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microRNAs: Key regulators of chemotherapy response and metastatic potential via complex control of target pathways in esophageal adenocarcinoma

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Title
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ABSTRACT

Introduction

Incidence of esophageal adenocarcinoma (EAC) increased significantly over the last decades. Lack of response to chemotherapy is a major problem in the treatment of this disease. This study aims to assess the biological relevance of characteristic microRNA profiles of chemotherapy resistant EAC cells with regards to response to chemotherapy and biological behavior.

Methods

We selected 3 microRNAs from characteristic microRNA profiles of resistant EAC (miR-27b-3p, miR-200b-3p, and miR-148a-3p). Expression of microRNAs was modified in 6 EAC cell lines. Effects on chemotherapy, adhesion, migration, apoptosis and cell cycle were assessed using standard assays. Target analyses were performed using Western Blot and Luciferase techniques.

Results

MiR-27b-3p significantly sensitized cells to 5FU and Cisplatin in 83% respectively in 33% of cell lines, miR-148a-3p in 67% respectively 33% of cases. MiR-200b-3p increased sensitivity only towards 5FU in 50% of cases. Co-transfections with miR-27b-3p/miR-148a-3p showed an additive effect on response to chemotherapy in 50% of cases. Upregulation of miR-148a-3p reduced protein expression levels of DNMT-1, MSK-1, Bcl-2 and Bim, and miR-27b upregulation led to downregulation of Sp1 and PPARy proteins implicating a potential negative post-transcriptional control via the respective microRNAs. Finally, we were able to confirm Bcl-2 for the first time as direct target of miR-148a-3p in EAC.

Conclusion
This study demonstrates that specific microRNA profiles of chemotherapy resistant EAC in fact determine their response to chemotherapy and biological behavior. Our data further show that microRNA-mediated regulation of chemotherapy resistance is complex, and several microRNAs seem to “co-operate” at various steps within a broad number of pathways what fits very well to our recently proposed understanding of microRNA-mediated regulation as function of cellular functional complexes. These data highlight the promising potential of microRNAs to predict or monitor treatment response to chemotherapy in EAC, and to potentially modulate tumor biology in a therapeutic approach.

**Keywords**

microRNA, chemotherapy, resistance, esophageal adenocarcinoma, metastasis
Abbreviations:

Bcl-2: B-cell lymphoma 2
BIM: Bcl-2-like protein 11
DNMT1: DNA-(cytosine-)-methyltransferase 1
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen
EAC Esophageal adenocarcinoma
ERK: Extracellular signal-regulated kinases;
ESCC: Esophageal squamous cell carcinoma
JNK: c-Jun N-terminal kinase
LIMK1: LIM domain kinase 1.
MEKK: Mitogen-activated protein kinase kinase
MiRs: microRNAs
MiRNA microRNA
MSK-1: Mitogen- and stress-activated protein kinase-1
PPARγ: Peroxisome proliferator-activated receptor gamma
Raf: Rapidly accelerated fibrosarcoma
Sp1: Specificity protein 1
Spry2: Sprouty homolog 2
TRAIL: TNF-related apoptosis-inducing ligand
TS: Thymidylate synthase
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Conflict of interest statement

There are no competing financial interests in relation to the work described for any of the authors.
1. **INTRODUCTION**

Esophageal cancer (EC) is the eighth most common cancer worldwide [1-3]. Predominant histological subtypes include squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) [4]. Over the past decades, the incidence of EAC has increased significantly, up to 6-fold, in most parts of the Western World [5]. Despite recent improvements in diagnostics and therapy - including better perioperative management or neoadjuvant chemo- or radiotherapy, general outcome of EAC patients remains very poor with an overall 5-year survival of approximately 20% [2]. This is mainly caused by a frequent delay in diagnosis due to the lack of early clinical symptoms. The most commonly used chemotherapy for EAC includes Cisplatin in combination with 5-fluorouracil (5-FU) [6]. Unfortunately, response rates in EAC are reported to be as low as 19% due to development of (multi-) drug resistance [2]. To date, there are no biomarkers available that can predict tumor response to chemotherapy in EAC. The availability of such markers might help to identify patients who would profit from neoadjuvant treatment.

