Human Cancer Biology

microRNA 223 Is Upregulated in the Multistep Progression of Barrett's Esophagus and Modulates Sensitivity to Chemotherapy by Targeting PARP1

Mirte Mayke Streppel1,4,5, Shweta Pai1, Nathaniel R. Campbell1, Chaoxin Hu1, Shinichi Yabuuchi1, Marcia Irene Canto2,3, Jean S. Wang6, Elizabeth A. Montgomery1, and Anirban Maitra1,3

Abstract

**Purpose:** Recent microarray and RNA-sequencing studies have uncovered aberrantly expressed micro-RNAs (miRNA) in Barrett's esophagus–associated esophageal adenocarcinoma. The functional significance of these miRNAs in esophageal adenocarcinoma initiation and progression is largely unknown.

**Experimental Design:** Expression levels of miR-199a/b-3p, -199a-5p, -199b-5p, -200b, -200c, -223, and -375 were determined in microdissected tissues from cardiac mucosa, Barrett's esophagus, dysplastic Barrett's esophagus, and esophageal adenocarcinoma using quantitative real-time PCR. miR-223 expression was validated in precursors and esophageal adenocarcinomas from 95 patients with esophageal adenocarcinoma by *in situ* hybridization (ISH). miR-223 was transfected into two esophageal adenocarcinoma cell lines, and *in vitro* assays were conducted. Target genes were identified using Illumina microarray, and results were validated in cell lines and human specimens.

**Results:** miR-199 family members and miR-223 were significantly overexpressed in esophageal adenocarcinoma, however, only miR-223 showed a stepwise increase during esophageal adenocarcinoma carcinogenesis. A similar trend was observed by ISH, which additionally showed that miR-223 is exclusively expressed by the epithelial compartment. miR-223–overexpressing cells had statistically significantly more migratory and invasive potential than scramble sequence–transfected cells. PARP1 was identified as a direct target gene of miR-223 in esophageal adenocarcinoma cells. Increased sensitivity to chemotherapy was observed in cells with enforced miR-223 expression and reduced PARP1.

**Conclusions:** miR-223 is significantly upregulated during the Barrett's esophagus–dysplasia–esophageal adenocarcinoma sequence. Although high miR-223 levels might contribute to an aggressive phenotype, our results also suggest that patients with esophageal adenocarcinoma with high miR-223 levels might benefit from treatment with DNA-damaging agents. *Clin Cancer Res;* 19(15); 4067–78. ©2013 AACR.

Introduction

Barrett’s esophagus has become a major public health problem in the United States and other Western countries over the past decade (1). Barrett’s esophagus is a risk factor for the subsequent development of esophageal adenocarcinoma (EAC). Esophageal adenocarcinomas arise on the background of metaplastic Barrett’s mucosa in the distal esophagus, following a multistep progression through non-dysplastic Barrett’s esophagus (BE-ND) to dysplasia, and culminating in invasive adenocarcinoma (2). Esophageal adenocarcinoma is one of the few cancers with an increase in incidence, particularly among Caucasian males, and has a dismal 5-year survival below 20% due to advanced presentation in most patients (1). Although neo-adjuvant chemoradiotherapy has shown to improve survival (3), it is not beneficial to all patients with esophageal adenocarcinoma. There is an unequivocal need for elucidating the molecular underpinnings of Barrett’s esophagus and esophageal adenocarcinoma pathogenesis to develop more potent therapeutic strategies.

miRNAs (miRNAs) are noncoding, 18- to 24-nucleotide long, single-stranded RNAs that have the ability to negatively regulate the expression of genes involved in several cellular processes, including cell proliferation, apoptosis, migration, invasion, and stress response (4–8). It has been shown that abnormal patterns of miRNA expression are present in many human carcinomas (6, 9), and are associated
with the pathogenesis, progression, and natural history of several cancers (4, 10). On the basis of their functional activity, cancer-related miRNAs can be divided into two groups: oncogenic miRNAs (onco-miRNA) and miRNAs with tumor suppressor activity (TS-miRNA; refs. 10, 11). Onco-miRNAs are usually silenced in normal tissues and overexpressed in neoplastic or cancerous lesions, causing a reduced expression of target genes with a tumor suppressor role. In contrast, TS-miRNAs are downregulated during carcinogenesis (11).

