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MicroRNA 223 is Up-regulated in the Multistep Progression of Barrett's Esophagus and Modulates Sensitivity to Chemotherapy by Targeting *PARP1*

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Abstract

PURPOSE—Recent microarray and RNA-sequencing studies have uncovered aberrantly expressed microRNAs (miRs) in Barrett's esophagus (BE)-associated esophageal adenocarcinoma (EAC). The functional significance of these miRs in EAC 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, BE, dysplastic BE, and EAC using quantitative real time PCR. MiR-223 expression was validated in precursors and EACs from 95 EAC patients by in situ hybridization (ISH). MiR-223 was transfected into two EAC cell lines, and *in vitro* assays were performed. 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 over-expressed in EAC, however only miR-223 showed a stepwise increase during EAC carcinogenesis. A similar trend was observed by ISH, which additionally showed that miR-223 is exclusively expressed by the epithelial compartment. MiR-223 over-expressing 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 EAC cells. Increased sensitivity to chemotherapy was observed in cells with enforced miR-223 expression and reduced *PARP1*.

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GEO accession number of repository for microarray expression data: GSE44120.

CONCLUSIONS—MiR-223 is significantly up-regulated during the BE-dysplasia-EAC sequence. Although high miR-223 levels might contribute to an aggressive phenotype, our results also suggest that EAC patients with high miR-223 levels might benefit from treatment with DNA-damaging agents.

Keywords

miR-223; PARP1; Barrett's esophagus; adenocarcinoma

INTRODUCTION

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

MicroRNAs (miRs) are non-coding, 18–24-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 miR expression are present in many human carcinomas(6, 9), and are associated with the pathogenesis, progression and natural history of several cancers(4, 10). Based on their functional activity, cancer-related miRs can be divided in two groups; oncogenic miRs (onco-miRs) and miRs with tumor suppressor activity (TS-miRs) (10, 11). Onco-miRs are usually silenced in normal tissues and over-expressed in neoplastic or cancerous lesions, causing a reduced expression of target genes with a tumor suppressor role. In contrast, TS-miRs are down regulated during carcinogenesis(11).

Previous microarray and “next-generation” RNA sequencing studies have uncovered aberrantly expressed miRs in EACs compared to normal squamous epithelium (NSE), BE-ND, low grade dysplasia (LGD), and high grade dysplasia (HGD) (12–21). Although numerous miRs have been identified as misexpressed during the multistep progression of BE, there is scant information vis-à-vis their functional role in BE and EAC pathogenesis and therapy thereof.

In the present study, we examined the expression of a limited panel of miRs in tissue samples of non-dysplastic and dysplastic BE, EACs and matched NSE obtained from a cohort of patients with treatment-naïve EAC. The miR panel was selected based on review of published literature for aberrantly expressed candidates during BE and EAC pathogenesis (12–14, 16, 18, 22, 23). Based on our initial screening, we further explored the expression patterns of miR-223 in an independent series of archival tissues, comprised of NSE, precursor lesions, invasive carcinomas and metastases by *in situ* hybridization (ISH). The function of deregulated miR-223 expression was then investigated using EAC 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-miR in EAC, whose expression is progressively

up-regulated in the multistep transition from BE to EAC. In addition, we identified the gene encoding the DNA damage repair protein poly(ADP-ribose) polymerase 1 (PARP1) as a *bona fide* target of miR-223, and demonstrate that miR-223 up-regulation is also associated with reduced *PARP1* transcripts, and an increased sensitivity to cis-diamminedichloroplatinum (II) (Cisplatin), Doxorubicin and Mitomycin C, providing a potential therapeutic vulnerability node for exploitation in the clinic.

MATERIALS AND METHODS

MicroRNA selection

A panel of seven miRs (miR-199a/b-3p, -199a-5p, -199b-5p, -200b, -200c, -223 and -375) that have been reported as misexpressed in EAC were chosen for validation by quantitative real-time PCR (qRT-PCR) in a cohort of chemo-radiation-naïve intramucosal carcinoma (IMC)/EAC patient tissue samples (Supplemental Table S1). These miRs were selected based on 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-miR or TS-miR.

