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A comparison between the effects of over-expression of miRNA-16 and miRNA-34a on cell cycle progression of mesothelioma cell lines and on their cisplatin sensitivity

S. Pinelli [a], R. Alinovi [a], M. Corradi [a,b], D. Poli [c], D. Cavallo [c], G. Pelosi [d], L. Ampollini [a,b], M. Goldoni [a], P Mozzoni [a,*]

[a] Department of Medicine and Surgery, University of Parma, via A. Gramsci 14, 43126 Parma, Italy
[b] University Hospital of Parma, Parma, Italy
[c] INAIL Research, Department of Occupational and Environmental Medicine, Epidemiology and Hygiene Via Fontana Candida1, 00078 Monte Porzio Catone, Rome, Italy
[d] Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 17/A, 43124 Parma, Italy

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ABSTRACT

The prognosis of patients affected by malignant pleural mesothelioma (MPM) is presently poor and no therapeutic strategies have improved their survival yet. Introduction of miRNA mimics to restore their reduced or absent functionality in cancer cells is considered an important opportunity and a combination of miR's might be even more effective. In the present study, miR-16 and miR-34a were transfected, singularly and in combination, in MPM cell lines H2052 and H28, and their effects on cell proliferation and sensitivity to cisplatin are reported. Interestingly, the overexpression of both miRs, alone or combined, slows down the cell cycle progression, modulates the p53 and HMGB1 expression and increases the sensitivity of cells to cisplatin, producing a marked impairment of cell proliferation and strengthening the apoptotic effect of the drug. However, the co-overexpression of the two miRs results more effective only in the regulation of the cell cycle, but does not enhance the sensitivity of MPM cells to cisplatin. Consequently, although the potential of miR-16 and miR-34a is confirmed, we must conclude that their combination does not improve the response of MPM to chemotherapy.

1. Introduction

Malignant mesothelioma is a rare, aggressive tumour originating from the pleura or serous layer of the peritoneum. Inhalation of asbestos fibers is the most important risk factor in the development of mesothelioma, and the incidence of mesothelioma due to asbestos exposure has shown an increase all over the world [1-3]. Treatment of malignant pleural mesothelioma (MPM) results in frustrating results: the average life expectancy is usually less than one year with a five-year survival of less than 5% [4,5]. This is due to various reasons amongst which the silent clinical progression, the late diagnosis and the resistance to current therapeutic treatments. Whilst some patients presenting with early disease will undergo aggressive surgery and multimodality therapy, for most patients with advanced disease palliative systemic therapy will be their mainstay of treatment. Despite amazing efforts devoted to understanding and treating MPM better, clinical practice has not changed over the past decades, the standard therapeutic strategies for MPM are (a) surgery for resectable tumours, often combined with radiotherapy and/or chemotherapy (trimodality treatment) or (b) chemotherapy or radiotherapy in unresectable tumour cases [6]. To date the only FDA- and EMA-approved and registered systemic treatment is platinum-based chemotherapy combined with premetrexed, with or without bevacizumab [7-9]. Several studies highlight the possibility of using immunotherapy for mesothelioma both at the first line then in association with chemotherapy [10]. Systemic therapy for mesothelioma has not yet benefited from the paradigm shift of personalized medicine and, therefore, early treatment approaches are needed. In recent years many efforts and attempts have been carried out and proposed to improve treatment possibilities based on immunotherapy [11], anti-angiogenic strategies [12], personalized vaccination [13], immune

* Corresponding author.
E-mail addresses: silvana.pinelli@unipr.it (S. Pinelli), rossella.alinovi@unipr.it (R. Alinovi), massimo.corradi@unipr.it (M. Corradi), d.poli@inail.it (D. Poli), d.cavallo@inail.it (D. Cavallo), giorgio.pelosi@unipr.it (G. Pelosi), luca.ampollini@unipr.it (L. Ampollini), matteo.goldoni@unipr.it (M. Goldoni), paola.mozzoni@unipr.it (P. Mozzoni).

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checkpoint inhibitors [14], oncoviral therapies [6] or microRNA manipulation [15]. Anti-angiogenic therapies or immune checkpoint inhibitors that showed impressive clinical responses in other solid malignancies have little impact on survival in MPM as single agents [6]. The therapeutic use of microRNAs (miRNAs) is much more promising [15-17]. miRNAs are an important class of non-coding RNA involved in post-transcriptional regulation and modulation of the expression of most protein-coding genes across multiple cellular pathways. Their aberrant expression in tumours contributes to each of the hallmarks of cancer. In MPM, as in most cancers, miRNA expression is disregulated and characterized by a global downregulation, although elevated levels of some miRNAs are also found [18]. Many of these changes in miRNA expression are related to the loss of tumour suppressor activity. Numerous *in vitro* studies have identified miRNAs that can function as conventional tumour suppressors or oncogenes and have demonstrated that the introduction or repression of a single miRNA can effectively contribute to tumorigenesis, tumour progression, or regression [15]. This opportunity provides a rationale for developing miRNA-based therapeutic strategy introducing a miRNA mimic to restore the functionality of a tumour suppressor miRNA that may be lost or expressed at reduced levels in cancer cells.