Previous microarray and "next-generation" RNA sequencing studies have uncovered aberrantly expressed miRNAs in esophageal adenocarcinomas compared with normal squamous epithelium (NSE), BE-ND, low-grade dysplasia (LGD), and high-grade dysplasia (HGD; refs. 12–21). Although numerous miRNAs have been identified as misexpressed during the multistep progression of Barrett’s esophagus, there is scant information concerning their functional role in Barrett’s esophagus and esophageal adenocarcinoma pathogenesis and therapy thereof.

In the present study, we examined the expression of a limited panel of miRNAs in tissue samples of nondysplastic and dysplastic Barrett’s esophagus, esophageal adenocarcinomas, and matched NSE obtained from a cohort of patients with treatment-naïve esophageal adenocarcinoma. The miRNA panel was selected on the basis of review of published literature for aberrantly expressed candidates during Barrett’s esophagus and esophageal adenocarcinoma pathogenesis (12–14, 16, 18, 22, 23). On the basis of our initial screening, we further explored the expression patterns of miR-223 in an independent series of archival tissues, composed of NSE, precursor lesions, invasive carcinomas, and metastases by in situ hybridization (ISH). The function of deregulated miR-223 expression was then investigated using esophageal adenocarcinoma cell lines. Putative targets of miR-223 were identified by transcriptomic profiling, and rigorously validated using in vitro cell-based systems. Our profiling and functional data led us to propose that miR-223 behaves as an onco-miRNA in esophageal adenocarcinoma, whose expression is progressively upregulated in the multistep transition from Barrett’s esophagus to esophageal adenocarcinoma. In addition, we identified the gene encoding the DNA damage repair protein PARP1 as a bona fide target of miR-223, and show that miR-223 upregulation is also associated with reduced PARP1 transcripts, and an increased sensitivity to cis-diaminedichloroplatinum (II; cisplatin), doxorubicin, and mitomycin C, providing a potential therapeutic vulnerability node for exploitation in the clinic.

Materials and Methods

miRNA selection

A panel of seven miRNAs (miR-199a/b-3p, -199a-5p, -199b-5p, -200b, -200c, -223, and -375) that have been reported as misexpressed in esophageal adenocarcinoma were chosen for validation by quantitative real-time PCR (qRT-PCR) in a cohort of chemoradiation-naïve intramuscular carcinoma (IMC)/esophageal adenocarcinoma patient tissue samples (Supplementary Table S1). These miRNAs were selected on the basis of several criteria, including their misexpression in multiple independent publications, demonstration of aberrant expression in primary tissue samples and not just cell lines, and functional evidence in other tumor types as either onco-miRNA or TS-miRNA.

Patient selection and tissue processing for qRT-PCR

Formalin-fixed paraffin-embedded (FFPE) cardio-oxyntic (cardiac; n = 11), BE-ND (n = 13), HGD (n = 17), IMC/EAC (n = 13), and matched NSE tissues were obtained from patients with treatment-naïve esophageal adenocarcinoma that had either undergone endoscopic mucosal resection or surgical resection of esophageal adenocarcinoma and precancer lesions. Hematoxylin and eosin (H&E) staining was reviewed by an expert pathologist (E.A. Montgomery), and all normal tissues and lesions were carefully microdissected. For the initial screening (set 1; Supplementary Fig. S1), all seven miRNAs were validated in the IMC/EAC and matched NSE tissues. The miRNAs that revealed a significantly different expression pattern in the IMC/EAC compared with the paired NSE tissues were subjected to further evaluation in cardiac, BE-ND and HGD lesions, additional esophageal adenocarcinoma (n = 8; frozen biopsies), and matched NSE tissues (set 2; Supplementary Fig. S1). On the basis of the results of the second screening, we decided to explore only miR-223 in a larger BE-ND (frozen biopsy) cohort (n = 10; set 3; Supplementary Fig. S1). Reference biopsies taken directly adjacent to the frozen research biopsies were examined by an expert pathologist (A. Maitra), and purity and high cellularity of the biopsies were confirmed. An overview...
of the study design for miRNA validation using FFPE and frozen specimens is provided in Supplementary Fig. S1.

**Cell culture of esophageal cell lines**

Two esophageal adenocarcinoma cell lines, OE33 (American Type Culture Collection) and JHesoAD1 (24), and one primary (nonimmortalized) squamous epithelial cell line, HEEpiC, were cultured for this study. OE33 and JHesoAD1 were authenticated using short-tandem repeat profiling less than 6 months ago and the cells have not been in culture for more than 2 months. HEEpiC cells were purchased from ScienCell. OE33 and JHesoAD1 were cultured in RPMI-1640 medium supplemented with 15% to 20% FBS. HEEpiC cells were grown in Epithelial Cell Medium-2 (ScienCell).