Patient selection and tissue processing for quantitative real-time PCR

Formalin-fixed paraffin-embedded (FFPE) cardio-oxytic (cardiac) (n=11), BE-ND (n=13), HGD (n=17), IMC/EAC (n=13) and matched NSE tissues were obtained from treatment-naïve EAC patients that had either undergone endoscopic mucosal resection or surgical resection of EAC and precursor lesions. Haematoxylin and eosin (H&E) staining was reviewed by an expert pathologist (EAM), and all normal tissues and lesions were carefully microdissected. For the initial screening (set 1, supplemental Figure S1), all seven miRs were validated in the IMC/EAC and matched NSE tissues. The miRs that revealed a significantly different expression pattern in the IMC/EAC compared to the paired NSE tissues were subjected to further evaluation in cardiac, BE-ND and HGD lesions, additional EAC (n=8, frozen biopsies), and matched NSE tissues (set 2, supplemental Figure S1). Based on 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, supplemental Figure S1). Reference biopsies taken directly adjacent to the frozen research biopsies were examined by an expert pathologist (AM), and purity and high cellularity of the biopsies were confirmed. An overview of the study design for miR validation using FFPE and frozen specimens is provided in supplemental Figure S1.

Cell culture of esophageal cell lines

Two EAC cell lines, OE33 (American Type Culture Collection (ATCC), Manassas, VA) and JHesoAD1(24), and one primary (non-immortalized) squamous epithelium cell line, HEEpiC, were cultured for this study. OE33 and JHesoAD1 were authenticated using short tandem repeat profiling less than six months ago and the cells have not been in culture for more than 2 months. HEEpiC cells were purchased from Sciencell (Carlsbad, CA). OE33 and JHesoAD1 were cultured in RPMI-1640 medium supplemented with 15–20% fetal bovin serum. HEEpiC cells were grown in Epithelial Cell Medium-2 (Sciencell).

RNA extraction, cDNA preparation and quantitative real-time PCR

Total RNA was extracted from FFPE tissues using the Recover All™ Total Nucleic Acid Isolation Kit for FFPE according to the manufacturer's handbook (Life technologies, Grand Island, NY). 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 carried out 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. MiR expression levels in the lesion tissues were directly compared to 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 performed on tissue microarrays (TMAs), which have previously been described (25), to investigate the clinical and biological relevance of miR-223 up-regulation in EAC carcinogenesis. Briefly, the TMAs consisted of 74 NSE, 37 BE-ND, 27 LGD, 43 HGD, 96 EAC, and 29 lymph node metastases (LNM) tissues obtained from a chemo-radiation-naïve EAC cohort. The staining was performed using the tyramide signal amplification system for ISH with biotinylated probes (DAKO North America, Carpinteria, CA) according to the manufacturer's instructions. The slides were hybridized with 10 nM hsa-miR-223 5'-biotinylated miRCURA LNA™ probes (Exiqon, Woburn, MA) for 16 hours at 56 °C. Staining patterns were reviewed by three reviewers (EAM, AM, MMS), semi-quantified and categorized into four groups; absent, focal (<25% of epithelial cells exhibit miR-223), moderate (25–50%), and diffuse (>50%).

Transfection of miR-223 mimics in EAC cells

In vitro functional studies were performed using transiently enforced expression of miR-223 in the two EAC cell lines. Specifically, hsa-miR-223 miRNA precursor (pre-miR) or pLV-[hsa-miR-223] plasmid (Biosettia, San Diego, CA) were introduced into OE33 (miR-223 OE33) and JHesoAD1 (miR-223 JHesoAD1) cells. Cells transfected with a non-targeting, negative control mimic (scramble transfected EAC cells) and empty LV vector, respectively, were utilized as controls in the functional analyses. In order to identify candidate gene targets of miR-223, we performed Illumina microarray analysis on OE33 cells transiently transfected with a miR-223 mimic. Specifically, 10 nM 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 *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) siRNA (Thermo Scientific, Pittsburgh, PA).

In vitro assays (cell growth, migration, invasion)

Cell growth, migration, and invasion assays were performed 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 performed in triplicates and repeated at least twice. For the migration and invasion assays, parallel plates were quantified with Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI) to correct for viability and cell growth variation, and five randomly selected 20x 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 EAC cells.