Multidrug resistance (MDR) is a major obstacle in the context of successful chemotherapy in cancer. MDR can be caused by a number of molecular mechanisms [7], including epigenetic regulation via microRNAs. MicroRNAs (miRNAs) are small non-coding, single-stranded, endogenous RNA molecules, which are approximately 21-25 ribonucleotides long and highly conserved in evolution [8]. MiRNAs control many fundamental cellular processes by silencing target gene expression via posttranscriptional inhibition of translation via miRNA-mRNA interactions [9-11]. Today, there is no doubt that miRNAs exert key roles in cancer initiation and progression. They have been also demonstrated to impact on both the ability to develop metastases via dysregulation of cell proliferation, migration, adhesion or apoptosis, and on MDR [12-18]. However, only few studies have investigated potential associations between miRNA dysregulation and resistance towards chemotherapy in EC [6,19-23, 24,25].
In this context, we showed with our previous work that chemotherapy resistant EAC tumor cell lines present characteristic miRNA profiles that discriminate them from chemotherapy sensitive cells [6,23]. With this current study, we now aimed to thoroughly assess the biological relevance of this characteristic miRNA expression profile of chemotherapy resistant EAC cells. In detail, we selected three miRNAs from these profiles and analyzed their impact on sensitivity to chemotherapy. Chemoresistant tumors often present a more aggressive behavior such as increased potential to metastasize [26,27]. Therefore, we also investigated the impact of these miRNA candidates on adhesion, migration and cell cycle control. Finally, we included target gene analyses to elucidate downstream pathways that are controlled by these miRNAs.
2. MATERIAL AND METHODS

2.1 Cell culture

Six human EAC cell lines (OE-19 and OE-33 purchased from DSMZ, Germany; Flo-1, SKGT4, OACM5.1 and OACP4C which were a gift of Prof. Dinjens / Erasmus MC / Netherlands) were selected for experiments and were cultured with standard medium (RPMI (GIBCO® Invitrogen, Germany, #11875), respectively DMEM (Lonza Group, Switzerland, #BE12-741F) for Flo-1 containing 1% L-Glutamine (Lonza Group, Switzerland, #17-605C) and 10% FCS (GIBCO® Invitrogen, Germany, #26140) without antibiotics in a humidified atmosphere containing 5% CO2 at 37° C as already described [28,29].

2.2 Selection of miRNAs from the characteristic profile of chemotherapy resistant EAC cells

Based on preliminary data, we selected miR-27b-3p and miR-200b-3p from the characteristic profiles of chemotherapy resistant EAC cells for this work [23]. In addition, we included miR-148a-3p into this study as our previous work showed that downregulation of miR-148a-3p leads to a sensitizing effect towards chemotherapy in sensitive and Cisplatin-resistant EAC cells [6].

2.3 Transient transfection protocols for single or co-transfection experiments

For transfection experiments, expression levels of the three selected miRNAs (miR-27b-3p (#MSY0000419), miR-148a-3p (#MSY0000243) and miR-200b-3p (#MSY0000318) (Qiagen, Germany) were upregulated in order to test their ability to modulate chemotherapy response. Six EAC cell lines (OE-19, OE-33, SKGT4, Flo-1, OACM5.1 and OACP4C) were transiently transfected using mentioned miRNAs and Lipofectamine™2000 transfection agent (Life Technologies, Carlsbad, CA, USA, #11668027)
according to a modified manufacturer’s protocol. Cells were plated in 6-well plates in culture medium at a cell-specific density (Supplement A) and allowed to attach for 24 h.

For single transfection experiments, medium was changed at a confluence of 50-60% to Opti-MEM® Reduced Serum Media (Invitrogen, Carlsbad, CA, USA, #11058-021), and cells were transfected for 24 h with 20 pm oligonucleotides using Opti-MEM® to prepare oligomer-Lipofectamine™2000 complexes. Medium was replaced 24 h after transfection. The negative scramble-control was selected to contain no homology to human gene sequences and miRNAs (Qiagen, Germany).

For co-transfection experiments, optimal oligonucleotide and Lipofectamine™2000 concentrations were tested in preliminary experiments in order to achieve similar miRNA expression levels after co-transfection as seen after single transfection of the respective miRNAs. In summary, 10 pm oligonucleotides were used per miRNA (Supplement A). For formation of the oligonucleotide-Lipofectamine complexes, transfection of both miRNAs was performed simultaneously. Medium was replaced 24 h after transfection.