**RNA extraction, cDNA preparation, and qRT-PCR**

Total RNA was extracted from FFPE tissues using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE according to the manufacturer’s handbook (Life Technologies). Total RNA was recovered from frozen biopsies and cell lines using the mirVana miRNA Isolation Kit (Life Technologies).

cDNA was prepared with the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) following the manufacturer’s protocol. qRT-PCR was conducted using TaqMan Universal Master Mix II no UNG (Life Technologies) and TaqMan assays hsa-miR-199a/b-3p, hsa-miR-199a-5p, hsa-miR-199b-5p, hsa-miR-200b, hsa-miR-200c, hsa-miR-223, and hsa-miR-375 (Life Technologies). RNU6B functioned as the endogenous control. miRNA expression levels in the lesion tissues were directly compared with the expression levels in the matched NSE tissues and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

**In situ hybridization**

ISH was conducted on tissue microarrays (TMA), which have been previously described (25), to investigate the clinical and biologic relevance of miR-223 upregulation in esophageal adenocarcinoma carcinogenesis. Briefly, the TMAs consisted of 74 NSE, 37 BE-ND, 43 HGD, 96 esophageal adenocarcinoma, and 29 lymph node metastases (LMN) tissues obtained from a chemoradiation-naive Type Culture Collection) and JHesoAD1 (24), and one primary esophageal adenocarcinoma cohort. The staining was conducted Illumina microarray analysis on OE33 cells transiently transfected with a miR-223 mimic. Specifically, 10 nmol/L of hsa-miR-223 miRNA precursor (Life Technologies) was transfected into OE33 cells using Lipofectamine 2000 (Life Technologies), and cells were harvested 24 hours after transfection. Transfection efficiencies of the cells were estimated by transfection experiments with glycolaldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (Thermo Scientific).

**In vitro assays (cell growth, migration, invasion)**

Cell growth, migration, and invasion assays were conducted as previously described (26). Briefly, miR-223 mimic and scramble-transfected OE33 and JHesoAD1 cells were harvested 24 hours after transfection and assays were conducted for an additional 48 hours. Experiments were carried out in triplicates and repeated at least twice. For the migration and invasion assays, parallel plates were quantified with CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega) to correct for viability and cell growth variation, and five randomly selected 20× fields per chamber were counted. The Mann–Whitney U-test was used to determine differences in cell growth, and migratory and invasion potential between miR-223 and scramble-transfected esophageal adenocarcinoma cells.

**Microarray and validation experiments**

The HT12 v4 Illumina microarray platform (Illumina Inc.) was used for transcriptomic analysis following enforced miR-223 expression in two biologically replicated experiments [Gene Expression Omnibus (GEO) accession link: GSE44120]. All genes that were downregulated at least 1.5-fold in miR-223 compared with scramble-transfected OE33 cells were considered as potential target genes of interest. Putative target genes of miR-223 were validated using qRT-PCR. The following criteria were used for this selection:

1. The gene expression was downregulated by at least 1.5-fold in miR-223–transfected OE33 cells compared with scramble-transfected OE33 cells.
2. Binding of miR-223 to the 3′-untranslated region (3′-UTR) of the downregulated gene was predicted by MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/home/targets/v5/) and/or TargetScan (27).

Candidate target genes were validated in OE33 and JHesoAD1 cells by conducting qRT-PCR on esophageal adenocarcinoma cells with enforced miR-223 expression. Both pre-miRNA hsa-miR-223 miRNA precursor and pLV-hsa-miR-223 were used for validation to exclude off-target effects.
effects. qRT-PCR was conducted using Fast SYBR Green Master Mix (Life Technologies) and customized primers (sequences are listed in Supplementary Table S2). The top candidate target gene PARP1 was further validated at the protein level by Western blot analysis [clone A6.4.12 (mouse): 1:1,000 dilution; Abcam]. To show the binding of miR-223 to the 3′-UTR seed sequence of PARP1, luciferase expression was measured following simultaneously transfecting OE33 cells with pLV-hsa-miR-223, the 3′-UTR of PARP1 fused to a firefly luciferase gene (OriGene), and Renilla luciferase. Furthermore, expression levels of selected target genes [PARP1 and SWI/SNF–related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 1 (SMARCD1)] were measured in eight frozen esophageal adenocarcinoma and matched NSE biopsies using qRT-PCR. Patients were divided into groups, higher (>17-fold miR-223 upregulation in esophageal adenocarcinoma compared with matched NSE sample) and lower (<4-fold upregulation), and correlations between miR-223 and target gene expression was tested using the Mann–Whitney U test. A P value less than 0.05 was considered statistically significant.

