ORIGINAL ARTICLE

Mir-148a Improves Response to Chemotherapy in Sensitive and Resistant Oesophageal Adenocarcinoma and Squamous Cell Carcinoma Cells

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Abstract

Background Response to chemotherapy varies widely in patients with advanced oesophageal cancer. We investigated the impact of manipulating certain microRNAs on response to cisplatin and 5-fluorouracil (5-FU) in oesophageal cancer cells.
Methods Cisplatin-/5-fluorouracil-resistant oesophageal squamous cell carcinoma (SCC) and adenocarcinoma (EAC) cell lines were established, and the impact of ectopic upregulation of miR-106a and miR-148a on response to both drugs was assessed.
Results The impact of miR-106a-upregulation was inconsistent. Upregulation was followed by reduced sensitivity to cisplatin in chemotherapy-sensitive EAC cells (cell survival, $+8.7 \pm 0.8\%$; $p = 0.003$) and an improved response to 5-FU in cisplatin-resistant EAC cells (cell survival, $-6.4 \pm 2.5\%$; $p = 0.011$). MiR-148a upregulation significantly increased sensitivity to chemotherapy in seven out of ten cell lines, represented by a decrease in cell viability of $22.6 \pm 7.9\%$ to $50.5 \pm 10.6\%$ after cisplatin ($p \leq 0.014$) and $6.0 \pm 0.8\%$ to $15.0 \pm 4.1\%$ after 5-FU treatment ($p \leq 0.012$). The only cell lines in which miR-148a upregulation had no effect were cisplatin-resistant EAC exposed to cisplatin and 5-FU-sensitive and 5-FU-resistant SCC cells exposed to 5-FU.
Conclusion MiR-148a sensitized chemotherapy-sensitive oesophageal cancer cell lines to cisplatin and, to a lesser extent, to 5-flurouracil and attenuated resistance in chemotherapy-resistant variants. Further experimental and clinical studies to investigate the exact mechanisms involved are warranted.

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Introduction

Oesophageal cancer is usually diagnosed at a locally advanced stage, and local lymph node metastases are common. Conse-

quently, its prognosis is generally poor, and there has been considerable interest over recent decades in using chemotherapy, with or without radiotherapy, for the treatment of patients with oesophageal cancer, either before surgery or in the definitive treatment of patients in whom surgery is not appropriate. Most clinical studies report a variable response to chemotherapy, with some tumours disappearing completely, and others responding poorly to such treatment. More recent meta-analyses suggest that patients who undergo esophagectomy, and in whom neoadjuvant treatment has achieved a "complete" response, have a much better survival outcome.[1–3] Unfortunately, however, only 20–40% of patients have such a response to neoadjuvant therapy,[4,5] and the development of methods to improve the response to neoadjuvant therapies seems worthwhile.

In this context, we have been interested in the potential of microRNAs (miRNAs) to impact on chemotherapy. MiRNAs are small non-coding RNA molecules which regulate gene expression posttranscriptionally and control many fundamental cellular processes.[6] In various cancers, miRNAs have been demonstrated to regulate oncogenes or tumour suppressor genes.[7–10] In oesophageal carcinoma, different levels of miRNAs can be used to discriminate between benign and malignant oesophageal tissues,[11–21] and miRNAs are involved in cell proliferation, invasion, migration, apoptosis, cell cycle and tumorigenesis.[14–16,18,21,22] Most importantly, expression of miRNAs or RNASEN (enzyme in the biogenesis of miRNAs) correlates with the risk of lymph node metastasis, venous invasion,[21] and prognosis.[17,23–25] Interestingly, in other cancer types, some miRNAs have been shown to be associated with sensitivity to,[26–28] or modification of, response to chemotherapy agents such as cisplatin or 5-fluorouracil (5-FU).[29–31] However, no studies have investigated the potential effect of altered miRNA expression on response to chemotherapy treatment in oesophageal cancer.

Recently, we demonstrated that two miRNAs, miR-106a and miR-148a, are negatively associated with oesophageal cancer recurrence after surgical treatment in patients with advanced oesophageal squamous cell carcinoma, as well as the likelihood of tumour-related death. Furthermore, miR-148a expression was inversely correlated with adenocarcinoma differentiation grade.[32] As recurrent disease and poor tumour differentiation both suggest more aggressive malignancies, and aggressive tumours might be increasingly resistant to chemotherapy, we hypothesized that altered levels of these two miRNAs might have an impact on the outcome after chemotherapy for oesophageal cancer. As the standard chemotherapy treatment for both forms of oesophageal cancer is based on cisplatin and 5-fluorouracil, we investigated the potential for these two miRNAs to modulate the cellular response to either cisplatin or 5-fluorouracil in adenocarcinoma and squamous cell oesophageal carcinoma cell lines.