2.4 RNA extraction and qRT-PCR

Success of transfection was confirmed with qRT-PCR using miScript PCR system (Qiagen, Germany, #218161). MiScript assays were performed according to the manufacturer’s instructions. Briefly, for each sample, 500 ng of DNase pre-treated RNA was used for reverse transcription into cDNA. Following the manufacturer’s protocol, we utilized 4 μl miScript 5X RT Buffer, 1 μl Reverse Transcriptase and 5 μl nuclease-free water. Incubation of reagents was performed in a thermocycler (protocol: 60 min at 37°C, 5 min at 95°C, then a hold at 4°C). For real-time PCR (using miScript Sybr Green PCR Kit, Qiagen, Germany, #204145), 2 μl of cDNA was mixed with 10 μl QuantiTect SYBR Green, 2 μl 10X miScript Universal Primer, 2 μl gene specific 10x miScript Primer Assay, and 4 μl nuclease-free water. All samples were assayed in triplicate reactions using a BioRad CFX 384 Real-Time System (Hercules, CA, USA).
Quantitative analysis was performed using Bio-Rad CFX Manager 2.1. MiRNA expression data of miR-27b-3p (hs_miR-27b_2; MS00031668), miR-148a-3p (hs_miR-148a_1; #MS00003556) and miR-200b-3p (hs_miR-200b_3; MS00009016) were normalized to the expression levels of SNORD25 (hs_SNORD25_11; #MS00014007), RNU6b (Hs_RNU6-2_11; #MS00033740) and SNORD68 (hs_SNORD68_11; #MS00033712), which displayed comparable expression across the different groups (data not shown).

2.5 Chemotherapy treatment

For chemotherapy treatment, transfected cells were seeded 24 h post-transfection onto 96-well plates in a cell type specific concentration (Supplement B) and allowed to attach for 24 h in order to reach a confluence of 50%. Then, chemotherapeutics (Cisplatin (TEVA GmbH, Germany) or 5FU (medac GmbH, United Kingdom)) were prepared at the respective IC50 doses for each cell line (identified in previous experiments; Supplement B) and added to the corresponding cells. After 72 hours exposure, viability assays were performed using Thiazolyl Blue Tetrazolonium Bromide (MTT, Sigma-Aldrich, St. Louis, Missouri, USA, #M2128-1G). Cell culture medium was removed and 100 µL MTT solution (1 mg/mL MTT in cell culture medium) was added per well. After two hours, the supernatant was removed and the MTT formazan crystals were solubilized in 100 µL/well 2-Propanol (AppliChem, Germany). Finally, the absorbance was measured (570 nm) on the spectrophotometer Dynatech MR5000 using the software MikroWin2000. One experiment was performed with 4 technical replicates, and ten experiments were repeated independently.

2.6 Adhesion and wound healing assays
EAC cell lines OE-19 and SKGT4 were used to analyze the effect of miRNA manipulation on metastatic potential. 48 hours after transfection, cells were trypsinized and underwent a 60 minutes reconstitution period using serum free medium containing 1% albumin.

For adhesion assay, cells were then transferred to 96-well plates (30,000 cells/well) coated with extracellular matrix (ECM) components type I collagen (2.5 µM; Sigma-Aldrich, St. Louis, Missouri, USA, #C8919-20ML) and fibronectin (50 nM; PromoCell, Germany, #C-43050). Cells were plated under the stimulation of 10% fetal bovine serum, and adhesion was assessed after 30, 60, and 90 minutes. After incubation, cells were fixed for 15 minutes with formalin, stained for 15 minutes using crystal violet (0.1%) and quantified by photospectrometer at 570nm. One experiment was performed with 4 technical replicates, and confirmed with five independent experiments.

For wound healing assays, cells were plated in 6-wells using 35 mm IBIDI-inserts (30,000 cells per insert-side; IBIDI, Germany, #80209). Inserts were removed after 24 hours incubation, cells were washed twice with PBS, culture medium was added and a photo was taken at time-point zero. A second photo was taken at 48 hours for OE-19 and 8 hours for SKGT4 after start of the experiment. Rate of migrated cells were determined using ImageJ. 6 experiments were repeated independently.

2.7 Apoptosis and cell cycle analysis

EAC cell lines OE-19 and SKGT4 were used to analyze the effect of miRNA manipulation on apoptosis and cell cycle control. Cells were pelleted 48 h after transfection and washed twice with PBS. Supernatant and wash solutions were collected as well. Experiments were performed in 6 independent replicates.

For apoptosis assays, transfected cells were resuspended in 100 µL Annexin V/7AAD-solution (BioLegend, United Kingdom, #640905) according to manufacturer´s instructions. Cells were analyzed via flow cytometry using Beckman Coulter FC500.
For cell cycle analysis, transfected cells were fixed in 70% ice-cold ethanol at room temperature for 30 minutes. Cells were washed with FACS buffer (0.2% albumin and 0.01% NaN3 in PBS) and resuspended in 300 µL FACS buffer. The samples were treated with 1 µL of 40 µg/mL RNase A for 30 minutes at 37°C and stained with 5 µL propidium iodide (7.5 mmol/L; Carl Roth, Germany, #CN74.1) for 5 minutes. The cell cycle analysis was carried out via flow cytometry using Beckman Coulter FC500 at 488 nm.