The significant inhibitory effects on tumour growth following targeted delivery of miRNA-16-based mimics in a xenograft model was the basis for a phase I clinical trial [19-21]. Several articles have also demonstrated the role of miRNA-16 (miR-16) in the control of cell cycle [22-23]. A phase I study was carried out also for the tumour suppressor microRNA-34a (miR-34a) that downregulates the expression of more than 30 oncogenes across multiple oncogenic pathways, as well as genes involved in tumour immune evasion, and is lost or under-expressed in many malignancies [24]. Several studies report the down-regulation of miR-34a and miRNA-16 in tissue samples of MPM patients compared to normal pleural tissue samples and the role of them in the regulation of sensitivity to cisplatin treatment [25].

Although the studies on these two miRNAs, sharing overlapping functions, and on their mirR-mimic are advanced, the effects of their combination are still poorly evaluated [26-27], especially in MPM. Therefore, in the present study we aimed to transfect MPM cells lines with a combination of these two promising miRNAs, miR-16 and miR-34a, in order to try to restore the molecular pathways impaired by their hypoexpression and improve their effects. As cellular models we have chosen two cell lines differing in histology and BAP1 mutations: H28 (epithelioid, BAP1 null) and H2052 (sarcomatoid, BAP1 wild type) [28]. Finally, since targeting multiple signalling pathways, miRNA transfection or co-transfection may also provide an effective way of overcoming drug resistance and improving tumour responses [29], we attempted to test the ability of miRNAs transfection to modulate the sensitivity of mesothelioma cells to cisplatin (CDDP).

2. Materials and methods

2.1. Cell culture, treatments and transfection

Human mesothelioma cell lines H28 and H2052 were obtained from American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (Euroclone) at 37 °C in an atmosphere with 5% CO_2. Exponentially growing cells were used for all assays. For cisplatin (CDDP) (Sigma) treatment cells were incubated with different drug concentrations.

The *mir*Vana™ miR-16-mimic, *mir*Vana™ miR-34a-mimic and control-mimic, were designed and synthesized by Life Technologies. miRNAs were transfected using Lipofectamine™ 2000 (Invitrogen) as recommended by the manufacturer at the final concentrations of 50 or 10 nM.

2.2. Cell viability assay

Cells seeded in 24 or 96-well plates (Costar Corning) were transfected with miR-16 or miR-34a mimics and/or were treated with CDDP. Viability was tested by counting viable cells in a haemocytometer (trypan blue exclusion) and/or by 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) (Sigma) added at a final concentration of 0.5 mg/ml for 3 h. After dissolution of precipitated formazan, absorbence was recorded using a Multiscan Ascent plate reader (Thermo Labsystems).

2.3. Cell cycle distribution

H28 and H2052 cells were trypsinized, fixed in 96% ethanol, washed once with PBS and then stained with propidium iodide (PI) in the presence of RNase A at 4 °C overnight. Samples were run on a FC500™ flow cytometer (Beckman Coulter) and the percentages of cells within each phase of the cell cycle were analysed using FlowJo software.

2.4. Apoptosis

Following treatments, all floating and adherent cells were collected and stained with Annexin V-fluorescein isothiocyanate (FITC) and PI in the dark (Bender MedSystems GmbH). Cells were immediately sorted using a FC500™ flow cytometer (Beckman Coulter). Twenty thousand cells were counted for each measurement and data were analysed using FlowJo.

Caspase 3 activity was measured by Caspase-Glo® 3/7 Assay System (Promega) adding a proluminescent DEVD-aminoluciferin substrate. Luminescence was measured by means of a Cary Eclipse fluorescence spectrophotometer (Varian).

2.5. RNA extraction, miRNAs and gene expressions quantification

RNA was isolated from cultured cells using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's instructions, digested with DNase I (DNA-free kit; Thermo Fisher Scientific) to remove any genomic DNA contamination and quantified using a NanoDrop spectrophotometer (Thermo Scientific).

miRNA quantification. Total RNA was reverse transcribed using a TaqMan MicroRNA RT kit (Thermo Fisher Scientific). The reaction included 3µl of stem-loop RT primer 50 nM, 1.5µl of 10 × RT buffer, 0.15µl of dNTPs 100 mM, 0.19µl RNase Inhibitor 20 U/µl, 1µl of MultiScribe reverse transcriptase 50 U/µl and 5µl of RNA sample in a total volume of 15µl. Quantitative real time-PCR was performed using an iCycler iQ Real-Time Detection System (Bio-Rad). All assays were performed in triplicate, using one no-template and two interpolate controls. The Ct values of the target miRNAs were normalized to sno-RNU6B and the fold-change in expression of each miRNA were calculated using the equation $2^{-\Delta\Delta Ct}$.