Sensitivity of miR-223 esophageal adenocarcinoma cells to chemotherapeutics

OE33 and JHesoAD1 cells were used to investigate the potential effects of miR-223 misexpression on chemotherapy sensitivity. Cells were harvested 24 hours after transfection with miR-223 mimic or scrambled miRNA, and plated in 96-well plates. Twenty-four hours later, cells were treated with cisplatin (range, 0–20 μmol/L), mitomycin C (range, 0–15 μmol/L), or doxorubicin (range, 0–15 μmol/L) for 24 to 48 hours. MTS assays were conducted and statistical tests (Mann–Whitney U test and two-way ANOVA) were run to determine differences in viability between miR-223 and scramble-transfected OE33 cells. Experiments were repeated at least three times in triplicates, and a P value less than 0.05 was considered statistically significant.

Clonogenic assay

Anchorage-independent clonogenic assays were conducted to determine the effect of miR-223 overexpression on cisplatin sensitivity. miR-223 and scramble-transfected OE33 cells in 0.5% agarose in normal growth media were plated in triplicates on top of a base layer of 1% agarose in normal growth media. The next day, 1 mL of 0 to 6 μmol/L cisplatin was added on top of the cell layer, and colonies were treated for 10 days. The colonies were stained with 0.005% crystal violet and manually counted. Statistical significant differences were tested using the Mann–Whitney U test. A P value less than 0.05 was considered as statistically significant.

Immunofluorescence for phospho-H2A.X and 53BP1

We further investigated whether DNA breaks are more often induced by treatment with doxorubicin in miR-223 compared with scramble-transfected OE33 cells. Immunofluorescence experiments were carried out to detect the phosphorylated histone H2A.X (pH2A.X, a.k.a. γH2A.X) and tumor suppressor protein-binding protein 1. DNA-damage response proteins that are recruited to DNA breaks and therefore can be used to visualize DNA breaks. miR-223 and scramble-transfected OE33 cells were plated on chamber slides and treated with 0 to 1.25 μmol/L doxorubicin for 24 hours. Primary antibodies against pH2A.X (clone JBW301; dilution 1:500; Millipore) and 53BP1 (NB100-304; dilution 1:250; Novus Biologicals) and Alexa Fluor secondary antibodies (dilutions 1:250; Invitrogen), respectively, were used. Reference photomicrographs were taken from five randomly selected fields. Cells were manually counted and categorized into cells with and without costaining. The Mann–Whitney U test was used to determine statistically significant differences between miR-223 and scramble-transfected OE33 cells. A P value less than 0.05 was considered as statistically significant.

Results

miRNA expression in the Barrett’s esophagus–dysplasia–esophageal adenocarcinoma sequence

A panel of seven miRNAs was selected for profiling based on their recurrent misexpression in Barrett’s esophagus and esophageal adenocarcinoma across multiple independent studies (Supplementary Table S1). All transcript profiling was conducted on chemoradiation-naïve esophageal adenocarcinoma patient samples. Five miRNAs, miR-199a/b-3p, -199a-5p, -199b-5p, -200b, and -223, were significantly differentially expressed between esophageal adenocarcinoma and paired NSE tissues (Fig. 1). The median relative expression values of the miR-199 family members and miR-223 were more than 2-fold higher in esophageal adenocarcinoma than matched NSE, and these miRNAs were subjected to further investigation (Fig. 2).

Expression of miR-199 family members and miR-223 was examined in gastric cardia, BE-ND, HGD, and an expanded group of treatment-naïve esophageal adenocarcinoma tissues, and compared with the expression of these miRNAs in matched NSE tissues from the same patient. The expression levels of gastric cardia and BE-ND tissues were remarkably similar for all four miRNAs, suggesting ontogenic similarities between the two differentiated epithelial subtypes. Significantly higher expression of the miR-199 family members was found in esophageal adenocarcinoma tissues compared with HGD, BE-ND, and paired NSE tissues (all P < 0.006), and among those, miR-199a-5p showed the highest median expression in esophageal adenocarcinomas (7.70; Fig. 2A–C).