Microarray and validation experiments

The HT12 v4 Illumina microarray platform (Illumina Inc, San Diego, CA) was utilized for transcriptomic analysis following enforced miR-223 expression in two biologically replicated experiments (GEO accession link: GSE44120). All genes that were down-

regulated at least 1.5 fold in miR-223 compared to 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 down-regulated by at least 1.5 fold in miR-223 transfected OE33 cells compared to the scramble transfected OE33 cells.
2. Binding of miR-223 to the 3'UTR of the down-regulated gene was predicted by MicroCosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) and/or TargetScan(27).

Candidate target genes were validated in OE33 and JHesoAD1 cells by performing qRT-PCR on EAC cells with enforced miR-223 expression. Both pre-miR hsa-miR-223 miRNA precursor and pLV-[hsa-miR-223] were used for validation to exclude off-target effects. qRT-PCR was carried out using Fast SYBR green master mix (Life technologies) and customized primers (sequences are listed in supplemental 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, Cambridge, MA). In order to demonstrate 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, Rockville, MD), 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 EAC and matched NSE biopsies using qRT-PCR. Patients were divided into groups, higher (>17 fold miR-223 up-regulation) in EAC compared to matched NSE sample) and lower (<4 fold up-regulation), and correlations between miR-223 and target gene expression was tested using the Mann-Whitney U test. A *P*-value <0.05 was considered statistically significant.

Sensitivity of miR-223 EAC 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 miR, and plated in 96-well plates. Twenty-four hours later, cells were treated with Cisplatin (range 0–20 μ M), Mitomycin C (range: 0–15 μ M) or Doxorubicin (DOX) (range: 0–15 μ M) for 24–48 hours. MTS assays were performed 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 <0.05 was considered statistically significant.

Clonogenic assay

Anchorage independent clonogenic assays were performed to determine the effect of miR-223 over-expression 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 0–6 μ M 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 <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 to scramble transfected OE33 cells.

Immunofluorescence experiments were performed to detect the phosphorylated histone H2A.X (pH2A.X, a.k.a. γ H2AX) and tumor suppressor protein 53 binding protein 1 (53BP1), DNA-damaging 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 0–1.25 μ M Doxorubicin for 24 hours. Primary antibodies against pH2A.X (clone JBW301, dilution 1:500, Millipore, Billerica, MA) and 53BP1 (NB100-304, dilution 1:250, Littleton, CO) 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 co-staining. The Mann-Whitney U test was used to determine statistically significant differences between miR-223 and scramble transfected OE33 cells. A P -value <0.05 was considered as statistically significant.

RESULTS

MicroRNA expression in the BE-dysplasia-EAC sequence

A panel of seven miRs was selected for profiling, based on their recurrent misexpression in BE and EAC across multiple independent studies (Supplemental Table S1). All transcript profiling was performed on chemo-radiation-naïve EAC patient samples. Five miRs, miR-199a/b-3p, -199a-5p, -199b-5p, -200b, and -223, were significantly differentially expressed between EAC and paired NSE tissues (Figure 1). The median relative expression values of the miR-199 family members and miR-223 were greater than 2-fold higher in EAC than matched NSE, and these miRs were subjected to further investigation (Figure 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 EAC tissues, and compared to the expression of these miRs in matched NSE tissues from the same patient. The expression levels of gastric cardia and BE-ND tissues were remarkably similar for all four miRs, suggesting ontogenic similarities between the two differentiated epithelial subtypes. Significantly higher expression of the miR-199 family members was found in EAC tissues compared to HGD, BE-ND and paired NSE tissues (all $P<0.006$), and among those, miR-199a-5p showed the highest median expression in EACs (7.70) (Figure 2A–C).