Gene expression. cDNA was synthesized using a commercial kit, based on the use of inverse transcriptase (Invitrogen), and amplified by quantitative real-time PCR using specific primers including exon-exon junctions specifically designed for CCND1, CCNE1, CDK4 and CDK6. The expression values of each mRNAs were normalized to expression of RPL13 housekeeping gene. The changes in expression of each mRNA respect to untreated controls were calculated using the equation $2^{-\Delta\Delta Ct.}$

2.6. Statistical analyses

All experiments were performed at least in triplicate. Data are presented as the mean±standard deviation (SD). One-way analysis of variance (ANOVA) with Dunnett's or Turkey's post hoc tests was performed to test the comparisons. $p<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. miR-16 and miR-34a expression after transfection

To investigate the biological roles and effects of miRNA-16 and miRNA-34a in human mesothelioma cells H28 and H2052, we selectively regulated their expression by miR-16 and miR-34a mimic transfection both individually and in combination up to 72 h. We tested two different concentration of miR mimic: 10 and 50 nM. The results indicated that in both cell lines the two miRNAs were overexpressed in comparison to the controls (untreated, lipofectamine treated or miR-control transfected cells) starting from 24 h after the transfection and that there were no differences between the two concentrations (Fig. S1). For this reason, in the subsequent tests we a 10 nM concentration was used. The expression of both miRNAs increases over time and reaches the maximum levels at the end of observation period (72 h).

The possibility that both miRNAs mutually regulate their expression was also evaluated. Our results demonstrate that neither miR-16 nor miR-34a was able to affect the expression of its counterpart.

3.2. Transfection affected cell proliferation

Viability and cell number were assessed up to 72 h after transfection or co-transfection. The counts showed reduced number of cells in all the three transfection conditions (Fig. 1A and B). This decrease started 48 h after transfection, was time dependant and more significant for miR-34a. The anti-proliferative effect was much more evident in H2052s. In this line, the co-transfection was also significantly more effective than the individual miRNA. In contrast co-transfected H28s did not differ from miR-34a-mimic transfected.

In both lines we did not detect any significant increase in apoptotic cells on transfection or co-transfection. Likewise, no significant increase in cleaved caspase-3-positive cells was observed (data not shown).

3.3. Upregulation of miRNA-16 and miRNA-34a inhibits cell cycle progression

Cell cycle was analysed to verify cytostatic effects of miRNA transfection. Transfections resulted in accumulation of H2052 cells in G0/G1 (Fig. 1C and S2), indicating that both miR-16 and miR-34a or their combination induced an arrest in this phase of the cell cycle, although at different times. This same trend also occurred for H28 but to a lesser extent (Fig. 1D and S2). The combination increased the effects of the individual miRNAs.

G0/G1 blockade was confirmed by monitoring the expression of the cyclins and cyclin-dependant kinases involved in G1/S transition starting from 24 h after transfection (Fig. 2). Transfection and co-transfection resulted in significant drops of the expression of Cyclin D1 (CCND1), Cyclin E1 (CCNE1), Cyclin-dependant kinase 4 (CDK4) and Cyclin-dependant kinase 6 (CDK6) albeit at different times and levels, with the exception of CCNE1 whose expression was not changed by miR-34a, of which it is not a specific target.

Combined effects of the two miRNAs occurred only in H28 and specifically for CCND1, CCNE1 and CK4.

3.4. miRNA-16 and miRNA-34a influenced expression of p53 and HMGB1

The expression of p53 was significantly stimulated after transfection of miRNA mimics in H28 cells, while in H2052 especially miR-16 was able to induce p53 over-expression (Fig. 3A).

For high mobility group box1 (HMGB1) an opposite behaviour was observed (Fig. 3B). In all three transfection conditions a HMGB1 hypo-expression trend was observed, even if in the two lines the activity of the