2.8 Western blot

Target gene expression analysis on protein levels was performed using Western Blot techniques. Based on in-silico approaches (TargetScan), a number of potential targets of miR-148a-3p and miR-27b-3p were selected. In this context, we focused on targets that were reported to influence metastatic potential and chemotherapy response in other tumors. In detail, protein levels for B-cell lymphoma 2 (Bcl-2) [30,31], Bim [32], mitogen and stress activated protein kinase 1 (MSK-1) [33] and DNA (cytosine-5)-methyltransferase 1 (DNMT-19) [34] for miR-148a-3p and protein levels for Peroxisome proliferator-activated receptor gamma (PPARγ), specificity protein 1 (Sp1) [35], Sprouty homolog 2 (Spry2) [36], and LIM domain kinase 1 (LIMK1) [37] for miR-27b-3p were analyzed.

Cells were collected 48 h after transfection and lysed in RIPA buffer containing protease inhibitor cocktail (100:1). Samples were shredded with 7 mm steel beads at 50 Hz for 2 x 2 minutes. Cell suspension was centrifuged at 14,000 g for 45 min at 4°C. The supernatant contained total protein. Protein concentrations were measured via photospectrometer using the Bradford method (Thermo Fisher, Germany, #23227). 15-35 µg protein was loaded onto 6-10% SDS denaturating polyacrylamide gel. Primary antibodies of anti-Bcl-2 (BD Bioscience, Germany, #551097), anti-Bim (BD Bioscience, Germany, #559685), anti-MSK-1 (Biomol, Germany, #A302-747A-T), anti-DNMT1 (Cell signaling, United Kingdom, #5119), anti-PPARγ (Cell signalling, United Kingdom, #2435), anti-Sp1 (BioLegend, United Kingdom,
#624801), anti-Spry2 (Cell signaling, United Kingdom, #13264) and anti-LIMK1 (Cell signaling, United Kingdom, #3842) were used.

Proteins were processed with an ECL Western blotting detection system. To determine the relative concentration of stained protein, staining intensity was measured using imageJ. Protein concentrations were normalized to the tubulin-β-3 (BioLegend, United Kingdom, #657401) signal of scramble-transfected cells.

2.9 Luciferase assay

Subcloning Efficiency™ DH5α™ Competent bacteria (Invitrogen, Germany) were transformed according to manufacturer’s instructions. Transformed bacteria were cultured in LB-medium overnight containing 1x ampicillin to amplify plasmids of interests. Plasmids were isolated from overnight cultures using the Zyppy Plasmid Isolation Kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer’s recommendations.

MiRNA control or miRNA precursor molecules were co-transfected with luciferase vectors in EAC cell lines (SKGT4 and OE-19). Cells were seeded into white 96-well plates at a density of 70-80% at day of transfection. 20 nmol miRNA mimics, 100 ng of the reporter vector (firefly luciferase) and 0,06 µg Renilla luciferase vector (Promega, Germany) were co-transfected by transient transfection via Lipofectamine2000. Luciferase activity was measured 24 hours after transfection with the Dual-Glo Luciferase Assay System (Promega, Germany). Activity of firefly and Renilla was measured in cells transfected without miRNA as internal control. 4 experiments were performed independently with 4 technical replicates each.

2.10 Statistical analysis
All data are presented as means ± standard deviation unless otherwise stated. The relative cell survival rate after transfection and chemotherapy treatment was calculated by normalizing the mean corrected absorbance of the treated cells to the corresponding untreated controls (given in %). For assessment of the effect of miRNA transfection on sensitivity to chemotherapy, the relative survival of the transfected cells was given in %, related to the survival of negative controls, which was set to the value “0”. Data were assessed for statistical significance using parametric tests (Student’s t-test for equal and unequal variances) and non-parametric tests (Mann-Whitney U test) as appropriate. P <0.05 was considered to be statistically significant. All analyses were performed using SPSS 21.0 (SPSS, Chicago, IL).
3. **RESULTS**