Significantly increased miR-223 levels were found in BE-ND (median: 2.21, $P=0.003$), HGD ($P=0.001$), and EAC ($P<0.001$) compared to their matched NSE tissue (Figure 2D). MiR-223 was up-regulated in all EAC tissues, and the median expression level in the EAC group (7.02) was significantly higher than in the HGD (2.73), BE (2.21) and cardiac group (1.81) (all $P<0.014$, Figure 2D). Up-regulation in a stepwise manner was revealed during EAC carcinogenesis ($P=0.002$, Figure 2D). From six patients two or more neoplastic lesions were available and analysis of miR-223 expression in these lesions confirmed that miR-223 is also up-regulated in a stepwise manner during the multistep progression of BE in any given individual (Figure 3A). MiR-223 expression was validated in a larger cohort of lesions obtained from treatment-naïve EAC patients using ISH. MiR-223 transcripts were present in 8.1%, 52.8%, 40.7%, 67.4%, 79.2%, 65.5% of the NSE, BE, LGD, HGD, EAC, and LNM tissues, respectively (Figure 3B). Diffuse expression was more often detected in EACs 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 (Figure 3C).

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

Low endogenous levels of miR-223 were detected in the EAC cell lines OE33 and JHesoAD1 compared to the normal esophageal cell line HEEpiC (data not shown). In order

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 EAC cells had lower cell numbers than scramble transfected EAC cells ($P<0.001$, data not shown). In contrast, additional *in vitro* assays showed a more aggressive phenotype in miR-223 than scramble transfected EAC cells; miR-223 transfected cells exhibited statistically significantly more migratory and invasive potential ($P<0.022$, Figure 4A).

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

In order to discover direct target genes of miR-223 that could explain the phenotype of miR-223 over-expressing cells in the esophagus, microarray experiments were performed in replicate on OE33 cells. The expression profiles of miR-223 transfected OE33 cells were compared with those of scramble transfected OE33 cells, and fifty-eight genes showed an average down-regulation of more than 1.5 fold in miR-223 relative to scramble transfected OE33 cells. The miR target gene prediction platforms, MicroCosm and Target Scan(27), were queried to determine whether binding of miR-223 to the 3 prime untranslated region (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 down-regulated genes (Supplemental Table S3).

Ten potential target genes were validated in replicate experiments in OE33 and JHesoAD1 cells using qRT-PCR. Nine genes were significantly down-regulated in miR-223 compared to scramble transfected OE33 cells, with *PARP1*, *CYB5A*, and *SMARCD1* as top candidates. The three top candidates were also found to be down-regulated upon miR-223 over-expression in JHesoAD1 cells (Table 1). We observed that the effect of miR-223 over-expression 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 EAC and matched NSE frozen biopsies. The patients were divided into two groups based on the miR-223 level in EAC: "low" (n=5, range: 1.5–3.6) and "high" (n=3, range: 17.1–137.8). *PARP1* and *SMARCD1* were significantly lower expressed in the "high miR-223" group ($P=0.036$, Figure 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. Co-transfection 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 to co-transfection with a scramble miR (Figure 4C). In addition, functional down-regulation of PARP1 protein was confirmed by Western blot analysis in OE33 and JHesoAD1 cells (Figure 4D).

PARP1 is involved in the repair of single stranded DNA 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* down-regulation, 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 ($P<0.001$, Figure 5A and B). MiR-223 transfected JHesoAD1 were also significantly less viable after Mitomycin C treatment at all tested concentrations ($P<0.001$, data not shown),

while miR-223 transfected OE33 cells were only more sensitive to 7.5–10 μ M Mitomycin C ($P=0.009$, data not shown) than scramble transfected cells. In longer term (T = 10 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 ($P<0.001$, Figure 5C). The IC₅₀ was reached at concentrations of 2.45 μ M and 6.00 μ M 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 co-staining of pH2A.X and 53BP1, are encountered in untreated ($P=0.032$) as well as with 1.25 μ M Doxorubicin treated ($P=0.008$) miR-223 expressing compared to scramble transfected OE33 cells (Figure 5D) indicating that the DNA repair mechanism is impaired in miR-223 OE33 cells. Together, these data suggest that EAC patients with high miR-223 levels in their EACs 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 miRs that are differentially expressed in EAC and BE; however, surprisingly little is known about the functional roles of these miRs in the multistep progression of BE. In this study we validated a panel of miRs that have previously been reported in the literature, on an independent cohort of treatment-naïve EAC patients. Only four miRs, miR-199a/b-3p, -199a-5p, -199b-5p, -223, showed a significant aberrant expression pattern in EAC compared to NSE, and these miRs were subjected to further analysis. For miR-223 a stepwise increase in expression was observed in the NSE-BE-dysplasia-EAC sequence. 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 up-regulated in gastric cancer tissues compared to normal gastric mucosae (29–31). Furthermore, it has been reported that miR-223 can be detected in serum of gastric cancer patients 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 over-expressed in the non-dysplastic precursors and HGD lesions in the esophagus, suggesting that we might be able to predict EAC 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, over-expression of miR-223 in EAC 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 over-expressing 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 over-expressing EAC cells. Other groups have previously also shown 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 EAC 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 appears 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 over-expression may lead to reduced chromatin remodeling activity, impaired transcription from the chromatin, and decreased p53 activity by directly inhibiting *SMARCD1* transcription.