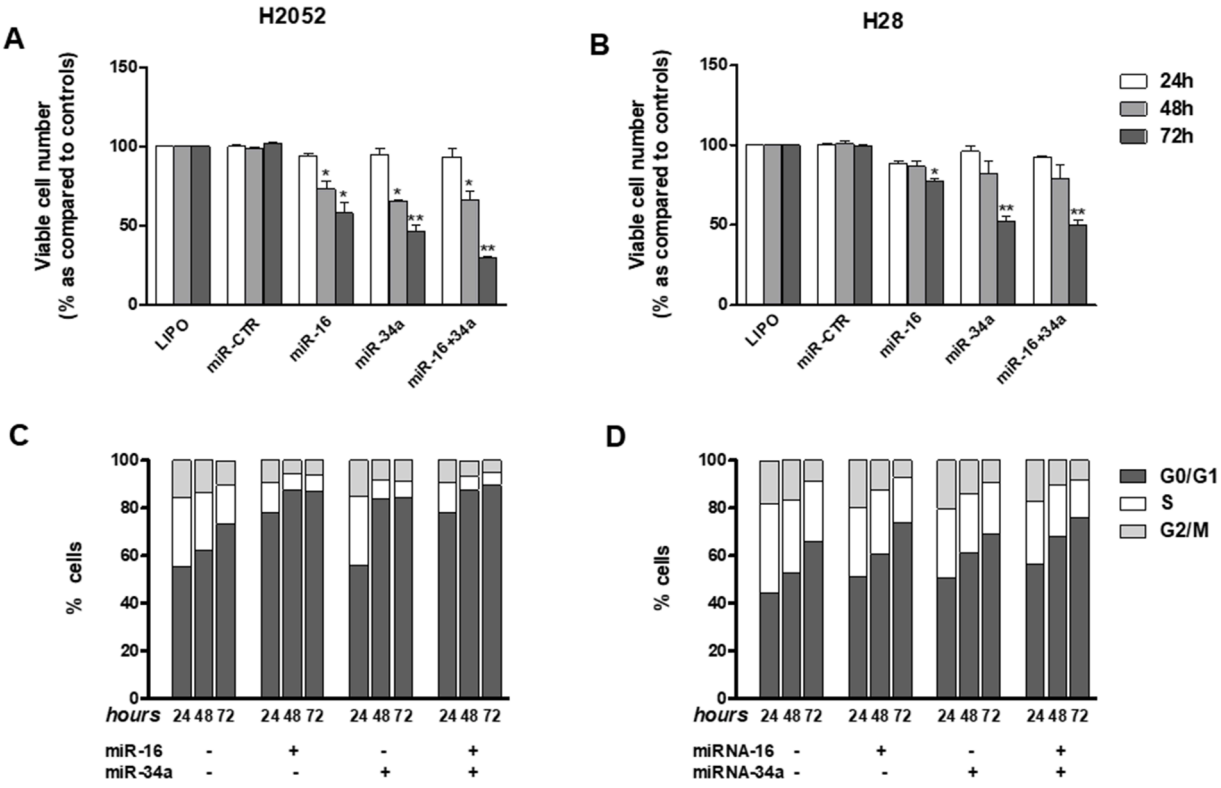

Fig. 1. Effects of transfections on cell viability/growth and cell cycle progression after miR-16 and miR-34a induction. (**A-B**) Relative cell proliferation after miR-16 and miR-34a induction in H2052 and H28 cells, respectively. Each value of cell growth refers to its non-induced condition. Statistical significance: $*p < 0.05$; $**p < 0.01$. (**C-D**) Cell cycle distribution in transfected cells. By cytofluorimetric analysis of DNA three distinct phases can be recognized in the proliferating cell population, corresponding to different peaks: the G_0/G_1, S (DNA synthesis phase), G_2M phase (mitosis).

