Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma

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Background: Malignant pleural mesothelioma (MPM) is recalcitrant to treatment and new approaches to therapy are needed. Reduced expression of miR-15/16 in a range of cancer types has suggested a tumour suppressor function for these microRNAs, and re-expression has been shown to inhibit tumour cell proliferation. The miR-15/16 status in MPM is largely unknown.

Materials and methods: MicroRNA expression was analysed by TaqMan-based RT-qPCR in MPM tumour specimens and cell lines. MicroRNA expression was restored in vitro using microRNA mimics, and effects on proliferation, drug sensitivity and target gene expression were assessed. Xenograft-bearing mice were treated with miR-16 mimic packaged in minicells targeted with epidermal growth factor receptor (EGFR)-specific antibodies.

Results: Expression of the miR-15 family was consistently downregulated in MPM tumour specimens and cell lines. A decrease of 4- to 22-fold was found when tumour specimens were compared with normal pleura. When MPM cell lines were compared with the normal mesothelial cell line MeT-5A, the downregulation of miR-15/16 was 2- to 10-fold. Using synthetic mimics to restore miR-15/16 expression led to growth inhibition in MPM cell lines but not in MeT-5A cells. Growth inhibition caused by miR-16 correlated with downregulation of target genes including Bcl-2 and CCND1, and miR-16 re-expression sensitised MPM cells to pemetrexed and gemcitabine. In xenograft-bearing nude mice, intravenous administration of miR-16 mimics packaged in minicells led to consistent and dose-dependent inhibition of MPM tumour growth.

Conclusions: The miR-15/16 family is downregulated and has tumour suppressor function in MPM. Restoring miR-16 expression represents a novel therapeutic approach for MPM.

Key words: mesothelioma, miR-16, microRNA, minicells

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Malignant pleural mesothelioma (MPM), caused by inhalation of asbestos fibres, is an aggressive tumour originating in the lining of the thoracic cavities. The prognosis for MPM patients is poor, with median survival around 12 months, and therapy is mainly palliative [1]. Selected patients presenting with early-stage disease may receive a combination of chemotherapy, surgery and radiotherapy. However, despite intensive combined modality therapy, the disease will recur in almost all cases. In 2004, the pemetrexed with cisplatin doublet was identified as the most effective chemotherapy combination [2], but there has been almost no progress since. Unlike the situation in non-small-cell lung cancer where driver mutations have been identified, trials of targeted therapies in MPM were not successful [3], and new treatment approaches are urgently required.

In common with other cancers, the microRNA expression in MPM displays characteristic alterations [4-9]. However, none of these changes were present in a large proportion of tumours or cell lines, limiting their clinical utility. In a previous study, we noted a significant downregulation of miR-16 expression in tumour samples from patients who underwent extrapleural pneumonectomy [10]. Here, we report that compared with normal mesothelium or mesothelial cell lines, a consistent downregulation of the miR-15/16 family was observed in all MPM tumours and cell lines investigated. Restoring expression of miR-16 resulted in inhibition of cell growth in vitro, and using epidermal growth factor receptor (EGFR)-targeted minicells to deliver miR-16 mimics to MPM xenografted tumours in vivo caused profound inhibition of tumour growth. Together, these results suggest that miR-16 replacement represents a novel approach to therapy for MPM patients.

MicroRNA mimics and transfection

Synthetic microRNA mimics (supplementary Table S2, available at Annals of Oncology online) were purchased from Shanghai GenePharma. Following reverse transfection (and where indicated, additional treatment with chemotherapeutic drugs), effects on proliferation, colony formation, cell cycle and apoptosis were analysed by standard assays (see supplementary Information, available at Annals of Oncology online, for detailed methods).

Xenograft studies

The effect on tumour growth of EGFR-targeted minicells loaded with miR-16 was evaluated in a subcutaneous human xenograft model of MPM in nude mice, in experiments approved by the EnGeneIC Animal Ethics Committee. See supplementary Information, available at Annals of Oncology online, for detailed methods.

