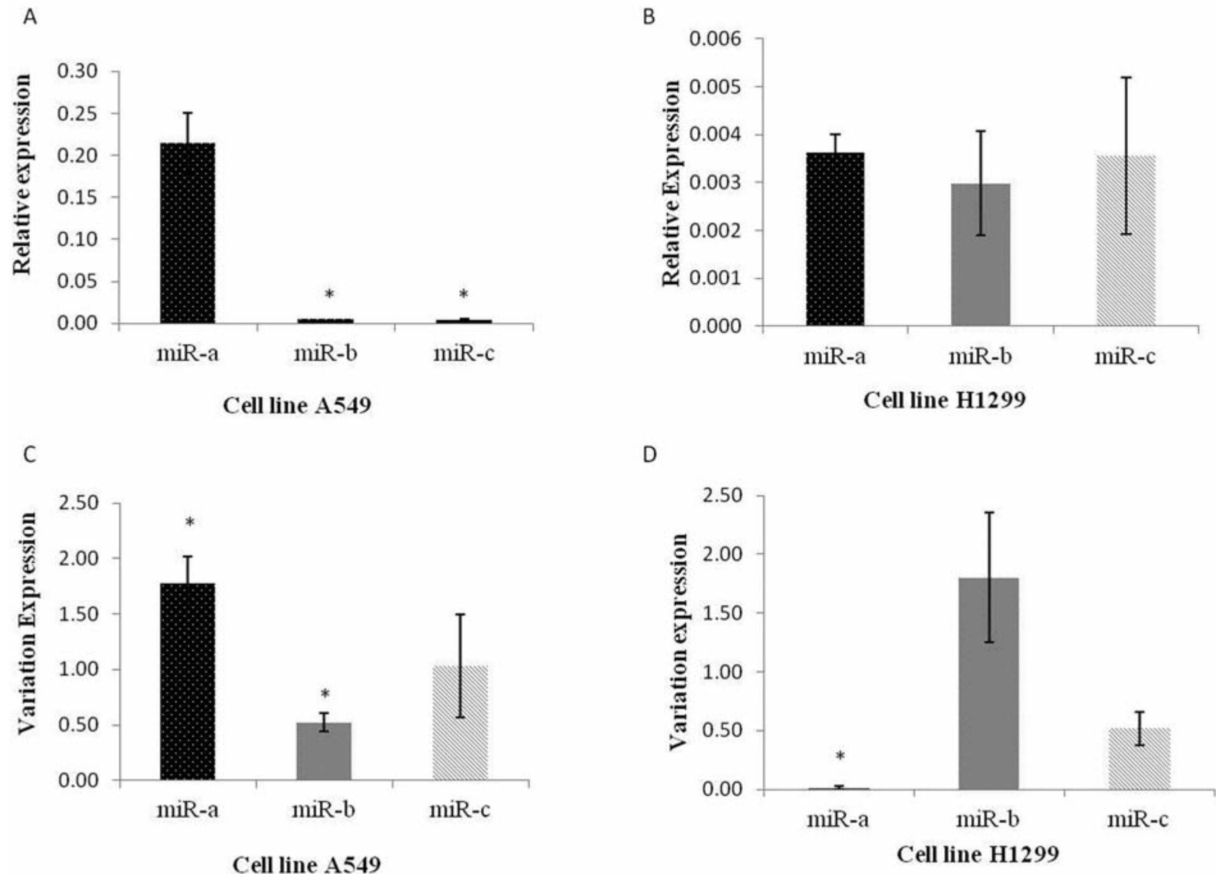

Figure 1. *Expression of miR-34 members in A549 and H1299 cell lines, before and after irradiation. A: basal expression of miR-34 a, b and c in A549 cell line in comparison to RNU48; B: basal expression of miR34 a, b and c in H1299 cell line in comparison to RNU48; C: expression of miR-34 members in the A549 cell line 24 h after irradiation with 12 Gy; D: expression of miR-34 members in the H1299 cell line 24 h after irradiation with 12 Gy.*