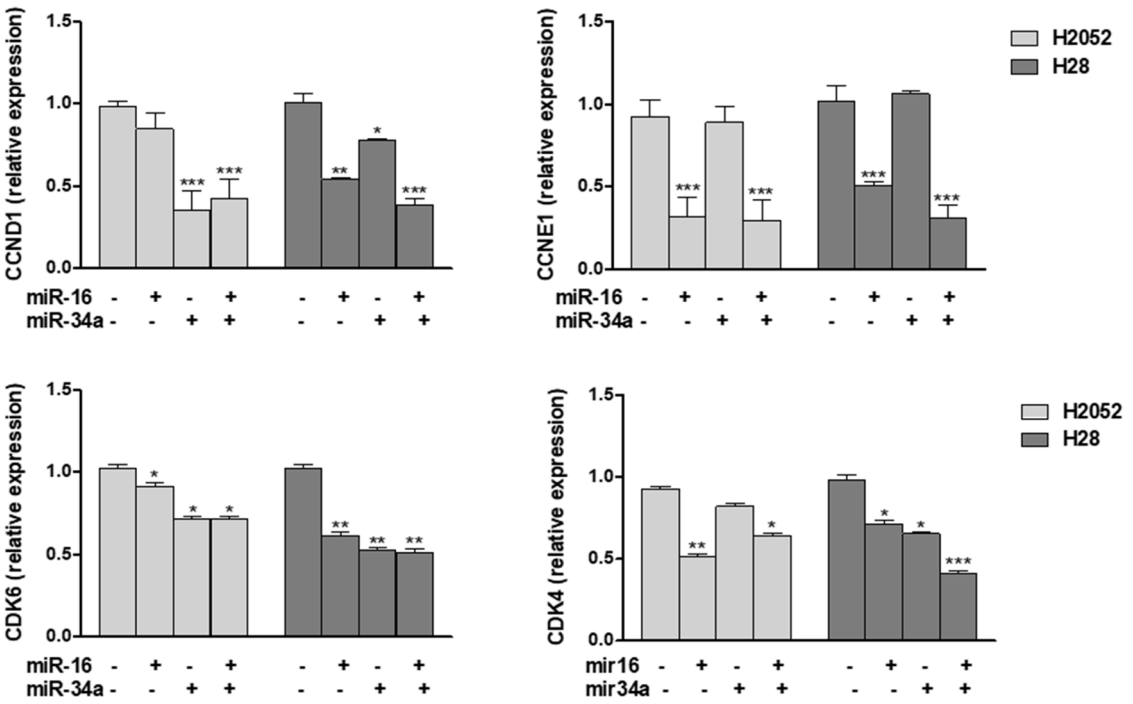

Fig. 2. Transfection of miR-16 and /or miR-34a modulated the expression of cyclins and cyclin dependant kinases. Relative expression of CCND1, CCNE1, CDK4 and CDK6 after 24 h transfection with miRNAs. Expression values refer to the control condition (miRNA control). Comparable results were also obtained 48 and 72 h post-transfection (data not shown). Statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

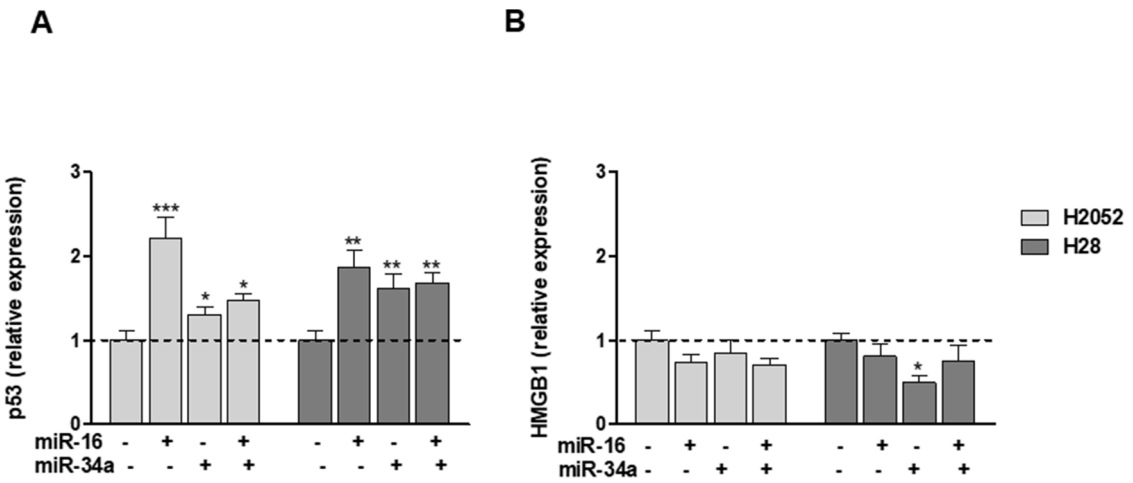

Fig. 3. Transfection of miR-16 and /or miR-34a modulate the expression of p53 and HMGB1 Relative expression of p53 **(A)** and HMGB1 **(B)** after (24 h) transfection with miRNAs. Expression values refer to the control condition (miRNA control). Comparable results were also obtained 48 and 72 h post-transfection (data not shown). Statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

individual miRNA was not parallel and concordant, and in H2052 miR-34a resulted more effective. For both the proteins co-transfection did not lead to significant changes compared to individual miRNAs.

3.5. miR-16 and miRNA 34a modulated cddp sensitivity of mesothelioma cells

In order to check their CDDP sensitivity, H28 and H2052 cells were exposed to different concentrations of CDDP (range 1–100 μM). As shown in Fig. 4, CDDP caused a dose and time dependant decrease of cell viability in both lines. H2052s resulted more sensitive than H28 cell line. At 48 h the IC_{50} of H2052 were less than half of those of H28 (17,83 *vs* 50,55).

To compare the biological activity of miR-16, miR-34a and of their combination, transfected or co-transfected cells were cultured for 48 h with IC_{50} of CDDP. Transfection significantly decreased the cell viability of both lines (Fig. 5A and B), demonstrating that both miRNAs could modulate the CDDP sensitivity of human mesothelioma cells. The highest effects were obtained for miR-16, while the combination of the two miRNAs had no additional effects on CDDP cytotoxicity.

As shown in Fig. 5C and D, the transfection of miRNA mimics significantly increased apoptotic effects of CDDP in both lines. These results were supported also by caspase 3 activity evaluation (Fig. 6A). Furthermore, this assay showed that the caspases cascade occurs earlier in transfected cells.