3.1 Characteristic miRNA expression profiles in resistant EAC cell lines impact on sensitivity towards chemotherapy

Based on our previous results showing that resistant EAC cell lines present characteristic miRNA expression profiles, we investigated effects of upregulation of miR-27b-3p, miR-148a-3p and miR-200b-3p on sensitivity towards 5FU and Cisplatin treatment. Upregulation of all three miRNAs had a significant impact on sensitivity towards 5FU. In detail, increased miR-27b-3p levels led to increased sensitivity towards 5FU in five out of six cell lines (p≤0.042), higher miR-148a-3p levels sensitized four out of six cell lines towards 5FU (p≤0.030), and upregulation of miR-200b increased sensitivity towards 5FU in three out of six cell lines (p≤0.002) (Figure 1). The effect of miRNA upregulation on sensitivity towards Cisplatin was less prominent. Higher expression levels of miR-148a-3p and miR-27b-3p led to increased sensitivity towards Cisplatin in two out of six cell lines (p≤0.028), and miR-200b-3p had no effect at all on chemotherapy response towards Cisplatin.

**Figure 1.** Characteristic miRNA expression profiles in resistant EAC cell lines impact on sensitivity towards chemotherapy.

Response to chemotherapy after manipulation of miRNA expression levels in six EAC cell lines for (A) miR-27b-3p mimic, (B) miR-148a-3p mimic and (C) miR-200b-3p. Cells were treated with chemotherapy
at the respective LD50 dose 24 hours after transfection with mimics of miR-27b-3p, miR-148a-3p or miR-200b-3p, and relative cell survival in relation to control cells was measured 72 hours after treatment with Cisplatin (Cis) or 5FU. * p<0.05; control = 0%.

3.2 Co-transfections with miR-27b-3p/miR-148a-3p impact significantly on chemosensitivity in EAC

In order to investigate if the selected miRNAs “co-operate” in the regulation of chemotherapy, we established a model of co-transfection with simultaneous upregulation of expression levels of 2 miRNAs in the same experiment. We then compared the effect of co-transfection with miR-27b-3p/miR-148a-3p to the single transfections in two cell lines, as these two miRNAs increased sensitivity towards 5-FU in more than 50% of tested cell lines.

Co-transfection with miR-27b-3p/miR-148a-3p mimics significantly increased sensitivity towards Cisplatin in both cell lines (p≤0.001), and towards 5FU in one out of two cell lines (p≤0.015), compared to scramble transfected controls. Compared to single transfected cells, co-transfection significantly increased sensitivity towards Cisplatin or 5FU each in one out of two cell lines with a decrease in cell survival after transfection at around 3-21% (Figure 2).

Figure 2. Impact of co-transfections with two miRNAs on chemosensitivity in EAC

Effect of co-transfection and single transfection with miR-27b-3p mimic and/or miR-148a-3p mimic on chemotherapy response in OE-19 cells and SKGT4 cells. * p<0.05; M = mimic; control = 0%.
3.3 Chemotherapy resistance relevant miRNAs impact on metastatic potential of EAC

In a next step, we then investigated the effect of the selected miRNAs on two main mechanisms of metastasis (cell adhesion and cell migration), as well as on apoptosis and cell cycle checkpoints, which are all important features of chemoresistant cells.

Cell adhesion was significantly affected by upregulation of all three miRNAs. In five out of six experiments, early and/or intermediate adhesion was significantly reduced by upregulation of miR-27b-3p, miR-200b-3p or miR-148a-3p. MiR-148a-3p and miR-200b-3p led further to increased late adhesion in one cell line each (Figure 3A). With regards to migration, only upregulation of miR-27b-3p inhibited migration significantly. MiR-148a-3p and miR-200b-3p on the other hand led to increased migration in one out of two cell lines (Figure 3B).

Figure 3. Analysis on the effect of chemotherapy resistance relevant miRNAs on biological behavior of EAC: adhesion and migration

Effect of miRNA modulation on migration (A) and adhesion (B) in EAC cell lines OE-19 and SKGT4. Relative migration and adhesion ratio compared to control was measured with crystal violet staining 48 hours after transfection. * p<0.05; M = mimic; I = inhibitor; control = 0% (B).

3.4 Specific miRNA signatures of resistant cell lines impact on apoptosis and cell cycle in EAC

Analysis of apoptosis after transfection showed that both miR-148a-3p and miR-200b-3p increased apoptosis by about 50-97% in one out of two cell lines while reducing apoptosis in the other cell line (Figure 4A). MiR-27b-3p on the other hand significantly reduced apoptosis in both cell lines (-20% to -42%). Cell cycle analysis showed that upregulation of miR-148a-3p resulted in a cell cycle arrest in S phase in only one out of two cell lines (Figure 4B). MiR-27b-3p resulted in cell cycle arrest (in G1...
respectively G2 phases) in both cell lines. MiR-200b-3p overexpression led to G1 arrest in OE-19 cells and to a S-cell arrest in SKGT4.