Expression of p53, BAX and BCL2 proteins. The percentage of cells expressing p53, BAX and BCL2 proteins was measured by flow cytometry using 1×10^5 cells and mouse monoclonal antibodies to human BCL-2 and p53 (FITC-conjugated) and human BAX (PE-conjugated) (BD Biosciences) and a fixation/ permeabilization kit (Invitrogen, Carlsbad, CA). Stained cells were analysed by FACSCalibur using the CellQuest software (Becton Dickinson, San Jose, CA). Evaluations were performed in non-transfected non-irradiated cells and at 24 h after irradiation. The assays were carried out in triplicate for each condition in three independent experiments. Results are expressed as mean fluorescence intensity (MFI).

Cell cycle analysis. Cell cycle analysis was performed by flow cytometry with PI staining. The assay was carried out 48 h after transfection and 24 h after irradiation. Adherent cells (1×10^5) were collected, fixed in 70% ethanol, for 30 min at 4˚C, washed in PBS with 2% FBS, and then stained with 0.5 ml of PI (50 μg/ml)/RNase A (0.1 μg/ml) solution. Each histogram was constructed with data from at least 1×10^4 events. Data were analyzed in order to calculate the percentage of the cell population in each cell cycle phase using CellQuest software.

Statistical analysis. Data are expressed as the mean±standard deviation (SD) and were analyzed by the *t*-test. $p<0.05$ was considered statistically significant. Analysis was performed using the software SPSS, version 16.0 (SPSS Inc, Chicago, IL).

Results

The three members of the miR-34 family showed highly reduced expression in both A549 and H1299 NSCLC cell lines, particularly of miR-34b and in the p53-negative H1299 cell line, when compared to the expression of gene control *RNU48* (Figures 1A and B). miRNA expression was also evaluated 24 h after cell irradiation with 12 Gy and levels were compared to those obtained in non-irradiated cells using the $2^{-\Delta\Delta Ct}$ method. In the A549 cell line, we observed a slight increase in the miR-34a expression (1.8±0.24; $p<0.05$), a decreased miR-34b expression (0.5±0.08; $p<0.05$) and no significant change in the miR-34c expression (1.03±0.46). However, in the H1299 cell line, irradiation induced a different