Material and Methods

Cell Lines and Cell Culture

The human squamous cell carcinoma cell line KYSE410 (obtained from the Microbiology Laboratory, University of Muenster, Germany) and the human adenocarcinoma cell line OE19 (obtained from the Department of Surgery, Flinders University Adelaide, Australia) were cultured using RPMI 1640 medium (GIBCO® Invitrogen, no. 11875) or Dulbecco's modified Eagle's medium (DMEM) high-glucose 1× medium (GIBCO® Invitrogen, no. 11995) respectively, supplemented with 10% foetal bovine serum (GIBCO® Invitrogen, no. 26140), 1% penicillin–streptomycin (GIBCO® Invitrogen, no. 15140; 10,000 U of penicillin and 10,000 μg of streptomycin per 1 mL) and 2‰ Normocin™ (InvivoGen, San Diego, USA, catalog no. ant-nr-1; 50 mg/mL) in a humidified atmosphere containing 5% CO_2 at 37°C. For drug sensitivity assays and transfection experiments, phenol red free medium (RPMI 1640: GIBCO® Invitrogen, no. 11835; DMEM/F12 1:1: GIBCO® Invitrogen, no. 11039) containing the same supplements was used. Drug-resistant variants of both cell lines were established using a repetitive pulsatile treatment with constant concentrations of cisplatin and 5-FU. Briefly, KYSE 410 cells were subjected to a 4-day exposure of 2 μM cisplatin (KYSE410/C2) or 5 μM 5-FU (KYSE410/5-FU5) and OE 19 cells were exposed to 5 μM cisplatin (OE19/C5) for 3 days; the medium was not changed during this period, providing a constant exposure to the drug. We were unable to establish a 5-FU-resistant variant of the OE19 cell line during this study. After removal of the chemotherapy agents, cells were allowed to recover and split when reaching approximately 70–80% confluency, followed by the next cycle of chemotherapy. Prior to transfection, the degree of chemotherapy resistance of the respective cell lines was assessed. All cell lines presented significant resistance to the corresponding chemotherapy agent (see Table 1).

In Vitro Drug Sensitivity Assay

Cells were seeded onto 96-well plates (2.5×10^3 and $5–6 \times 10^3$ viable cells/well for KYSE410 and OE19, respectively) and allowed to attach. After cellular adhesion, phenol red free medium containing cisplatin or 5-FU at distinct concentrations (5 μM cisplatin or 5 μM 5-FU for KYSE410 cell lines; 20 μM cisplatin or 100 μM 5-FU for OE19 cell lines) was freshly prepared and added to the corresponding cells. The concentration of drugs represented the approximate median lethal doses (LD_{50}) in the respective cell lines following 72 h of exposure to cisplatin and 5-FU. This was estimated in previous experiments in our laboratory which

Table 1 Relative cell survival after cisplatin and 5-FU treatment in different sensitive and resistant oesophageal squamous cell carcinoma and adenocarcinoma cell lines

	Sensitive SCC	5-FU-resistant SCC	Cisplatin-resistant SCC	Sensitive EAC	Cisplatin-resistant EAC
Cisplatin	53.4 ± 6.1	54.8 ± 10.8	$68.9 \pm 4.2 \ (p = 0.035)$[a]	43.6 ± 6.3	$64.1 \pm 8.6 \ (p = 0.028)$[a]
5-FU	62.5 ± 6.0	$92.0 \pm 3.3 \ (p = 0.003)$[a]	57.0 ± 2.6	49.8 ± 8.7	57.6 ± 3.1

Data were presented as percentage of viable cells related to untreated controls and expressed as means ± standard deviation. Doses of chemotherapy agents used for the assay were as follows: cisplatin, 5 μM for SCC and 20 μM for EAC cell lines; 5-FU, 5 μM for SCC and 100 μM for EAC cell lines (see "Material and Methods")

[a] Statistical comparison to corresponding sensitive cell line

tested various drug concentrations over 24-, 48-, 72- and 96-h periods (data not shown). After 72 h, cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), inner salt; Promega). Cells were washed with PBS. MTS reagent was prepared in fresh medium (100 μL phenol red free medium +20 μL MTS solution) and applied to the cells. The absorbance at 490 nm for each well was read on a spectrophotometer after 2 h, and the absorbance of the background (wells with medium and MTS solution) was subtracted from experimental wells to provide corrected absorbance readings. For the assessment of the effect of transfection on sensitivity to drug treatment, three independent experiments were performed with nine technical replicates each. Drug resistance was assessed in a minimum of two independent experiments.

Establishment of Transfection

Hsa-miR-106a mimic, hsa-miR-148a mimic and negative controls were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The negative control was designed to contain no homology to human gene sequences and miRNAs. Cells were transfected using Lipofectamine™ 2000 (Invitrogen, cat. no. 11668-019) according to a slightly modified manufacturer's protocol as follows: Cells were plated in 24-well plates in antibiotics containing phenol red free medium at a density of 1.9×10^4 KYSE410 cells/well or 5×10^4 OE19 cells/well and allowed to attach for 24 or 48 h, respectively. At a confluency of 15–20%, antibiotics containing phenol red free medium were changed and cells were transfected with 20 pmol oligonucleotides using Opti-MEM® I medium (GIBCO® Invitrogen, no. 31985) to prepare oligomer–Lipofectamine™ 2000 complexes. The medium was replaced 24 h after transfection, cells were harvested 48 h after transfection, and the lysate was stored at −20°C. Three independent experiments were performed in triplicate. RNA from the triplicates was pooled for the determination of miRNA levels.