Significantly increased miR-223 levels were found in BE-ND (median, 2.21; P = 0.003), HGD (P = 0.001), and esophageal adenocarcinoma (P < 0.001) compared with their matched NSE tissue (Fig. 2D). miR-223 was upregulated in all esophageal adenocarcinoma tissues, and the median expression level in the esophageal adenocarcinoma group (7.02) was significantly higher than in the HGD (2.73). Barrett’s esophagus (2.21), and cardiac group (1.81; all P < 0.014; Fig. 2D). Upregulation in a stepwise manner was revealed during esophageal adenocarcinoma...
carcinogenesis ($P = 0.002$; Fig. 2D). From 6 patients, 2 or more neoplastic lesions were available and analysis of miR-223 expression in these lesions confirmed that miR-223 is also upregulated in a stepwise manner during the multistep progression of Barrett’s esophagus in any given individual (Fig. 3A). miR-223 expression was validated in a larger cohort of lesions obtained from patients with treatment-naive esophageal adenocarcinoma using ISH. Moderate to diffuse miR-223 expression was present in 5.4%, 52.8%, 40.7%, 67.4%, 79.2%, and 65.5% of the NSE, Barrett’s esophagus, LGD, HGD, esophageal adenocarcinoma, and LNM tissues, respectively (Fig. 3B). Diffuse expression was more often detected in esophageal adenocarcinomas and LNMs. ISH furthermore showed that miR-223 is exclusively expressed by metaplastic, dysplastic, and neoplastic epithelial cells, and not by the inflammatory or stromal cells adjacent to the lesion (Fig. 3C).

The role of miR-223 in cell growth, invasion, and migration

Low endogenous levels of miR-223 were detected in the esophageal adenocarcinoma cell lines OE33 and JHesoAD1 compared with the normal esophageal cell line HEEpiC (data not shown). To explore the function of miR-223 in the esophagus, we ectopically expressed miR-223 in OE33 and JHesoAD1 cells. In short-term viability assays, miR-223-transfected esophageal adenocarcinoma cells had lower cell numbers than scramble-transfected esophageal adenocarcinoma cells ($P < 0.001$; data not shown). In contrast, additional in vitro assays showed a more aggressive phenotype in miR-223 than scramble-transfected esophageal adenocarcinoma cells; miR-223–transfected cells exhibited statistically significantly more migratory and invasive potential ($P < 0.022$; Fig. 4A).

**PARP1 and SMARCD1 are target genes of miR-223 in the esophagus**

To discover direct target genes of miR-223 that could explain the phenotype of miR-223–overexpressing cells in the esophagus, microarray experiments were carried out in replicate on OE33 cells. The expression profiles of miR-223–transfected OE33 cells were compared with those of scramble-transfected OE33 cells, and 58 genes showed an average downregulation of more than 1.5-fold in miR-223 relative to scramble-transfected OE33 cells. The miRNA target gene prediction platforms, MicroCosm and TargetScan (27), were queried to determine whether binding of miR-223 to the 3'-UTR was predicted by the respective in silico algorithms. A match between miR-223 and 3'-UTR of the putative target gene was found in 51.7% (30/58) of the >1.5-fold downregulated genes (Supplementary Table S3).

Ten potential target genes were validated in replicate experiments in OE33 and JHesoAD1 cells using qRT-PCR. Nine genes were significantly downregulated in miR-223 compared with scramble-transfected OE33 cells, with PARP1, CYB5A, and SMARCD1 as top candidates. The three top candidates were also found to be downregulated upon miR-223 overexpression in JHesoAD1 cells (Table 1). We observed that the effect of miR-223 overexpression on target gene expression was more modest in JHesoAD1 than OE33 cells due to poorer transfection efficiency. Nonetheless, in both cell lines, we established that miR-223 expression...
leads to reduction in PARP1, CYB5A, and SMARCD1 transcripts (Table 1).

The expression levels of PARP1 and SMARCD1 were examined in eight treatment-naïve esophageal adenocarcinoma and matched NSE frozen biopsies. The patients were divided into two groups based on the miR-223 level in esophageal adenocarcinoma: "low" \( (n = 5; \text{range}, 1.5–3.6) \) and "high" \( (n = 3; \text{range}, 17.1–137.8) \). PARP1 and SMARCD1 were significantly lower expressed in the "high miR-223" group \( (P = 0.036; \text{Fig. 4B}) \).