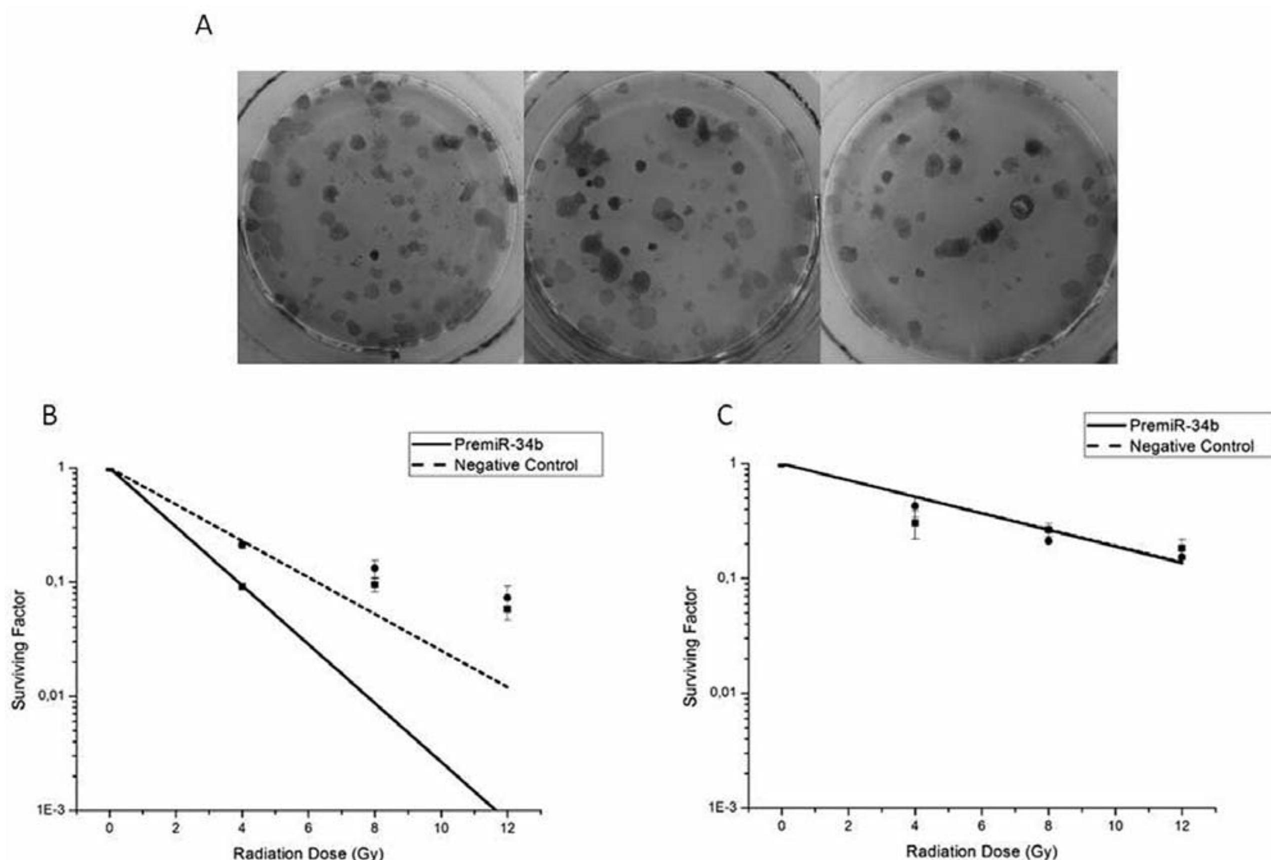

Figure 2. *Long-term cell survival after irradiation. A: Example of clonogenic assay in the A549 cell line after irradiation with 12 Gy: 1: colonies formed in non-transfected samples; 2: colonies formed from cells transfected with the negative control; 3: colonies formed from cells transfected with pre-miR-34b. B: Cell survival curves for A549 cell line. C: Cell survival curves for H1299 cell line. R2>0.99 for both cell lines.*

microRNAs expression profile: there was decreased expression of miR-34a (0.013±0.012; $p<0.05$) and miR-34c (0.52±0.14; $p<0.05$) and a non-statistically significant increase only in the expression of miR-34b (1.8±0.55) (Figures 1C and, D).

The clonogenic assay (Figure 2A) depicted the long-term irradiation effects on cell survival. In the A549 cell line, overexpression of miR-34b slightly, but significantly ($p<0.05$) increased the sensitivity of cells to a low radiation dose (4 Gy), when compared to cells transfected with the negative control. For other radiation doses, there were no significant differences between cells transfected with miR-34b precursor or negative control (Figure 2B). The SER at 4 Gy was 1.6 (as the radiation-survival data were fitted to a linear model, SER is constant). For the H1299 cell line, the transfection with miR-34b did not influence long-term cell survival after irradiation (Figure 2C).

Cell death analysis was performed in non-treated cells, 24 h after exposure to 4 and 12 Gy radiation and 48 h after transfection. For both cell lines, in non-irradiated cells, there was no statistically significant difference in cell death after miR-34b transfection (Figure 3A). After exposure to radiation, with 12 Gy, a slight but significant ($p=0.047$) increase in apoptotic cells, of 31% (from 4.33±0.58% to 5.67±0.57%), was observed for the H1299 cell line (Figure 3B).