Assessment of Effect of Transfection on Sensitivity to Anticancer Drug Treatment

Cells were plated in six-well plates in antibiotics containing phenol red free medium at a density of 9.5×10^4 or 2×10^5 cells/well and allowed to attach for 24 or 48 h (KYSE410 or OE19). Transfection was then performed as described above using the same miRNA mimics and negative controls and applying 100 pmol oligonucleotides to each well. Twenty-four hours after transfection, cells were seeded onto 96-well plates and allowed to attach overnight. Chemotherapy agents were applied 48 h after transfection, and in vitro drug sensitivity assays were then performed as described above. Cells in the remaining pellet after re-plating were harvested for confirmation of successful transfection.

RNA Harvest and Isolation

Just prior to harvest, cells were examined under the microscope to rule out contamination or other anomalies. RNA/cell harvest was then performed by applying TRIzol® (Invitrogen Life Technologies, NY, USA) either directly to the well/flask (transfection experiments: 500 μL per 24-well plate; resistant cell lines: 3 mL per T25 flask) or to the remaining pellet after re-plating experimental groups onto 96-well plates. The lysate was then transferred to 1.5-mL tubes and stored at −20°C until extraction of total RNA was performed according to the manufacturer's protocol. The concentration of RNA was quantified by UV spectrophotometry (NanoDrop® ND-8000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, USA). RNA quality was determined by electrophoresis through a 1% agarose gel. All RNA samples were confirmed to be undegraded by visualization of distinct 28S and 18S rRNA species. The final RNA solution was stored at −20°C until required for cDNA synthesis.

RT-PCR and TaqMan® miRNA Assay

For the determination of miRNA levels, TaqMan® miRNA Assays (Applied Biosystems, Foster City, CA, USA) were

used. These assays detect only the mature form of the specific miRNAs. Assay IDs were as follows: hsa-miR-148a: ID 000470; hsa-miR-106a: ID 002169; RNU44: ID 001094. For each sample, 5 ng of total RNA was used for reverse transcription into cDNA. Following the manufacturer's protocol, we utilized 100 nM stem-loop RT primer, 100 mM dNTPs, 50 U/μL multiscribe reverse transcriptase, 20 U/μL RNase inhibitor, 1.5 μL 10× RT Buffer (all purchased from PE Applied Biosystems) and nuclease-free water. Incubation of reagents was performed in a thermocycler (Eppendorf Mastercycler, Eppendorf, North Ryde, NSW, Australia; protocol: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, then hold at 4°C). For real-time PCR, 5 μL of respective cDNA was mixed with 1 μL of gene-specific primers, 10 μL of Taqman® Universal PCR Mastermix (Applied Biosystems) and 4 μL of nuclease-free water. All samples were assayed in triplicate reactions using a Rotorgene 6000 thermocycler (Corbett Life Science, Sydney, NSW, Australia). Quantitative analysis was performed using Q-Gene software. MiRNA expression data were normalized to the expression levels of RNU44, which displayed comparable expression across the different groups (data not shown).

Statistical Analysis

The relative survival of resistant cell lines and mimic or negative control transfected cells, after treatment with anticancer drugs, was calculated by adjusting the mean corrected absorbance of the treated cells to the corresponding untreated controls (given in percent). For an assessment of the effect of transfection on sensitivity to chemotherapy drug treatment, the relative survival of the negative controls was then set to 0 and the effect of transfection was presented as relative survival of miRNA mimic-transfected groups compared to negative control-transfected groups (given in percent). Gene expression data for miR-106a and miR-148a were expressed as means of normalized expression with standard deviation. Data were assessed for statistical significance using one-way analysis of variance with post hoc testing/Student's t test for equal and unequal variances as appropriate. A value of $p < 0.05$ was considered to be statistically significant. All analyses were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL).