**miR-223 regulates chemotherapy sensitivity via PARP1**

Direct binding of miR-223 to the 3'-UTR of PARP1 was further confirmed by a firefly luciferase assay. Cotransfection of the miR-223 mimic, plasmid DNA consisting of a 3'-UTR clone of PARP1 fused to the firefly luciferase gene, and the Renilla luciferase vector as transfection control resulted in a 2.7-fold decreased firefly luciferase activity compared with cotransfection with a scramble miRNA (Fig. 4C). In addition, functional downregulation of PARP1 protein was confirmed by Western blot analysis in OE33 and JHesoAD1 cells (Fig. 4D).

PARP1 is involved in the repair of ssDNA breaks, and studies have shown that PARP1 inhibitors improve the sensitivity of carcinomas to chemotherapeutics (28). We investigated whether ectopic miR-223 expression, and thus PARP1 downregulation, can increase the sensitivity of OE33 and JHesoAD1 cells to the DNA-damaging agents cisplatin, mitomycin C, and doxorubicin. In comparison with scramble-transfected cells, miR-223–transfected OE33 and JHesoAD1 cells were significantly more sensitive to cisplatin and doxorubicin at all tested concentrations, as assessed by in vitro viability assays \( (P < 0.001; \text{Fig. 5A and B}) \). miR-223–transfected JHesoAD1 were also significantly less viable after mitomycin C treatment at all tested concentrations \( (P < 0.001; \text{data not shown}) \), whereas miR-223–transfected OE33 cells were only more sensitive to 7.5 to 10 \( \mu \text{mol/L} \) mitomycin C \( (P = 0.009; \text{data not shown}) \) than scramble-transfected cells. In long-term \( (T = 10 \text{ days}) \) clonogenic survival assays, we observed that miR-223 colonies were significantly more sensitive to all tested cisplatin treatments relative to scramble-transfected OE33 colonies \( (P < 0.001; \text{Fig. 5C}) \). The IC\(_{50}\) was reached at concentrations of 2.45 and 6.00 \( \mu \text{mol/L} \) for miR-223 and scramble colonies, respectively, suggesting that miR-223 colonies are approximately 2.45 times more sensitive to cisplatin than scramble sequence–expressing colonies. We further determined that significantly more DNA breaks, as visualized by costaining of pH2A.X and 53BP1, are encountered in untreated \( (P = 0.032) \) as well as with 1.25 \( \mu \text{mol/L} \) doxorubicin-treated \( (P = 0.008) \) miR-223–expressing compared with scramble-transfected OE33 cells (Fig. 5D) indicating that the DNA repair mechanism is impaired in miR-223 OE33 cells. Together, these data suggest that patients with esophageal adenocarcinoma with high miR-223 levels in their esophageal adenocarcinomas might benefit more from treatment with
DNA-damaging agents than patients with lower miR-223 levels in their tumors.

Discussion

Recent microarray and "next-generation" RNA-sequencing studies have uncovered numerous miRNAs that are differentially expressed in esophageal adenocarcinoma and Barrett's esophagus; however, surprisingly little is known about the functional roles of these miRNAs in the multistep progression of Barrett's esophagus. In this study, we validated a panel of miRNAs that have previously been reported in the literature, on an independent cohort of patients with treatment-naive esophageal adenocarcinoma and LNM tissues, respectively (B). Representative ISH photos in (C) illustrate the stepwise increased miR-223 expression from NSE, to BE-ND, to HGD, and ultimately esophageal adenocarcinoma. miR-223 expression was limited to the (non-)dysplastic/neoplastic epithelial cells, and completely undetectable in inflammatory and stromal cells.

A similar trend was observed on a larger cohort using ISH hybridization.

In the context of upper gastrointestinal tract malignancies, miR-223 has been extensively studied in gastric cancer pathogenesis, where it is significantly upregulated in gastric cancer tissues compared with normal gastric mucosae (29–31). Furthermore, it has been reported that miR-223 can be detected in serum of patients with gastric cancer and implicates that miR-223 might function as a diagnostic biomarker for gastric cancer (32). In agreement with these studies, we showed that miR-223 is already overexpressed in the nondysplastic precursors and HGD lesions in the esophagus, suggesting that we might be able to predict esophageal adenocarcinoma development in an early, curable stage using miR-223 detection.