In non-irradiated cells, overexpression of miR-34b induced a significant decrease in BCL2 expression when compared to the negative control transfection: from 73.8±14.5 MFI to 33.37±5 MFI in the A549 cell line ($p<0.029$), and from 37.3±6.43 MFI to 19.33±2.08 MFI in the H1299 cell line ($p<0.03$) (Figure 4A). After irradiation, the differences in BCL2 expression were only significant in the A549 cell line, and with 12 Gy, there was a decrease from 68.1±8.07 MFI to 47.13±9.68 MFI ($p<0.05$), as depicted in Figure 4B. In irradiated and non-irradiated cells, overexpression of miR-34b induced no changes in the expression levels of BAX, for both cell lines, nor in p53 for the A459 cell line (results not shown).

Cell cycle analysis by flow cytometry revealed no significant changes related to miR-34b transfection, to cell radiation or to the association of both (Figures 5A and B).

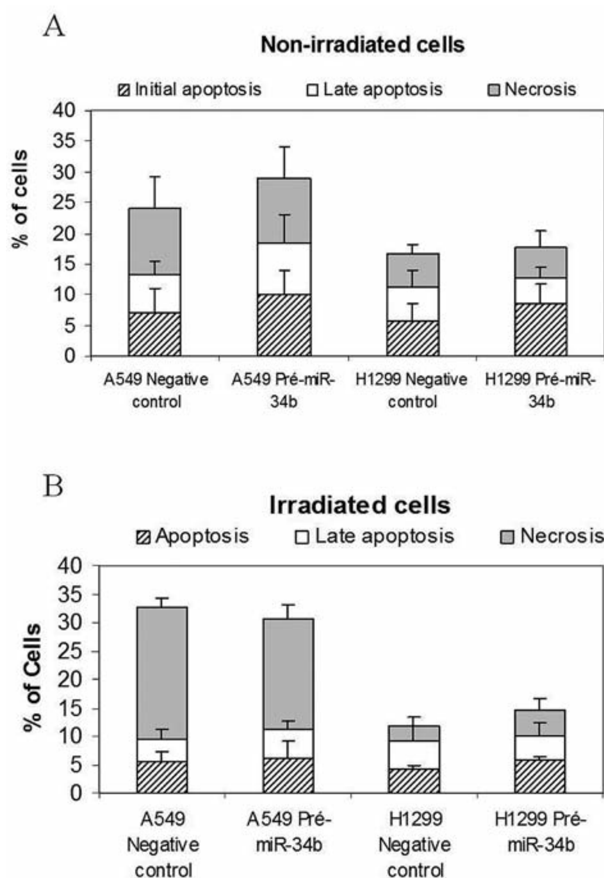

Figure 3. *Evaluation of cell death by flow cytometry. A: A549 and H1299 cell lines not exposed to radiation. Assays were performed 48 h after transfection with the negative control or with the miR-34b precursor; B: A549 and H1299 cell lines after irradiation with 12 Gy. For the H1299 cell line, there was a significant increase of 31% of apoptotic cells on radiation.*