Moreover, CDDP treatment resulted in a significant increase in the expression of p53 (Fig. 6B) and the overexpression of miRNA single or in combination strengthened its expression especially linked to miR-16,

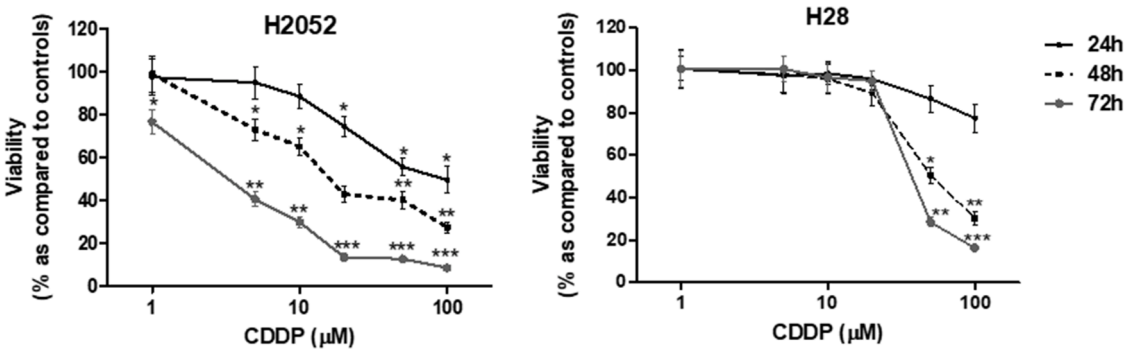

Fig. 4. Cytotoxic effects of cisplatin. Exponentially growing cells H2052 and H28 were cultured in medium containing increasing concentrations of CDDP up to 72 h. Statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

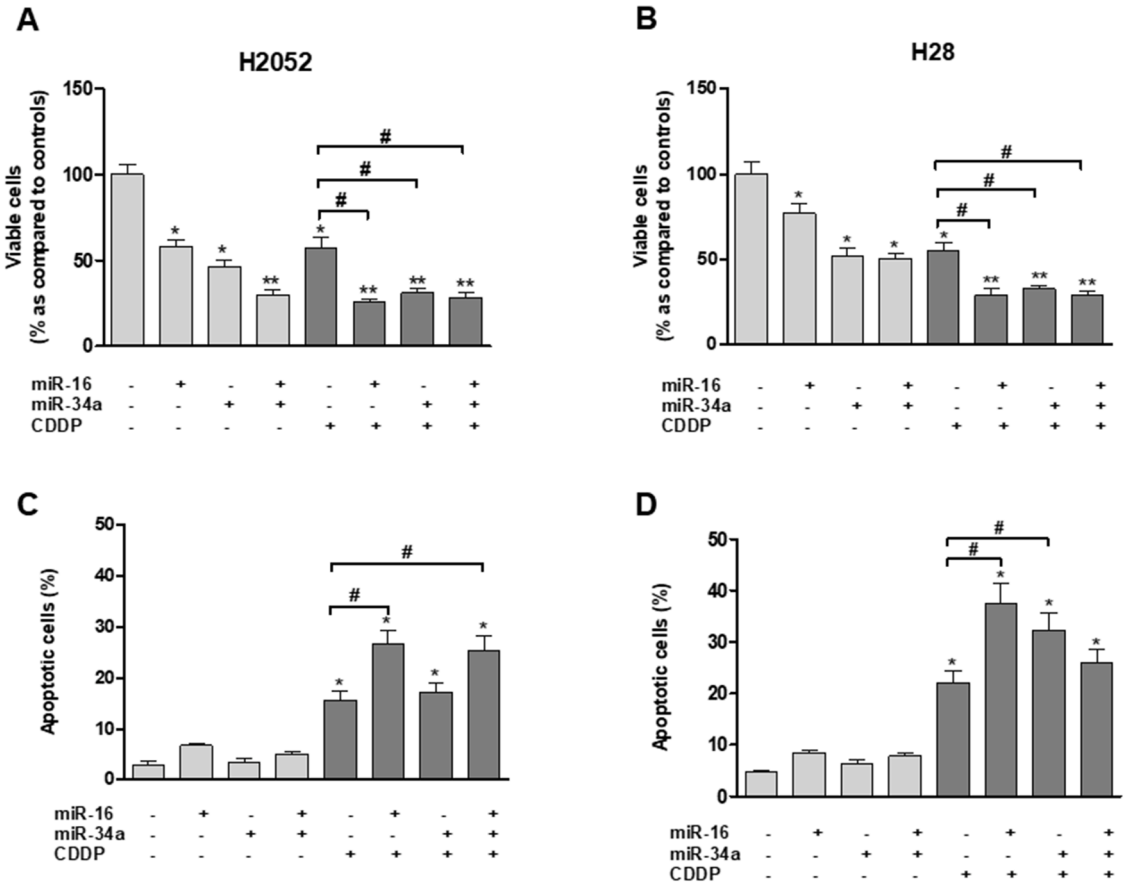

Fig. 5. Transfection of miR-16 and/or miR-34a affect cell viability and increased apoptosis in CDDP treated cells. Exponentially growing H2052 and H28 cells were transfected and after 24 h treated for 48 h with CDDP at the respective IC50. Viable **(A-B)** and apoptotic cells **(C-D)** were evaluated. Values refer to the control condition (miRNA control). Statistical significance *vs* control ($*p < 0.05$; $**p < 0.01***$; $p < 0.001$) and *vs* the CDDP treatment ($^{\#}p < 0.05$).

while the effects of the miRNA mixture were unequal in the two lines. During treatment with CDDP the expression of HMGB1 in the two lines was very different: in H2052s the drug alone did not cause significant changes in expression and in cells transfected with miR-16 or miR-34a alone there was a significant decrease in expression of this protein, while in H28s cisplatin halved the HMGB1 expression and the three conditions of transfection were not able to further reduce its levels (Fig. 6C).