PARP1 was down-regulated at both the transcript and protein level upon miR-223 over-expression in EAC 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 EACs with high miR-223 levels than lower miR-223 levels, indicating that this regulatory mechanism is intact *in vivo* in human tissue samples. PARP1 is a poly(ADP-ribose) polymerase, and 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, amongst 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 over-expressing EAC cells the DNA damage repair system is significantly impaired through direct down-regulation of *PARP1*. Induction of DNA damage by Doxorubicin resulted in significantly more DNA breaks in miR-223 over-expressing 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 since 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 EAC 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 EAC cells to Cisplatin, Mitomycin C and Doxorubicin, and found that miR-223 EAC cells were significantly more sensitive to these chemotherapeutics than scramble transfected OE33 cells in short term viability and longer term anchorage independent survival assays. These data suggest that EAC patients 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 since mRNA levels are greatly degraded in FFPE tissues. Secondly, the specimens must be obtained from treatment-naïve EAC patients, 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 up-regulated in the multistep progression of BE-ND to EAC in the majority of patients. MiR-223 over-expressing 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 endogenous levels of miR-223

in EACs were significantly associated with lower *PARP1* levels, and those patients might benefit more from treatment with DNA-damaging agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

53BP1	tumor suppressor protein 53 binding protein 1
BE	Barrett's esophagus
BE-ND	non-dysplastic Barrett's esophagus
cardiac	cardio-oxynitic
Cisplatin	cis-diamminedichloroplatinum (II)
CYB5A	cytochrome b5 type A
EAC	esophageal adenocarcinoma
FFPE	formalin-fixed paraffin-embedded
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IMC	intramucosal carcinoma
ISH	<i>in situ</i> hybridization
miR	microRNA
MTS	Cell Titer 96 [®] Aqueous One Solution Cell Proliferation Assay (Promega)
onco-miR	oncogenic microRNA
PARP1	poly(ADP-ribose) polymerase 1
pH2A.X	phosphorylated histone H2A.X
qRT-PCR	quantitative real-time PCR
SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1
TMA	tissue microarray
TS-miR	tumor suppressor microRNA

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STATEMENT OF TRANSLATIONAL RELEVANCE

Esophageal adenocarcinomas (EAC) typically present at an advanced stage of disease, and current chemo-radiation therapies have only modest impact on survival. In order to develop more effective therapeutic strategies, there is an urgent need for elucidating the molecular basis underlying EAC. MicroRNAs (miRs) could potentially help in identifying patients who would benefit from existing cancer therapy or represent targets for future therapy. Although prior studies have discovered aberrant miR signatures during EAC carcinogenesis, there is a lack of functional studies that could lead to improvements in therapeutic strategies.

In this study, we showed that miR-223, whose expression is progressively up-regulated during EAC carcinogenesis, behaves as an onco-miR in EAC. We identified the DNA damage repair gene *PARP1* as a direct target of miR-223, and demonstrated that miR-223 up-regulation results in increased sensitivity to chemotherapy by down-regulating *PARP1*, providing a potential therapeutic vulnerability node for exploitation in the clinic.