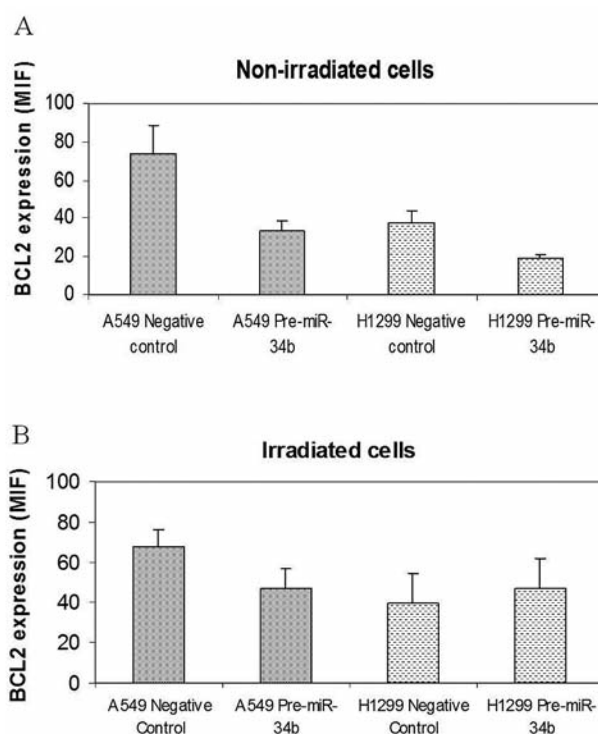

Figure 4. *B-cell lymphoma 2 (BCL2) expression by flow cytometry. A: A549 and H1299 cell lines not exposed to radiation. Assays were performed 48 h after transfection with the negative control or with the miR-34b precursor; B: A549 and H1299 cell lines after irradiation with 12 Gy. Cells were irradiated 24 h after transfection with the negative control or with the miR-34b precursor. Cytometry was performed 24 h after irradiation.*

Discussion

Inhibition of BCL2 and restoration of the p53 pathway represents a promising strategy in order to overcome chemo- and radioresistance of cancer cells. miR-34 family members are tumor suppressor microRNAs up-regulated by p53, which inhibit expression of multiple genes, including *BCL2*, and have been implicated in cell-cycle arrest and apoptosis in cells submitted to genotoxic stress (12, 17-22). The broad action of miR34s could provide a therapeutic advantage over re-expression of wild-type p53 in tumours with mutated p53, due to a dominant negative effect of the mutant.

miR-34 members, a, b and c, are highly homologous and though they certainly share most mRNAs targets, each one probably has specific features. In this work, we analyzed how miR-34b overexpression influenced the radiosensitivity of two radioresistant NSCLC cell lines, A549, expressing wild-type p53 and H1299, not expressing p53.

In accordance with previous studies of miRNA expression profiles in lung cancer (14, 28), in our experiments, we found that both cell lines had extremely reduced levels of expression of all three members of miR-34 family, especially the $p53^{-/-}$ cell line, H1299. We observed that in the $p53^{WT}$ A549 cell line, radiation induced a significant increase in the expression levels of miR-34a and a reduction in miR-34b, while in the H1299 cell line, levels of miR-34a decreased and those of miR34-b did not change significantly. miR-34c expression remained unchanged after irradiation. These results show that basal and radiation-induced expression of miR-34 family members is abnormally and differentially regulated in both cell lines.

Clonogenic cell survival assay evaluates the long-term effects of cytotoxic stress in cell survival, from cell death to cell senescence or mitosis arrest. It also seems to have the best correlation with the metastatic potential of treated cancer cells and is recognized as a standard method for radiation sensitivity assays. In the $p53^{WT}$ A549 cell line, overexpression of miR-34b potentiated the loss of cell survival at the lower radiation dose (4 Gy), with an SER of 1.6. On the other hand, in the H1299 cell line, transfection of miR-34b did not induce any specific radiosensitizing effect when compared to the negative