Our in vitro data suggest that miR-223 plays a role in the regulation of cell proliferation and viability. Paradoxically, overexpression of miR-223 in esophageal adenocarcinoma cells resulted in significantly reduced cell growth. These
results are in agreement with miR-223 expression studies in other cancer cell lines: significantly less proliferative potential was revealed in miR-223–overexpressing osteosarcoma, hepatoma, hepatocellular cancer, colorectal cancer, cervical cancer, and gastric cancer cells (31, 33–35). This seemingly tumor-suppressive role of miR-223 is greatly opposed to the aggressive behavior, characterized by enhanced migratory and invasive capacity, of miR-223–overexpressing esophageal adenocarcinoma cells. Other groups have also shown previously in esophageal squamous cell carcinoma and gastric cancer that miR-223 is able to promote migration, invasion, and metastasis formation (30, 31, 36). These studies have furthermore reported that high miR-223 levels are correlated with advanced disease and poor outcome in these cancers (31, 37, 38). To further explore how miR-223 regulates cancer-promoting cellular processes, we profiled esophageal adenocarcinoma cells in the setting of ectopic miR-223 expression.

We identified two novel target genes of miR-223, namely SMARCD1 and PARP1, the inhibition of which may contribute to cancer development and/or progression as well as to chemotherapy sensitivity in the esophagus. SMARCD1, also known as BRG1-associated factor 60a, has binding sites for the glucocorticoid receptor and BRG1, and seems to be essential for chromatin-remodeling processes (39). In addition, it has been shown that interaction of the SNF/SWI complex and p53 is required for p53-mediated transcription and cell-cycle regulation (40). SMARCD1 possesses a binding site for p53, and inhibition of SMARCD1 reduces the SWI/SNF complex-mediated transcriptional activity of p53 (41). We showed in this study that miR-223 expression is negatively correlated with SMARCD1 expression. miR-223 overexpression may lead to reduced chromatin-remodeling activity, impaired transcription from the chromatin, and decreased p53 activity by directly inhibiting SMARCD1 transcription.

PARP1 was downregulated at both the transcript and protein level upon miR-223 overexpression in esophageal adenocarcinoma cells, and we confirmed the direct binding of miR-223 to the 3' UTR of PARP1 using a luciferase assay. Furthermore, we showed that lower PARP1 levels are significantly associated with esophageal adenocarcinomas.

Figure 4. miR-223 functional studies in esophageal adenocarcinoma cell lines. miR-223 esophageal adenocarcinoma cells showed significantly increased migratory and invasive potential compared with scramble esophageal adenocarcinoma cells (A). Validation of the Illumina microarray results in OE33 and JHesoAD1 cells by qRT-PCR confirmed that PARP1 and SMARCD1 are prone to be direct target genes of miR-223 in the esophagus. Correlation of miR-223 and target gene expression in 8 patients with esophageal adenocarcinoma provided additional evidence that PARP1 and SMARCD1 are targeted by miR-223. PARP1 and SMARCD1 levels were significantly lower among patients with high miR-223 expression levels (B). We next focused on PARP1, and established that firefly luciferase activity is significantly decreased when miR-223 and plasmid DNA consisting of PARP1 3' UTR and a firefly luciferase gene are cotransfected in OE33 cells (C). Complete knockdown of PARP1 and significant reduction of PARP1 activity at protein level was detected upon miR-223 transfection in OE33 and JHesoAD1 cells, respectively (D). The error bars in panels (A)–(C) depict the SEM.
miR-223 Modulates Chemotherapy Sensitivity

Table 1. Putative target genes of miR-223

<table>
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<tr>
<th>Gene</th>
<th>Full name</th>
<th>Average fold change miR-223 vs. mock OE33 cells</th>
<th>Binding of miR-223 to 3'-UTR gene predicted by:</th>
<th>Match miR-223 and 3'-UTR gene</th>
<th>Fold change qRT-PCR validation</th>
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<tr>
<td>PARP1</td>
<td>Poly (ADP-ribose) polymerase 1</td>
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NOTE: Illumina microarray on OE33 cells identified candidate target genes. Publicly available databases were queried to search for binding sites of miR-223 in the 3'-UTR of the putative targets. Ten potential target genes were selected for further validation in OE33 and JHesoAD1 cells using qRT-PCR. All putative targets except LMO2 were significantly downregulated in OE33 cells upon miR-223 transfection. Expression of PARP1, CYB5A, and SMARCD1 was significantly inhibited in miR-223 compared with scramble JHesoAD1 cells. 3'UTR, 3' untranslated region.

8mer: an exact match to positions 2–8 of miR-223 (the seed plus position 8) followed by an "A".
7mer-m8: an exact match to positions 2–8 of miR-223 (the seed plus position 8).
7mer-1A: an exact match to positions 2–7 of miR-223 followed by an "A".