Results

the miR-15 family is downregulated in MPM

In a previous report, we observed a downregulation of miR-16 in a small series of MPM tumour specimens [10]. Here, we compared miR-16 expression in a larger sample set consisting of 60 archival FFPE blocks, and 23 normal pleural tissue samples. This revealed that miR-16 expression was 22-fold lower in tumours than normal tissue (Figure 1A). Similarly, the levels of the co-expressed miR-15a and miR-15b were also reduced in tumours, by 4- and 10-fold, respectively (Figure 1A). The expression of miR-195, the remaining member of the miR-15 family, was 11-fold lower in tumours (Figure 1A). We then profiled expression of 760 microRNAs in the mesothelial cell line MeT-5A and four MPM cell lines (supplementary Figure S3, available at Annals of Oncology online). This revealed that expression of the miR-15 family members was lower in MPM cell lines. These data were confirmed by RT-qPCR, showing a two- to fivefold decrease for each microRNA (Figure 1B) across six MPM cell lines.

A comparison of MeT-5A cells and MPM cell lines revealed a relationship between downregulation of the miR-15 family microRNAs (all of which contain the AGCAGC seed) and higher expression of a number of their predicted target mRNAs (Figure 1C). This relationship was confirmed experimentally by the observed decrease in the mRNA expression of a number of predicted targets of this microRNA following transfection with miR-16 mimic (Figure 1D). In addition, the protein expression of the well-characterised targets of miR-16- Bcl-2 and CCND1 – was decreased following transfection with miR-16 mimic (Figure 1E).

Restoring expression of miR-15 or miR-16 inhibits MPM cell growth and miR-16 sensitises to antimetabolites

The microRNAs of the miR-15/16 family share extensive sequence identity with identical seed sequences, and there is overlap in their predicted mRNA targets. Transfecting with a synthetic mimic of miR-16, miR-15a or miR-15b led to growth inhibition in MPM cell lines but not in normal MeT-5A cells (Figure 2A). Growth inhibition was most effective following transfection with miR-16 mimic, and we focused on this microRNA in subsequent experiments. The effects of miR-16

Materials and methods

Clinical samples

The 60 tumour samples used in this study were formalin-fixed paraffin-embedded (FFPE) specimens from patients undergoing extrapleural pneumonectomy, and are part of a series described previously [11]. Formalin-fixed normal pleural tissues were collected from 23 patients undergoing cardiac or aortic surgery at Royal Prince Alfred Hospital (RPAH), Sydney. A written, informed consent was obtained from all patients and the study was approved by the Human Research Ethics Committees at RPAH and Concord Repatriation General Hospital, Sydney (supplementary Table S1, available at Annals of Oncology online).

RNA isolation, reverse transcription and real-time qPCR

Total RNA from cell lines (see supplementary Information, available at Annals of Oncology online, for details) was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. RNA isolation from FFPE sections was carried out after laser-capture microdissection (LCM) as described [10]. Measurement of microRNA expression was carried out using microRNA-specific TaqMan assays, with expression of target gene mRNA analysed by standard RT and SYBR Green-based real-time qPCR (see supplementary Information, available at Annals of Oncology online, for details).
levels were dose-dependent and evident following transfection with 1 nM mimic (supplementary Figure S4, available at *Annals of Oncology* online). Transfection with miR-16 also reduced the ability of MPM cells to form colonies when plated at low density, but did not affect normal cells (Figure 2B). Further investigation revealed that restoring miR-16 in MSTO-211H cells resulted in a G0/G1 accumulation (Figure 2C), and a more striking induction of apoptosis evidenced by DNA fragmentation (Figure 2D) and increased annexin V staining (Figure 2E).