Results

miRNA Expression in Sensitive and Resistant Variants

The expression of miR-106a and miR-148a in the different cell lines is summarized in Fig. 1a, b. MiR-106a was significantly downregulated in 5-FU-resistant but not in cisplatin-resistant SCC cells compared to sensitive controls (relative miR-106a expression in sensitive SCC, 0.88 ± 0.06; cisplatin-resistant SCC, 0.85 ± 0.24; 5-FU-resistant SCC cells, 0.47 ± 0.13), and there was no difference in miR-106a expression between cisplatin-resistant (relative expression, 0.47 ± 0.02) and sensitive (relative expression, 0.56 ± 0.06) EAC cells. The relative expression of miR-148a was very low in our samples, and there was no statistically significant difference in levels between sensitive and resistant cell line variants (relative miR-148a-expression in SCC cells: sensitive vs. cisplatin-resistant vs. 5-FU-resistant cells, 0.0009 ± 0.0001 vs. 0.003 ± 0.001 vs. 0.001 ± 0.0001; relative miR-148a-expression in EAC cells: sensitive vs. cisplatin-resistant cells, 0.026 ± 0.008 vs. 0.016 ± 0.002).
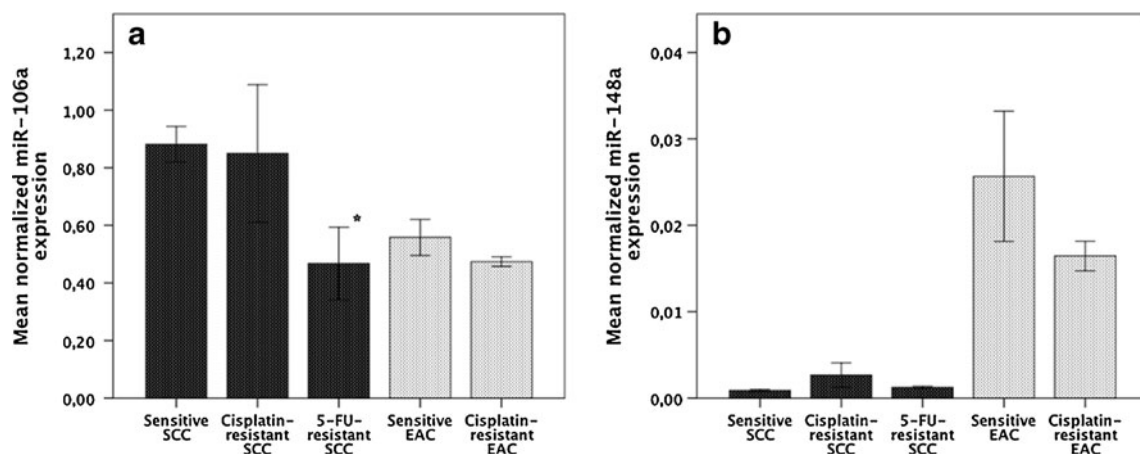

Fig. 1 Normalized expression of miR-106a (**a**) and miR-148a (**b**) in sensitive and resistant oesophageal squamous cell carcinoma and adenocarcinoma cell lines. SCC cell lines in *dark grey*, EAC cell lines in *light grey*. *Statistically significant compared to sensitive SCC ($p = 0.007$)

Transfection Experiments

Before testing the response to chemotherapy, pilot transfection experiments were performed to assess the level of overexpression of each miRNA after transfection. Forty-eight hours after transfection, PCR analysis demonstrated a successful increase in the levels of the transfected miRNAs (there were no significant differences in the increase of miRNA levels after transfection between the groups for either miRNA; Fig. 2a, b).

In the chemotherapy-sensitive maternal SCC and EAC cell lines, transfection with miR-106a did not affect chemotherapy treatment response, except in EAC cells where there was a slight increase in resistance to cisplatin (cell viability, compared to negative control, increased by $8.7 \pm 0.8\%$, $p = 0.003$)). In contrast, miR-148a overexpression resulted in an improved response to 5-FU and cisplatin treatment in both maternal cell lines. Whilst effects of 5-FU on treatment were of relatively low magnitude (cell viability compared to negative control: SCC, $-7.8 \pm 9.0\%$, $p = 0.273$; EAC, $-6.0 \pm 0.8\%$, $p = 0.006$), miR-148a transfection led to a marked increase in sensitivity to cisplatin in both cell lines (cell viability compared to negative control: SCC, $-50.5 \pm 10.6\%$, $p = 0.014$; EAC, $-22.6 \pm 7.9\%$, $p = 0.008$; see Fig. 3).

In most chemotherapy-resistant cell lines, miR-106a upregulation had no significant effect on 5-FU or cisplatin treatment, although cisplatin-resistant EAC cells had a slightly greater sensitivity to 5-FU after transfection (cell viability compared to negative control, $-6.4 \pm 2.5\%$, $p = 0.011$; see Fig. 4).

In contrast to miR-106a transfection, miR-148a transfection was followed by an improved response to anticancer treatment in four of six resistant cell lines. Sensitivity to 5-

FU was increased in cisplatin-resistant SCC (cell viability compared to negative control, $-15.0 \pm 4.1\%$, $p = 0.003$) and EAC cells (cell viability compared to negative control, $-10.9 \pm 2.1\%$, $p = 0.012$). Furthermore, the effect of increased miR-148a levels was again more pronounced with cisplatin treatment, with the decrease in cell viability being $-25.0 \pm 9.3\%$ (cisplatin-resistant SCC: $p = 0.009$) and $-30.6 \pm 6.4\%$ (5-FU-resistant SCC: $p = 0.014$) after treatment. MiR-148a did not have a sensitizing effect on cisplatin treatment in cisplatin-resistant EAC cells or on 5-FU treatment in 5-FU-resistant SCC cells (see Fig. 5).