<sup>a</sup>Average of 2 biologic replicated experiments.
<sup>b</sup>P < 0.05.

with high miR-223 levels, indicating that this regulatory mechanism is intact in vivo in human tissue samples. PARP1 plays a role in processes involving DNA recognition (42). PARP1 interacts with single and double DNA breaks, which results in automodification, self-activation, and the addition of poly (ADP-ribose) polymers to itself and surrounding histones (28, 43). It recruits DNA repair proteins to the site of DNA breaks by, among other mechanisms, changing local chromatin structures to admit DNA repair proteins to the DNA breaks (28). We showed in the current study that in miR-223-overexpressing esophageal adenocarcinoma cells the DNA damage repair system is significantly impaired through direct downregulation of PARP1. Induction of DNA damage by doxorubicin resulted in significantly more DNA breaks in miR-223 overexpressing than scramble-transfected OE33 cells.

Interestingly, PARP1 also seems to play an essential role in the maintenance of genetic stability. Reduced PARP1 activity promotes homologous recombination, and it has been extensively reported that homologous-deficient cells, for instance BRCA1/2-deficient cells, are sensitive to PARP1 inhibitors as these cells are incapable of repairing recombinogenic lesions induced by PARP1 inhibition (28). A rational therapeutic approach for cancers that are not homologous deficient such as esophageal adenocarcinoma is to combine PARP1 inhibitors with DNA-damaging agents (42), or recruit patients who already exhibit low PARP1 levels for therapy with DNA-damaging agents. To test the latter approach, we investigated the sensitivity of miR-223 esophageal adenocarcinoma cells to cisplatin, mitomycin C, and doxorubicin, and found that miR-223 esophageal adenocarcinoma cells were significantly more sensitive to these chemotherapeutics than scramble-transfected OE33 cells in short-term viability and long-term anchorage-independent survival assays. These data suggest that patients with esophageal adenocarcinoma with high miR-223 levels might benefit more from treatment with DNA-damaging agents.

Our study has two related limitations. First, the correlation of miR-223 and PARP1 expression could only be confirmed in snap-frozen specimens as miRNA levels are greatly degraded in FFPE tissues. Secondly, the sample size is relatively small because the specimens could only be obtained from patients with treatment-naïve esophageal adenocarcinoma, and these specimens are scarce. Therefore, studies in larger cohorts are required before miR-223 can be implemented as predictive biomarker for chemotherapy sensitivity in the clinics.
In conclusion, we showed that miR-223 is upregulated in the multistep progression of BE-ND to esophageal adenocarcinoma in the majority of patients. miR-223–overexpressing cells exhibited an aggressive phenotype; however, they were also more sensitive to DNA-damaging agents due to the direct interaction between miR-223 and PARP1. High

Figure 5. Because PARP1 inhibition has been previously linked to increased chemotherapy sensitivity, we tested cisplatin (A) and doxorubicin (B) sensitivity of miR-223 OE33 and JHesoAD1 cells. miR-223 OE33 and JHesoAD1 cells were significantly more sensitive to both chemotherapeutics compared with scramble cells in short-term MTS assays (B and C). Long-term (10 days) sensitivity to cisplatin was evaluated in miR-223 and scramble-transfected OE33 colonies using a drug clonogenic survival assay (C). The concentration on the X-axis depicts the concentration of cisplatin in the media (1 mL) that was added on top of the cell-containing agarose layer. miR-223–transfected OE33 cells were significantly more sensitive to cisplatin after 10 days than scramble-transfected cells at all concentrations (C). D, example photomicrographs of the immunofluorescence experiment. Colocalization of pH2A.X and 53BP1 indicates the presence of a DNA break. Significantly more DNA breaks were detected in miR-223–transfected untreated and treated OE33 cells compared with scramble-transfected OE33 cells ($P < 0.05$).
endogenous levels of miR-223 in esophageal adenocarcinomas were significantly associated with lower PARP1 levels, and those patients might benefit more from treatment with DNA-damaging agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: M.M. Streppel, A. Maitra
Development of methodology: M.M. Streppel, N.R. Campbell, C. Hu, A. Maitra
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Streppel, N.R. Campbell, S. Yabuuchi, M.I. Canto, J.S. Wang, E.A. Montgomery
Analysis and interpretation of data (e.g., statistical analysis, biography, computational analysis): M.M. Streppel, S. Yabuuchi, A. Maitra
Writing, review, and/or revision of the manuscript: M.M. Streppel, N.R. Campbell, C. Hu, S. Yabuuchi, M.I. Canto, J.S. Wang, E.A. Montgomery, A. Maitra

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