4. Discussion

After thousands of researches on the role of miRNAs in the biology of MPM, their potential value as biomarkers and therapeutic targets is no longer in question [6,15-17,30]. Numerous miRNAs have been demonstrated to contribute to cancer hallmarks in MPM cells *in vitro*, and manipulating their expression using miRNA mimics or inhibitors can inhibit the proliferation and invasion of MPM cells, their interaction with stromal and immune cells and may have the potential to alter the course of disease. miRNA replacement therapy for MPM could be an effective inhibitor of tumour. Increasing evidence has been accumulated demonstrating the emerging role of miRNAs also in the diagnosis and therapeutics of the four principal cancer immunotherapy approaches: immune checkpoint blockade, adoptive cell therapy, cancer vaccines, and cytokine therapy. For example, *in vitro* studies revealed not only that

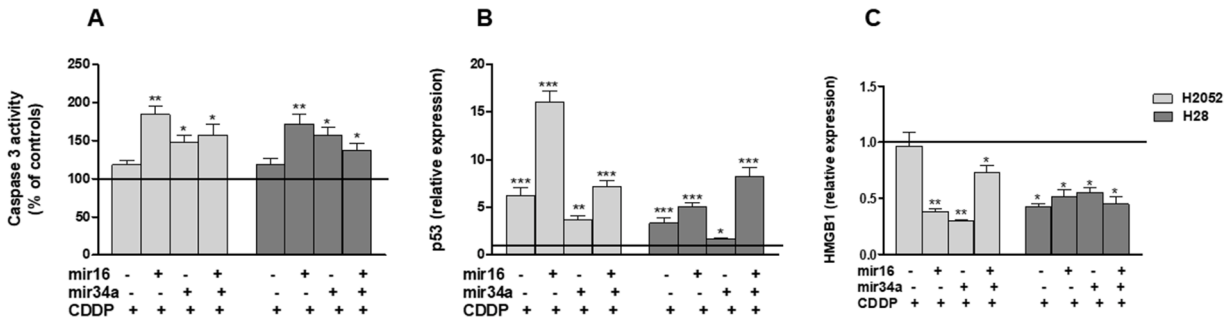

Fig. 6. miR-16 and**/or miR-34a transfection promote activation of caspase and p53 expression and reduceHMGB1 expression during CDDP treatment.** Caspase 3 activity in transfected cells after 24 h of treatment with CDDP **(A)**. Relative expression of p53 **(B)** and HMGB1 **(C)** after transfection with miRNAs and treatment with CDDP (48 h) in H2052 and H28 cells. Expression values refer to the control condition (miRNA control, solid line). Comparable results were also obtained 48 and 72 h post-transfection (data not shown). Statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

miR-34a inhibition induces PD-L1 expression in AML cells, but also that transfection with miR-34a rendered them more sensitive to T cell killing, even after PD-L1 induction by chemotherapy or IFN-γ [31]. Moreover, in malignant pleural mesothelioma (MPM), low levels of the miR-34, miR-200, as well as miR-15/16 families have been recently associated with high PD-L1 expression and poor prognosis. When one or more of the miRNAs were re-introduced in MPM cell lines, PD-L1 expression was effectively decreased [32].

To date, at least 20 miRNAs are being evaluated in clinical trials [15,24] such as TargomiRs, loaded with the miR-16 mimic and conjugated with an anti-EGFR antibody [21] and MRX34, a liposomal formulation of the naturally occurring tumour suppressor miR-34a [24,33]. Although this latter trial was closed early due to four patient deaths, dose-dependant modulation of relevant target genes provided proof-of-concept for miRNA-based cancer therapy.

Although located in separate chromosomal regions (13q14 and 1p34), miR-16 and miR-34a are functionally related because they can inhibit cell cycle progression in G0/G1 phase through a G1 checkpoint, modulating common targets (CCND1, CK4, CK6). CCNE1 and CCND2 are unique targets of miR-16 [26]. Therefore, in the absence of miR-16, cancer cells can grow uncontrollably [22,23].

To investigate if these miRNAs are able to interact with each other for the regulation of cellular processes also in MPM, as already reported in non-small cell lung cancer [27], they were co-transfected to induce overexpression in H28 and H2052 cell lines. Our results demonstrate that miR-16 and miR-34 reduce cell proliferation and induce cycle arrest in both lines, although to a different extent. The combination of these miRNAs, involved in the same pathway potentiates the effect of each individual miRNA and resulted in a G1 block greater than each microRNA alone, but not to an additive or synergistic extent. Their combined effect can be explained by the fact that their concerted action is able to regulate more targets than each miRNA alone. The effects are not only attributable to the investigated targets. Other genes might be regulated by the pooled mimetics but not by the individual microRNAs.