Figure 4. Analysis on the effect of chemotherapy resistance relevant miRNAs on biological behavior of EAC: apoptosis and cell cycle control

Effect of miRNA manipulation on apoptosis (A) and cell cycle control (B) in EAC cell lines OE-19 and SKGT4. (A) Relative late and early apoptosis rate in OE19 and SKGT4 cells, (B) effect of miRNA transfection of cell cycle. Relative apoptosis rate and cell cycle ratio compared to control was measured with 7AAD/Annexin-FITC respectively PI staining 48 hours after transfection. * p<0.05; M = mimic; I = inhibitor.

3.5 Analysis of expression of potential target genes and downstream pathways

In a next step, we selected potential targets for miR-148a-3p and miR-27b-3p and investigated their protein expression levels after transfection via Western Blotting. In this context, we focused on targets that were reported to influence cell survival and chemotherapy response (see above).

We found a downregulation of DNMT-1, MSK-1, Bcl-2 and Bim on protein levels after upregulation of miR-148a-3p, consistent with negative post-transcriptional control via the respective miRNA. Similarly, reduced protein levels of the transcription factor Sp1 and PPARy were found after upregulation of miR-27b-3p (Figure 5A). Figure 5C presents an overview about potential downstream effects of these putative targets on cellular control of apoptosis, cell motility, gene transcription amongst others.

In order to verify if some of these targets are direct targets of the respective miRNAs, we further applied Luciferase assay techniques. In this context, we focused on Bcl-2, which is a potential target of miR-148a-
3p. We could show a negative correlation between increased expression of miR-148a-3p and luciferase activity on Bcl-2 plasmid, confirming that miR-148a-3p targets Bcl-2 directly (-40% reduction; Figure 5B).

Figure 5. Analysis of expression of potential target genes: Western Blotting (A), Luciferase Assay (B) and potential pathway network (C).

(A) Effect of miRNA manipulation on protein levels of putative targets in EAC cell lines. Relative protein levels compared to controls was measured in Western blot analysis using antibodies 48 hours after transfection. Proteins are listed according to size. (B) Luciferase assays for target validation of Bcl-2 in EAC cells. Relative Renilla concentration of the specific target protein was measured by Dual Glo Luciferase Kit 24 hours after transfection with miRNA precursor molecules. (C) Potential interactions of miRNAs in different pathways (adapted from Qiagen). Raf: rapidly accelerated fibrosarcoma; MEKK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinases; JNK: c-Jun N-terminal kinase; DNMT-1: DNA (cytosine-5)-methyltransferase 1; MSK-1: mitogen and stress activated protein kinase 1; Bcl-2: B-cell lymphoma 2; PPARy: Peroxisome proliferator-activated receptor gamma; Sp1: specificity protein 1; Spry2: Sprouty homolog 2; LIMK1: LIM domain kinase 1; M: mimic. * p<0.05.
4. DISCUSSION

The fact that response to chemotherapy varies widely in patients with esophageal adenocarcinoma highlights the enormous importance of valid biomarkers, which may be able to either predict or even modulate therapy response in this tumor type.

With this current study, we have demonstrated that our previously reported miRNA expression profiles of Cisplatin and 5FU resistant esophageal cancer cells play an important role in regulation of chemotherapy resistance in EAC [6,38]. In detail, miR-27b-3p sensitized cells to 5FU and Cisplatin in 83% respectively in 33%, miR-148a-3p sensitized cells to these chemotherapeutics in 67% respectively 33% of cases. MiR-200b-3p increased sensitivity only towards 5FU treatment in 50% of cases. Co-transfection experiments with miR-27b-3p and miR-148a-3p further showed that a significantly better response to chemotherapy was achieved in 75% of experiments after co-transfections compared to scramble transfected controls, and in 50% of cases an additive effect of both miRNAs was observed. Moreover, co-transfections seemed to provide a more stable sensitizing effect of transfections on chemotherapy as we found a decrease in standard deviation in these experiments compared to single transfected cells.