Discussion

There is increasing evidence that the expression of miRNAs affects sensitivity to various chemotherapy agents across a broad variety of tumour types. Interestingly, so far, only a limited number of miRNAs (e.g. members of the let-7 family, miR-16, miR-21, or miR-451) had been confirmed to impact on more than one anticancer drug and/or to play a role in more than one tumour type, [33] and most importantly, little is known about synergies between these miRNAs in this context. Our study demonstrates, for the first time, an effect of miRNA modulation on sensitivity to anticancer treatment in oesophageal cancer. We have determined the effect of increasing the expression of miR-148a and miR-106a on sensitivity to cisplatin and 5-FU treatment in cisplatin- and 5-FU-sensitive and -resistant oesophageal cancer cell lines. In sensitive cells, transfection with miR-148a resulted in a marked increase in sensitivity to cisplatin in both oesophageal adenocarcinoma and squamous cell carcinoma cell lines, as well as a smaller but statistically significant increase in sensitivity to 5-FU treatment in
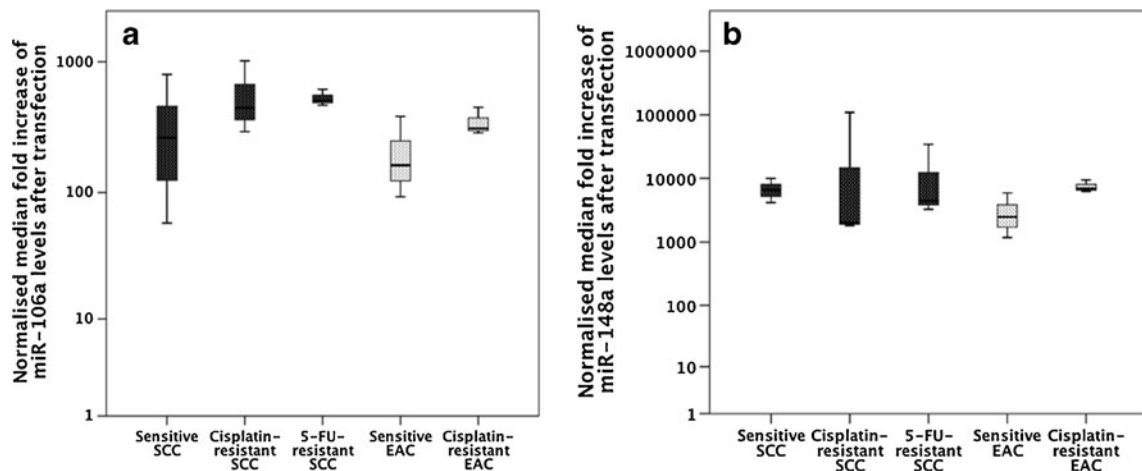

Fig. 2 Normalized median fold increase of miR-106a (**a**) and miR-148a levels (**b**) after transfection with the respective mimics. Scramble-transfected controls were set to 1 and the increase of miRNA levels of mimic-transfected groups was calculated as the ratio between expression in mimic and in scramble-transfected cells. SCC cell lines in *dark grey*; EAC cell lines in *light grey*

Fig. 3 Effect of transfection with miR-106a and miR-148a on sensitivity to 5-FU and cisplatin treatment in chemotherapy-sensitive squamous cell carcinoma and adenocarcinoma cell lines. Relative cell survival of negative control cells was set to 0 and the effect of transfection was presented as relative survival of transfected cells compared to negative control in percent. SCC cell lines in *dark grey*; EAC cell lines in *light grey*. *Statistically significant compared to respective negative controls (*p* values: see "Results")
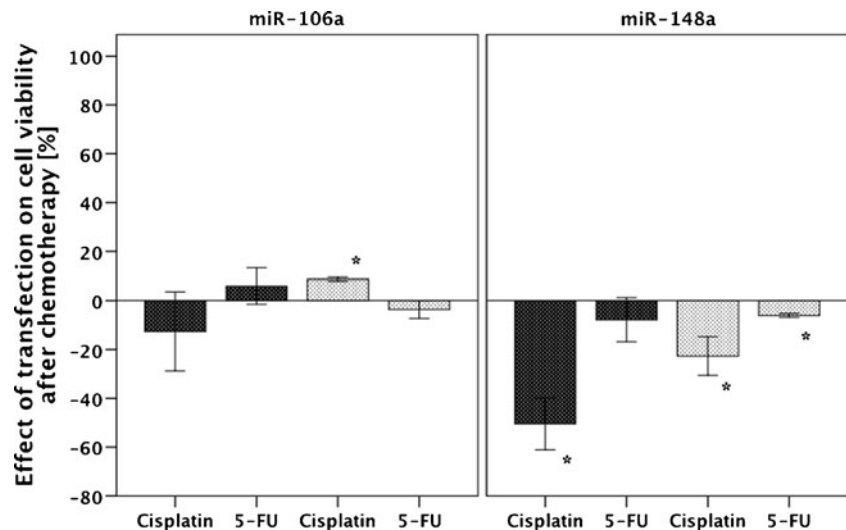
oesophageal adenocarcinoma cells. The upregulation of miR-106a, on the other hand, did not impact on treatment, except for a slight increase in resistance to cisplatin in oesophageal adenocarcinoma cells. In general, these results were replicated in the chemotherapy-resistant variants of both cell lines, with upregulation of miR-148a improving the response to treatment with 5-FU in cisplatin-resistant squamous cell carcinoma and adenocarcinoma cells even more than the response observed in the sensitive cell lines. Only in the 5-FU-resistant squamous cell carcinoma cell line did the effect of miR-148a transfection fail to reach significance (*p* = 0.117). The distinct effect of miR-148a transfection on cisplatin treatment was also observed for cisplatin- and 5-FU-resistant variants of the squamous cell carcinoma cell line, but not in the cisplatin-resistant oesophageal adenocarcinoma cells. MiR-106a transfection showed, except a slight improvement of sensitivity to 5-FU

in cisplatin-resistant EAC cells, no effect on cisplatin or 5-FU treatment in resistant variants.