MicroRNA expression levels in 13 EAC tissues (qRT-PCR)

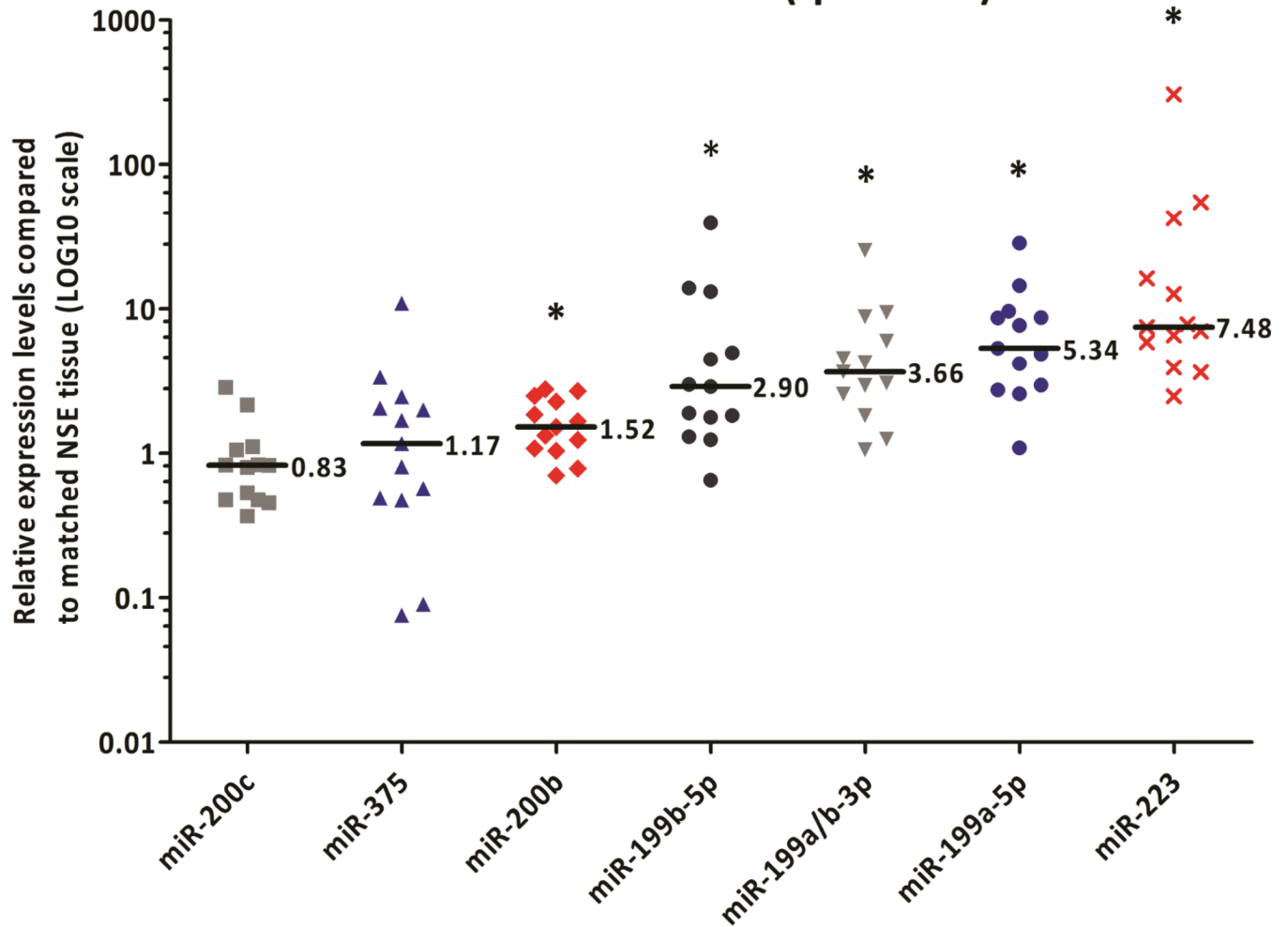


Figure 1.

MicroRNA validation in set 1. The miR-199 family members, miR-200b, and miR-223 were significantly up-regulated in EAC tissues compared to matched NSE tissues. The bar indicates the median expression levels, whereas the asterisk (*) depicts that the median significantly differs ($P < 0.05$) from the median in the matched NSE tissue.