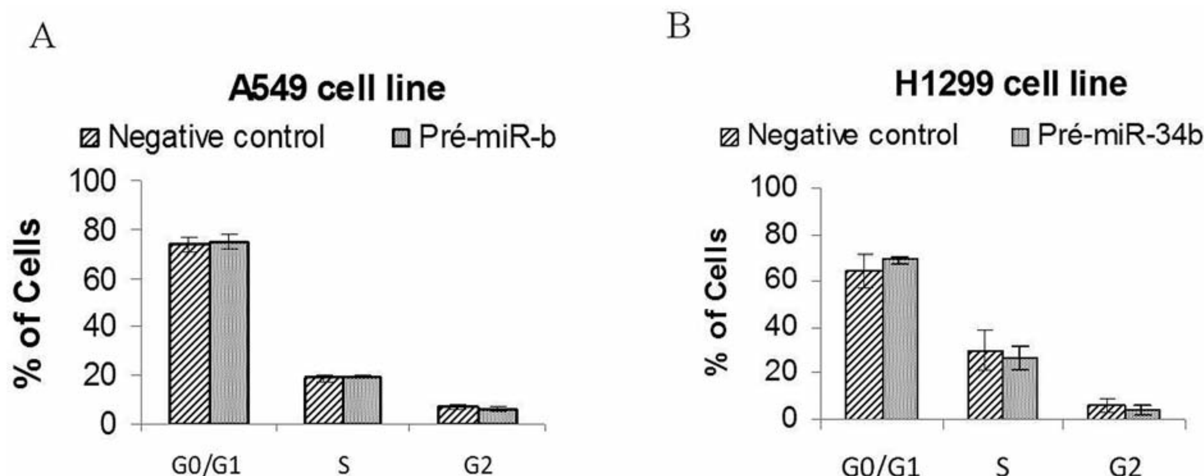

Figure 5. *Cell cycle analysis by flow cytometry. Results were obtained 24 h after irradiation with 12 Gy, and 48 h after transfection with the negative control or with the miR-34b precursor. A: A549 cell line; B: H1299 cell line.*

control. The different behavior of the two cell lines suggests that the effects of miR-34b in radiosensitization is p53-dependent and demonstrates that overexpression of miR-34b cannot completely overcome the p53 deficiency. Differences in cancer cell response to miR34a transfection were also reported, with the effects in cell survival being dependent on the cell background, namely on high basal levels of BCL2 (29) and on the presence of a functional p53 (19).

The expression of the anti-apoptotic protein BCL2 and the pro-apoptotic BAX have been reported as being negatively regulated by miR-34s in different types of cancer (29). In our experiments, in non-irradiated cells, miR-34b overexpression only reduced BCL2 levels. In irradiated cells, miR-34b overexpression was associated with reduced levels of BCL2 in the A549 cell line irradiated with 12 Gy, but not in the H1299 cells. Apart from the absence of p53 expression in the H1299 cells, the different behaviour may be explained by a more general and complex irradiation-induced cell-specifc transcriptome profile, including microRNAs, as previously described (30-31).

Overexpression of miR-34b induced only a slight but significant increase in the apoptosis levels in irradiated cells for the H1299 cell line, and no changes in the cell cycle profile, neither in irradiated nor in non-irradiated cells, were observed in either cell line. The negative effect on cell survival of A549 cell line, without apoptosis enhancement, may be explained by other cell death mechanisms, such as autophagy, as both mechanisms are associated with BCL2 expression (32). In fact, interference with pathways involved in autophagy has recently been suggested to modulate response to radiotherapy (33). A reduced but significant radiosensitizing effect, demonstrated by the clonogenic assay, also without inducing apoptosis, has been described for the A549 cell line when transfected with *let-7*, a miRNA targeting the mRNA of the V-KI-RAS2 Kirsten Rat Sarcoma viral oncogene (*KRAS*) (25). Apoptosis promotion and cell-cycle arrest in G_1 after miR-34a overexpression has mainly been described in cancer cells exposed to chemotherapy agents (19-20).

The radiosensitizing effect of miR-34b overexpression in the A549 cell line at 4 Gy was slight, but nevertheless it may be of benefit as this cell line represents a subset of lung carcinomas with *KRAS* mutations that are also resistant to the new therapies targeting EGFR (34).

In conclusion, in the $p53^{WT}$ A549 cell line, the overexpression of miR-34b increased radioresistance at low levels of radiation, an effect not observed in the p53-negative H1299 cell line. For both cell lines, miR-34b restoration was unable to abrogate the p53 tumor-suppressing effect, in regard to apoptosis or cell cycle regulation.

Conflict of Interest

There are no conflicts of interest to declare.

Acknowledgements

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