Previous work investigating the role of miR-106a and its impact on sensitivity to anticancer medications has been conflicting. On the one hand, miR-106a expression has been shown to be reduced with increasing resistance to anticancer drug treatments in ovarian and multidrug-resistant gastric cancer cell lines.[27,28] Kovalchuk et al.[26], on the other hand, found an opposite effect, with miR-106a being upregulated in doxorubicin-resistant breast cancer cells. However, we could not confirm different expression patterns of miR-106a in most of the resistant cell lines we generated (except the 5-FU-resistant SCC variant) compared to chemotherapy-sensitive controls, and varying its expression had little impact on cisplatin and 5-FU chemotherapy treatment in the oesophageal cancer cell lines we evaluated. We are unable to explain why transfection resulted in lower levels of

Fig. 4 Effect of transfection with miR-106a on sensitivity to chemotherapy treatment with cisplatin and 5-fluorouracil in resistant oesophageal squamous cell carcinoma and adenocarcinoma cell lines. Relative cell survival of negative control cells was set to 0 and the effect of transfection was presented as relative survival of transfected cells compared to negative controls in percent. SCC cell lines in *dark grey*; EAC cell lines in *light grey*. *Statistically significant compared to respective negative controls (*p* values: see "Results")
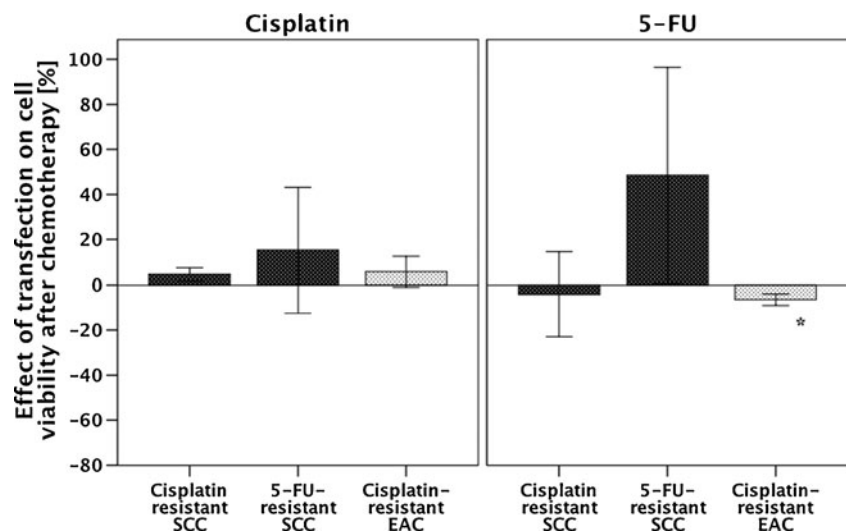
Fig. 5 Effect of transfection with miR-148a on sensitivity to chemotherapy treatment with cisplatin and 5-fluorouracil in chemotherapy-resistant oesophageal squamous cell carcinoma and adenocarcinoma cell lines. Relative cell survival of negative control cells was set to 0 and effect of transfection was presented as relative survival of transfected cells compared to negative controls in percent. SCC cell lines in *dark grey*; EAC cell lines in *light grey*. *Statistically significant compared to respective negative controls (*p* values: see "Results")
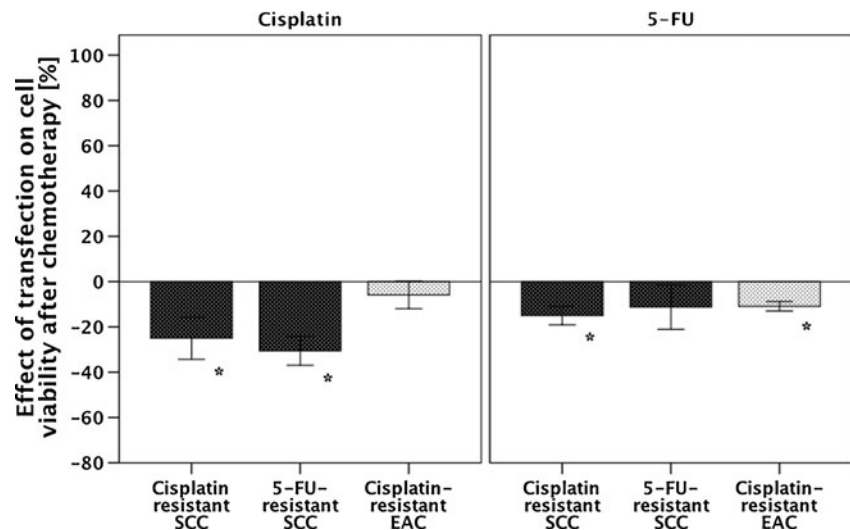
miR-106a expression compared with miR-148a expression, although this could be due to a difference in transfection efficiency between the two mimic molecules. In this context, however, the fact that miR-106a levels did not increase after transfection to the same extent as miR-148a levels (see Fig. 2a, b) is unlikely to affect the relevance of our findings for the following reason: miR-106a was found to be 2.3-fold upregulated in doxorubicin-resistant breast cancer cells,[26] more than 2-fold downregulated in multidrug-resistant gastric cancer cells,[28] and 1.9-fold downregulated in our own 5-FU-resistant SCC cell line. We observed a median increase of miR-106a levels of at least 162-fold, which is far higher than these reported pathological variations, and it is therefore reasonable to expect that the miR-106a levels obtained in our experimental regime are high enough to detect any possible effect on chemosensitivity.