Literature on the impact of miRNA expression on chemotherapy response in esophageal cancer in-vitro is limited so far, and most studies selected single miRNAs and focused on their specific effect on chemotherapeutic treatment. With regards to EAC, only two authors investigated the impact of miRNAs on chemoresistance yet. Derouet et al. showed that overexpression of miR-145 induced resistance to cell apoptosis in OE-33, SKGT4 and Flo-1 cells [39], and Streppel et al. showed that elevated miR-223 levels induced an increase in chemotherapy response towards Cisplatin treatment in OE-33 cells by targeting Poly[ADP-ribose]-polymerase 1 (PARP1) [40]. However, with regards to other tumor entities, all three miRNAs investigated in this current study have been linked to chemotherapy response in recent literature. Upregulation of miR-27b was demonstrated to sensitize gastric [41], liver and kidney [42]
tumors to chemotherapy *in-vitro* and *in-vivo*. Joshi et al. reported that increased expression of miR-148a sensitized non-small cell lung cancer (NSCLC) cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligands (TRAILs) [43]. In addition, Fujita et al. showed that miR-148a expression levels were downregulated in paclitaxel-resistant prostate cancer cells, and transfection of miR-148a precursor attenuated the resistance to paclitaxel [44]. For miR-200b, upregulation induced sensitivity towards chemotherapeutic treatment *in-vitro* and/or *in-vivo* in lung adenocarcinoma, small cell lung cancer and tongue squamous cell carcinoma [45-49].

As outlined above, worse response to chemotherapy clinically seems to be often associated with more aggressive biological behavior of tumors [26,27]. Therefore, we further investigated if the three selected miRNAs affect metastatic potential and apoptosis in EAC cell lines. As expected, cell adhesion was indeed significantly inhibited by upregulation of all three miRNAs. Surprisingly, however, only upregulation of miR-27b-3p inhibited migration significantly whereas miR-148a-3p and miR-200b-3p seemed to increase migration rates in some cell lines. Furthermore, all three miRNAs led to cell cycle arrests at different stages as expected. However, again, effects of miRNA upregulation on apoptosis were partly inconsistent as miR-27b-3p reduced apoptosis, and miR-148a-3p and miR-200b-3p increased apoptosis only in 50% of experiments.

With regards to esophageal cancer, none of the selected miRNAs have been investigated in either *in-vitro* or *in-vivo* studies focusing on metastatic potential so far. However, an impact of these miRNA candidates on tumor cell migration or apoptotic potential was demonstrated in other tumor entities. For miR-27b-3p, upregulation was associated with repressed cell proliferation, adhesion potential and colony forming in colorectal- and colon cancer *in-vitro* and *in-vivo* [50,51] in cervical carcinoma [52] and in gastric cancer [53]. Increased expression of miR-148a-3p reduced proliferation, migration and invasion in pancreatic [54], breast [55-57], HCC and gastric cancer cells [58-60]. Regarding miR-200b-3p, upregulation was shown to inhibit sphere formation, cell proliferation and migration capacities of
cholangiocarcinoma cells [61], in triple-negative breast cancer [62], nasopharyngeal carcinoma [63] and in gastric cancer [64] in-vitro and in-vivo. In addition, high expression of miR-200c level in serum was demonstrated to correlate with poor response to neoadjuvant triplet chemotherapy (cisplatin, 5-fluorouracil, and Adriamycin or cisplatin, 5-fluorouracil, and docetaxel) in patients suffering from esophageal squamous cell carcinoma [65].

Finally, we analyzed potential miRNA targets and the related downstream pathways of the selected miRNAs with an emphasis on pathways relevant for chemotherapy resistance or metastasis. As previously noted, miRNAs are involved in important cellular processes including proliferation and cell death [18]. And in fact, we could show that upregulation of miR-148a-3p reduced protein expression levels of DNMT-1, MSK-1, Bcl-2 and Bim, and miR-27b upregulation led to downregulation of Sp1 and PPARγ proteins. These data fit to a negative post-transcriptional control via the respective miRNAs. Finally, we were able to confirm Bcl-2 for the first time as a direct target of miR-148a-3p in EAC cells.

These data fit very well to the existing literature. For miR-148a for example, DNMT-1 was described as a potential target in breast cancer cell lines [66], MSK-1 in human epithelial teratocarcinoma cells [33], Bcl-2 in colorectal [67] and pancreatic cancer cells [31], and Bim in glioblastoma cell lines [68]. Or with regards to miR-27b, Sp1 was shown as potential target in NSCLC [69], and PPARγ in neuroblastoma cells [70].