To determine whether microRNA expression was related to drug resistance, we combined mimics with the chemotherapeutic drugs frequently used for the treatment of MPM. Transfection of MSTO-211H or MM05 cells with miR-16 mimic increased sensitivity of the cells to treatment with gemcitabine as well as pemetrexed, and these effects were
Figure 2. Transfection with microRNA mimics inhibits MPM cell proliferation. (A) Cells were transfected with miR-15b, miR-16 or control mimics at a final concentration of 5 nM. Growth was measured at 48, 72, 96 and 120 h after transfection (*P < 0.05, **P < 0.01). (B) Colony-forming ability was measured in cells transfected with 0.2 or 1 nM miR-16 mimic in MSTO-211H and MeT-5A cells. Data are representative of three experiments giving equivalent results. (C) MSTO-211H cells were transfected with 5 nM control or miR-16 mimic, and cell cycle distribution was analysed 72 h later. For each population, 10 000 events were counted and the proportion of the cells in each phase of the cell cycle is shown. The data are representative of three experiments giving similar results. MSTO-H211 cells were transfected with miR-16 or control mimic (1 or 5 nM) and apoptosis analysed by DNA fragmentation at 72 and 96 h (D) or annexin V/PI staining at 96 h (E). Genomic DNA revealed fragmentation with band sizes indicating typical apoptotic DNA fragmentation, and PI staining increased with miR-16 mimic concentration.
dependent on mimic dose (Figure 3). At a concentration of 5 nM, miR-16 increased the sensitivity of cells to gemcitabine and pemetrexed by three and sevenfold, respectively, but did not affect sensitivity of MeT-5A cells to either drug (Figure 3). In contrast, restoring levels of miR-16 restoration did not affect cisplatin sensitivity of MPM cells (supplementary Figure S5, available at Annals of Oncology online).

**Figure 3.** Restoring miR-16 increases sensitivity of MPM cells to gemcitabine and pemetrexed. MSTO-211H (A, B), MM05 (C, D) and Met-5A cells (E, F) were transfected with miR-16 mimic (at the indicated concentrations as labelled in (F)) and 24 h later exposed to pemetrexed (A, C, E) or gemcitabine (B, D, F) for 72 h. Compared with control mimic, transfection with miR-16 mimic increased toxicity of both pemetrexed and gemcitabine in MPM cells but not control MeT-5A cells. Data are mean ± SD of triplicate measurements, and are representative of three independent experiments giving similar results.

The strong growth inhibitory response to restoration of miR-16 expression in MPM cell lines observed in vitro suggested that a similar approach might have a potential clinical application. To explore this, we administered miR-16-containing minicells to nude mice bearing MSTO-211H-derived xenografts via intravenous injection. Minicells are formed through asymmetric bacterial cell division and have been shown to deliver drugs and siRNAs to tumour xenografts, via a combination of passive accumulation at sites of leaky vasculature and specific targeting using antibodies to tumour surface antigens [12, 13]. We used an EGFR-specific antibody for targeting, and to increase the stability of the miR-16 mimic the sequence included four 2’O-methyl-modified nucleotides at each end of the passenger strand (supplementary Figure S6, available at Annals of Oncology online).
Dosing mice with $1 \times 10^9$ minicells containing miR-16 mimic ($\sim 0.3 \mu g$ [12]) one, two or four times per week for 3 weeks resulted in a significant and dose-dependent inhibition of tumour growth compared with controls (Figure 4A). Increasing the dose to $2 \times 10^9$ minicells four times per week led to even greater inhibition of tumour growth (Figure 4B). In tumours from mice receiving the higher dose of miR-16-containing minicells, there was a consistent grade 3 or 4 lymphocyte infiltration observed (compared with grades 1–2 in control mice), and a twofold reduction in the number of mitoses observed (supplementary Table S7, available at Annals of Oncology online), but otherwise, no non-specific effects were observed (supplementary Figure S8, available at Annals of Oncology online). The results from DNA fragmentation assays revealed an increase in DNA fragmentation in sections from miR-16-treated mice compared with controls (Figure 4C).

**discussion**

MPM is remarkably resistant to therapy and so far, the evaluation of targeted agents and experimental (immuno) therapy approaches in clinical trials has not provided new treatment opportunities [3]. The marked heterogeneity of MPM tumours and global upregulation of multiple genes involved in metabolism, proliferation and avoidance of apoptosis may explain the lack of success observed with single-target agents in the clinic. In light of this heterogeneity, targeting aberrant...
microRNA expression to reassert control over entire gene expression networks provides an attractive means to simultaneously control multiple genes within the same and/or related pathways. Several studies have investigated the effects of restoring the expression of downregulated microRNAs in MPM. The expression of miR-29c-5p was decreased twofold in MPM cell lines compared with mesothelial cells, and re-expression led to inhibition of growth and invasion [9]. Increasing expression of miR-31, co-deleted with the CDKN2A locus in 32% of tumours, had similar effects [7]. A downregulation in miR-34b and miR-34c expression was observed in 50% of tumours analysed, and cells stably transfected with a miR-34b/c construct exhibited reduced colony formation, migration and invasion [14]. An adenoviral-based miR-34b/c vector reduced MPM cell viability. None of these studies, however, investigated the effects of microRNA replacement strategies on the growth of normal mesothelial cells, nor did they demonstrate the effects of microRNA changes in tumours in vivo. Here, we report that downregulation in expression of the miR-15 family in MPM seems to be more frequent than changes in other microRNAs, and is a more common event than the most frequent single-genetic events such as deletion of the CDKN2A locus. These observations, together with those reported previously, suggest that targeting microRNA expression may have applicability in the treatment of MPM patients presenting with an epithelioid or biphasic subtype.