In contrast, our results regarding miR-148a fit well with current knowledge about this miRNA. MiR-148a is considered an anti-oncogenic miRNA.[34,35] It has been shown that this miRNA is downregulated in a variety of human tumours and that its expression negatively affects tumour growth, cell motility, invasion, migration and metastasis.[36] In accordance with these findings, previous array data from our lab suggest that miR-148a is downregulated in EAC compared to its precursor lesion, Barrett's oesophagus.[37] From the current data, however, we cannot conclude that miR-148a is further downregulated in our resistant variants when compared to the chemotherapy-sensitive tumour cell lines. Nevertheless, our experiments have shown a consistent improvement in response to cisplatin and 5-FU treatment in most chemotherapy-sensitive and -resistant oesophageal adenocarcinoma and squamous cell carcinoma cell lines after miR-148a overexpression.

The discrepancy between the sensitizing effect of miR-148a and the lack of a miR-106a effect in our study (or desensitizing effect in the case of cisplatin and the sensitive EAC cell line) is somewhat surprising. As we chose these miRNAs based on our previous findings of an inverse association between clinical signs of more aggressive tumours vs. miRNA expression, we expected a positive result for both miRNAs (at least in SCC). However, in our previous study, only miR-148a was also associated with tumour staging parameters in EAC. Furthermore, the literature supports roles for miR-106a both as a tumour-promoting miRNA and a tumour-suppressive miRNA, depending on the context.[32] It is also possible that other miRNAs, not assessed in this study, may impact upon the roles of miR-106a and miR-148a in resistance to chemotherapy and that these may differ between squamous carcinoma cell and adenocarcinoma cell lines. Overall, we conclude from our results that in this context, miR-148a is a more powerful tumour suppressor and affects anticancer treatment to a greater extent.

In a clinical context, our data suggest a possible application for miR-148a as a "supplement" to conventional chemotherapy. Applied together with cisplatin and 5-fluorouracil in patients with chemotherapy-sensitive tumours, miR-148a could allow a reduction of both agents whilst providing the same therapeutic effect. With this reduction, side effects of chemotherapy might be lowered. Furthermore, this might increase the overall response rate to chemotherapy, as in the case of therapy-resistant tumours, i.e. the effect of treatment could be restored by overcoming the resistance of the malignancy toward either of the drugs. However, our results are very preliminary and a lot more work will need to be done before any clinical application can be considered.

Our data are supported by recent work from Japan. Whilst preparing our paper for publication, Fujita et al.[36] published first evidence that miR-148a upregulation enhances sensitivity to pacitaxel treatment in pacitaxel-sensitive

and -resistant prostate cancer cell lines. Therefore, when considered with our results for oesophageal cancer cells, it seems reasonable to conclude that miR-148a plays an important role in the cellular response to various chemotherapeutic agents including cisplatin, 5-FU and pacitaxel. In this context, the most reasonable explanation for the smaller effect of miR-148a transfection on 5-FU treatment in our study might lie in the differences in mechanism of action between cisplatin and 5-FU. Whilst cisplatin has cytostatic and cytotoxic effects, 5-FU presents mainly cytostatic properties. The relative cell survival after chemotherapy might therefore be affected earlier by cisplatin than by 5-FU treatment, and our assessment 72 h after induction of chemotherapy might underrepresent the impact of miR-148a expression on 5-FU therapy.