There are a few limitations of the current study that need consideration for proper interpretation of our findings. First, this study is an in-vitro study with all its typical limitations for potential transfer of results into clinical settings. However, we tried to address this argument by including 6 different EAC cell lines originating from different patients in order to answer the question if the selected miRNAs impact on chemotherapy response, and our results strongly support that our findings are not just incidental to one immortalized cell line, and indicate that these miRNAs indeed affect chemotherapy response in general. Another important aspect is the inclusion of chemotherapy treatment only without
radiotherapy in our experiments. Therefore, the results might not be completely transferable into clinical settings where patients receive often-combined radiochemotherapy. However, the application and relevance of radiotherapy in esophageal adenocarcinoma is still a matter of intensive debate, and our data definitively provide evidence for the complex role of epigenetics in the control of chemotherapy resistance. Finally, we used in this study only Cisplatin and 5FU, whereas there are numerous different chemotherapy regimens clinically in use at this stage (MAGIC, CROSS and others) which include additional drugs such as epirubicin, capecitabine, carboplatin, or paclitaxel [70]. However, platin-based chemotherapy with/without 5FU is still the basis for most chemotherapy approaches [71].

In summary, this study provides strong evidence that epigenetics – and specifically miRNAs – play a key role in chemotherapy response in esophageal adenocarcinoma. We showed that specific miRNA expression profiles of chemotherapy resistant cancer cells determine their response to chemotherapy treatment, which is potentially related to their ability to develop metastasis, escape apoptosis and control cell cycle. Together with the target analyses which confirmed Bcl-2 for the first time as a direct target of miR-148a-3p in EAC cells, our data show that miRNA-mediated regulation of chemotherapy resistance is complex, and several miRNAs seem to “co-operate” at various steps within a broad number of pathways. This fits very well to our recently proposed understanding of miRNA mediated regulation as a function of cellular functional complexes [72]. MiRNA profiling has therefore in our opinion the highly promising potential to predict treatment response to chemotherapy in esophageal adenocarcinoma, to monitor success of treatment and – most importantly - to modulate tumor biology in a therapeutic approach.
REFERENCES


71 http://www.cancer.org/cancer/esophaguscancer/detailedguide/esophagus-cancer-treating-chemotherapy (10.01.2016 01:12 pm)

SUPPLEMENTARY INFORMATION

Supplement A. Establishment of co-transfection protocol

Influence of different concentrations of miRNA mimics (oligonucleotides) in single and co-transfection settings on final miRNA expression levels measured by qRT-PCR (10 pm oligonucleotide versus 20 pm oligonucleotides). M: Mimic

Supplement B. Establishment of LC50 doses for chemotherapy with Cisplatin and 5-FU in the specific cell lines

EAC cell lines including cell number per 96-well plates and LD50 doses of Cisplatin / 5FU after 72 hour chemotherapy treatment. Cis: Cisplatin, 5FU: 5-fluorouracil.
Supplement B.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell number per 96-well</th>
<th>LD50 CIS</th>
<th>LD50 5FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>OE-19</td>
<td>6000</td>
<td>27,5 µM</td>
<td>25 µM</td>
</tr>
<tr>
<td>SKGT4</td>
<td>2500</td>
<td>5 µM</td>
<td>15 µM</td>
</tr>
<tr>
<td>OE-33</td>
<td>4500</td>
<td>1,5 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>Flo-1</td>
<td>4000</td>
<td>5 µM</td>
<td>5 µM</td>
</tr>
<tr>
<td>OACM5.1</td>
<td>8000</td>
<td>7,5 µM</td>
<td>20 µM</td>
</tr>
<tr>
<td>OACP4C</td>
<td>4000</td>
<td>3,5 µM</td>
<td>20 µM</td>
</tr>
</tbody>
</table>
A

![Chart A](chart_a.png)

B

![Chart B](chart_b.png)

C

![Chart C](chart_c.png)
**A**

- Relative protein concentration
- DNMT1, MSK-1, Bcl-2, Bim
- miR-148a-3p M
- OE-19 and SKGT4

**B**

- OE-19 and SKGT4
- Protein expression levels for DNMT-1, MSK-1, Bcl-2, Bim, Sp1, LIMK1, PPARγ, Spry2
- β-Tubulin

**C**

- Growth Factors
- Ras / Raf
- MEKKs
- p38, ERK 1/2, JNKs
- miR-148a-5p
- miR-27b-3p
- DNMT-1
- Sp1
- DNA-Methylation
- Gene Transcription
- Cell Motility
- Inflammation
- Osmoregulation
- Apoptosis
- Caspase 9/3, XIAP
- Withdrawal of Growth Factors