The consistent downregulation of the miR-15 family that we have observed in MPM patient samples and cell lines mirrors the changes seen in these microRNAs in other solid tumours—most notably in cancers of the prostate [15], lung [16] ovary [17] and colon [18]. Consistent with the other tumour types, multiple transcripts dysregulated in MPM are predicted to be targets of the miR-15 family. We observed a number of these potential targets to be expressed at higher levels in MPM cell lines compared with MeT-5A cells (concordant with downregulation of microRNA expression), and also demonstrated a number to be downregulated upon ectopic expression of miR-16. Together with the similar levels of growth inhibition we observed following re-expression of miR-16, miR-15a or miR-15b in MPM cells (but not normal mesothelial cells), this suggests that they are likely to be controlling proliferation through their effects on the cell cycle and apoptosis-related genes. The regulation of a related set of cell cycle- and apoptosis-related genes by the miR-34 family, and the frequent methylation-induced silencing of miR-34b/c in MPM tumours [14], further underline the importance of these pathways in MPM.

As is the case for other tumours, microRNAs are likely to contribute to the drug sensitivities of MPM cells. In the case of miR-15b and miR-16, both have been linked to the multidrug resistance of gastric cancer cells to vincristine, etoposide and Adriamycin [19]. Following miR-16 re-expression in MPM cells, we observed sensitisation to gemcitabine and pemetrexed. While there appears to be no obvious overlap between targets of these drugs (RM1 and TYMS) and the predicted gene targets of miR-16, it is noteworthy that the chemopreventive agent curcumin sensitises pancreatic cancer cells to gemcitabine. Curcumin has been shown to downregulate Bcl-2 and cyclins through inhibition of NF-kB in pancreatic cancer [20], and to stimulate expression of miR-15a/16-1 leading to downregulation of WT1 and growth inhibition in CLL cell lines and tumour cells [21]. Regardless of the mechanism, the ability of miR-16 to sensitise cells to antimetabolite drugs used to treat MPM patients has potential clinical implications.

The application of microRNA replacement as a therapeutic option for MPM patients is dependent on the ability to effectively deliver synthetic microRNA mimics in vivo. In previously reported pre-clinical studies, inhibition of tumour growth following the restoration of miR-16 expression in vivo has been reported in a prostate cancer model using lentivirus [15] or atelocollagen [22], and in multiple myeloma using neutral lipid emulsion [23]. In our study, we made use of the tumour targeting afforded by EGFR-coated minicells [12] to deliver synthetic miR-16 mimic to established MPM xenografts derived from MSTO-211H cells, which in common with most MPM tumours, express EGFR [24]. Our results confirm the tumour inhibitory effects of miR-16 re-expression and, importantly, we show that tumour growth can be effectively inhibited with systemic administration of doses of <1 µg miR-16 mimic. This compares very favourably with the 50 µg miR-16 mimic [22, 23] or 20 µg miR-34a mimic [25] required to achieve growth inhibition in previous studies.

In conclusion, we have demonstrated a consistent downregulation of multiple members of the miR-15/16 family across MPM cell lines and tumour samples. Restoring miR-16 in vitro resulted in growth inhibitory and drug sensitising effects in MPM cell lines, but not normal mesothelial cells. Furthermore, our demonstration that miR-16 mimics can be effectively delivered using EGFR-targeted minicells resulting in tumour regression in vivo, together with the safety profile of minicells and their current assessment in a phase I clinical trial, suggests that this approach may bear fruit in the clinic.

funding

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disclosure

NM, JW, HB and JM hold stock in, EnGeneIC Ltd. All other authors have declared no conflicts of interest.

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