Other published studies reveal several interesting downstream targets for miR-148a, which might explain the observed improvement in sensitivity to anticancer treatment. First, the study of Fujita et al.[36] demonstrated that miR-148a directly targets mitogen- and stress-activated kinase 1 (MSK1). MSK1 knockdown was shown to reduce resistance to paclitaxel in their experiments, indicating that miR-148a acts, at least in part, via the regulation of MSK1 expression. Second, miR-148a-mediated modulation of response to chemotherapy might partly be explained by the regulation of de novo DNA methylation (miR-148a targets include DNA methyltransferase 3B (DNMT3B)[38,39] and DNA methyltransferase-1 (DNMT-1) or regulating the expression of methylation-dependent tumour suppressor genes.[40] Another interesting target for miR-148a is the pregnane X receptor (PXR). PXR is a nuclear receptor that belongs to the family of ligand-activated transcription factors and can be activated by a large number of compounds. This receptor upregulates several important drug-metabolizing enzymes or drug efflux transporters, including CYP3A4, MDR1 (P-gp) and MRP3, which consequently leads to enhanced biotransformation and/or clearance of drugs.[41,42] Therefore, PXR is believed to be "a novel master regulator of multidrug resistance in cancers,"[43] and elevated PXR expression is associated with resistance to anticancer drug treatment in several cancers, including prostate and colorectal cancer.[42,44] Takagi and colleagues[45] were the first to demonstrate that PXR is directly targeted by miR-148a and that the miR-148a-dependent decrease of PXR protein attenuated the induction CYP3A4 mRNA. One known substrate of CYP3A4 is cisplatin,[41] and overexpression of MDR1 with consequent elevated expression of its product P-gp has been shown to result in an increased efflux of, for example, 5-FU in malignant cells.[46]

There are limitations which should be considered when interpreting the results of our study. The most important limitation is the restriction to only two oesophageal cancer cell lines (OE19 and KYSE410, respectively). The reason behind this restriction was the hypothesis that the examination of sensitive and derivative, resistant, cells provides more crucial information about the effect of miRNA modification on response to anticancer treatment than the inclusion of multiple sensitive cell lines only. Only about 20–40% of patients with oesophageal cancer present a major response after neoadjuvant treatment (i.e. sensitive tumours), and only these patients benefit from treatment.[4,5] Hence, we chose to focus on a model representing those patients who do not achieve a complete response to neoadjuvant treatment (i.e. chemotherapy-resistant cells). Even though we evaluated only two oesophageal cells lines, the consistent miR-148a-mediated enhancement of chemotherapy response in both the original cell lines, and their chemotherapy-resistant derivatives, suggests that common mechanisms may be conserved in the two different tumour types. Whilst this requires further verification, both in other oesophageal cell lines and in vivo, it provides a foundation for understanding such mechanisms in oesophageal cancer. It also identifies the need for broader investigative studies that may identify other regulatory pathways which distinguish responses in squamous- and adenocarcinoma-derived cells.

Furthermore, our current study did not include a thorough validation of possible gene expression targets for miR-148a influencing resistance to chemotherapy in our oesophageal cancer cell lines. This was not one of the aims of our study as we were primarily interested in whether the reported effect of miR-148a on chemosensitivity in other tumour types was applicable in oesophageal cancer types, and our study has shown such an effect. However, elucidation of the mechanisms behind the effect of miR-148a, and whether the same mechanisms apply across all tumour types, is an important question for future studies.

There are two major options for establishing chemotherapy-resistant cell lines: pulsatile treatment with constant doses vs. continuous application of drugs with increasing doses. The mechanism of resistance development might differ between these two approaches. As pulsatile treatment might be a better approximation of the clinical situation, we chose this technique. However, pulsatile treatment is usually applied for very short periods (3–24 h) and uses high concentrations of drugs.[47] In our current study, we tried to imitate the clinical situation of cisplatin and 5-FU application in patients with oesophageal cancer more precisely by a 3- to 4-day exposure to the drugs. In order to prevent total cell death during this exposure time, we had to use 5-FU and cisplatin doses which corresponded to the lower limit of clinically relevant doses. Therefore, the resistance development under these conditions might slightly differ from the clinical situation. However, we were able to show that the cell lines generated do have resistance to these chemotherapy agents.

Unfortunately, we were unable to establish a 5-FU-resistant variant of the oesophageal adenocarcinoma cell line, OE19, due to technical problems. This missing cell line might inform further on the observed impact of miR-148a upregulation especially on 5-FU treatment. As the cisplatin-resistant variant of OE19 did not respond to miR-148a transfection with the expected increase in sensitivity to anticancer treatment, it would be very interesting to see if this also occurs in 5-FU-resistant cells. However, despite the limitations inherent in our study, it does provide the first good evidence that miRNAs provide a very promising target for new therapeutic strategies to support and improve existing anticancer treatments in oesophageal cancer patients.

In conclusion, we have shown for the first time that miR-148a upregulation sensitizes chemotherapy-resistant variants of both oesophageal adenocarcinoma and squamous cell carcinoma cell lines, to cisplatin and 5-FU in vitro, and further improves sensitivity in the corresponding chemotherapy-sensitive maternal cell lines. A review of the literature highlighted MSK1, de novo DNA methylation and PXR as potential mediators of these observations. These findings provide a basis for future studies to determine the altered chemotherapy response in other oesophageal lines following miR-148a administration. They also highlight a need to determine which pathways, affected by miR-148a in oesophageal adenocarcinoma and squamous cell carcinoma, modulate response to chemotherapy, and clinical studies using human tissue samples are required to confirm that this miRNA plays an important role in chemotherapy resistance in oesophageal cancer in vivo. Although therapeutic delivery of miRNAs is still a developing field, and there is much more work to be done before these molecules can be securely applied in clinical settings, miR-148a may one day have a therapeutic application in patients undergoing chemotherapy for oesophageal cancer.

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