In cycle arrest, the network-axis between miRNAs and p53 should not be underestimated based on its pivotal role in regulating cellular processes. Our data confirmed this interplay and demonstrated that restoring normal intracellular miRNA levels may also increase the levels of this tumour suppressor.

As already observed, activation of p53 effectively induced cell death in mesothelioma, a tumour characterized by a high intrinsic resistance to apoptosis and suggested the use of p53-reactivating agents alone, or in combination regimens, to improve the outcome of patients [34].

MiR-34a is also reported to repress tumour development by targeting and downregulating HMGB1 expression in various cancers [35,36], but there is still no or little evidence for the functions of miR-34a/HMGB1 axis in MPM. This highly conserved protein regulates DNA replication, recombination, repair and transcription: binding to minor grooves and bends and so increasing DNA interaction with several transcription factors, such as p53, might modulate activation or repression. Secreted HMGB1 acts as an extracellular signalling molecule during cell migration, differentiation and apoptotic or necrotic deaths, in a context-dependant manner. HMGB1 is also a damage-associated molecular pattern molecule released during infection, injury and inflammation, and it activates innate immune response, playing a crucial role in a variety of inflammatory disorders including cancer and MPM [37,38].

Several studies have found evidence of crosstalk between the signalling pathways involving HMGB1 and cell cycle [39,40]. Our data proved that miR-34a and also miR-16, targeting cyclins and cyclins kinase dependant, mediated HMGB1 downregulation and this could lead to therapeutic advantage due to its role as a 'master switch' by which the chronic inflammation, that drives mesothelioma growth, is maintained.

By regulation of cell proliferation and apoptosis, microRNAs can also influence the response to cancer treatments and also to cisplatin, the most widely used drug in cancer therapy and the first FDA-approved platinum compound for MPM treatments [17,29,41-44]. A large proportion of patients are insensitive to drug or acquire resistance to chemotherapy. Our data confirmed that upregulation of miR-34a and miR-16 could modulate CDDP sensitivity by influencing cell proliferation, apoptosis also via targeting p53, potentially benefiting human MPM treatment. However, no further advantages were observed in the simultaneous transfection compared to the individual miRNA's, probably due to the complex intracellular interactions and alterations induced by the different treatments.

Despite some differences in the extent of the responses, no discrepant behaviours were observed between the two lines chosen, differing both in histopathological type and in BAP1 mutation. The mainly difference concerns drug resistance: epithelial H28 resulted more resistant to cisplatin than sarcomatoid H2052s, but this may be due to BAP1 mutation, whose loss-of-function is strongly associated with epithelioid differentiation and may have a role to predict prolonged survival or chemosensitivity [28]. Lost expression of BAP1 seems to promote the survival of H28 cells, reducing effects of transfection on cell cycle block, while normal levels of BAP1 in wild type cells H2052 caused a S-phase retardation/delay, as well as influencing sensitivity to CDDP. Similar conclusions were reported by a comparison aimed to evaluate the potential involvement of BAP1 in the chemosensitivity of human mesothelioma cell lines carrying wild-type, mutant or silenced BAP1 [45].

In conclusion, the concerted action of miR-16 and miR-34a was limited to cell cycle regulation, significantly reducing the number of cells. The combination of these miRNAs, involved in the same network rather than individual miRNAs, seems to be more effective and should be considered preferable. More controversial and unsatisfactory results were obtained on the other issues evaluated, particularly on the sensitivity to cisplatin. However, this observation does not argue against the combined use of miRNA in therapeutic strategies, in order to reactivate cellular pathways and/or potentiate the therapeutic impact of drugs.

MPM progression is driven by multiple cellular networks that act in concert and combinatorial treatments targeting multiple pathways simultaneously could reduce the risk of the development of resistance. This approach however will require a greater understanding of how drug and miRNAs work and how they interact with each other to ensure that the most appropriate miRNAs are chosen and matched.

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Institution and ethics approval and informed consent

Authors declare that approval and informed consent were not required for this manuscript, as it does not involve human subjects or animals.

Authors' contributions

Pinelli Silvana, Alinovi Rossella and Mozzoni Paola conceived of the presented idea.

Pinelli Silvana, Alinovi Rossella and Mozzoni Paola planned and carried out the experiments.

Alinovi Rossella wrote the manuscript with support from Pinelli Silvana, Mozzoni Paola, Pelosi Giorgio and Diana Poli.

Diana Poli, Delia Cavallo, Luca Ampollini, Massimo Corradi, Matteo Goldoni and Giorgio Pelosi revised critically the manuscript for important intellectual content.

All authors contributed to the interpretation of the results and revised critically the manuscript for important intellectual content.

All authors have approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2020.100276.

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