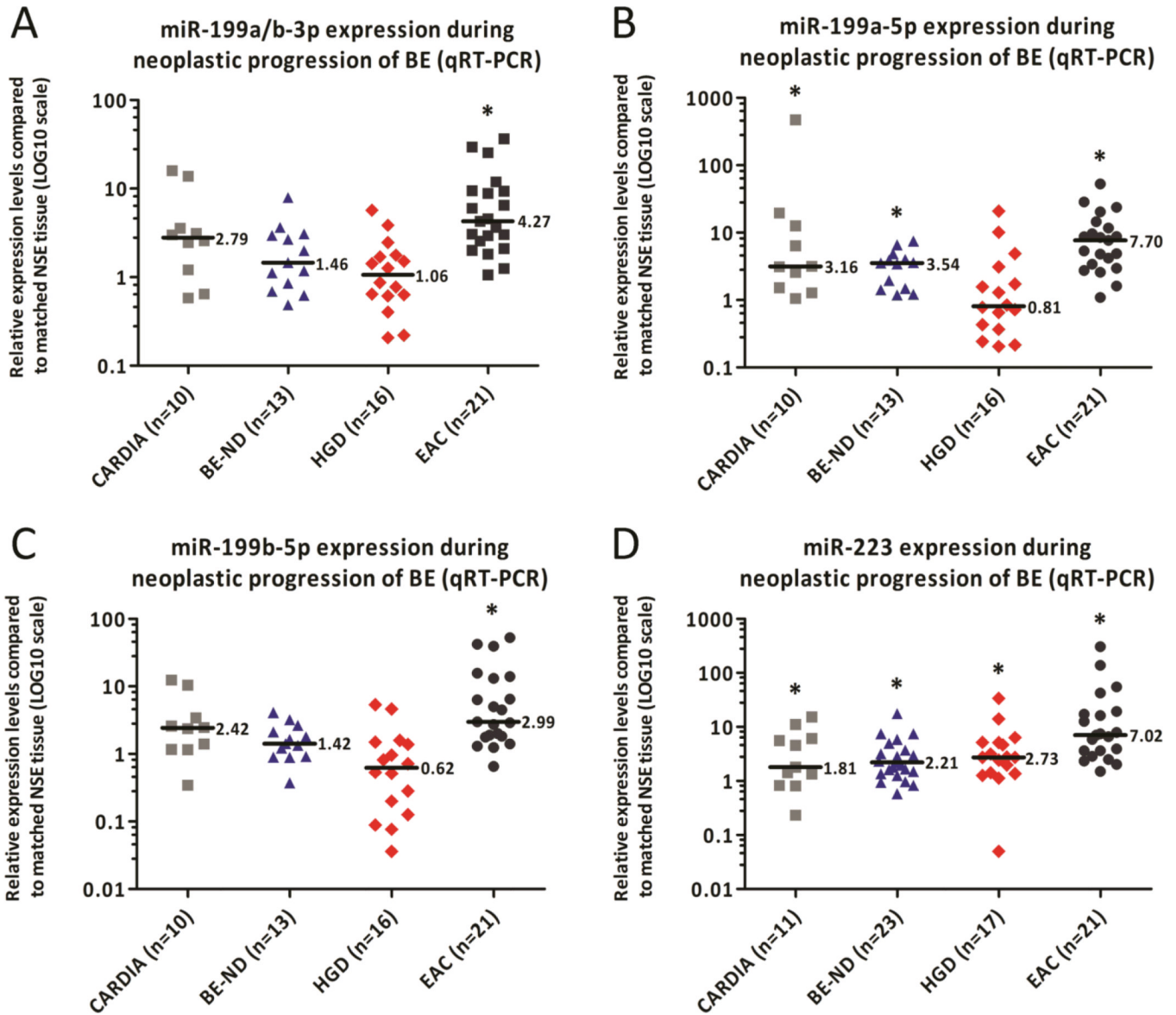


Figure 2. Validation of four microRNAs in set 2. MiR-199a/b-3p (A), -199a-5p (B), -199b-5p (C), and -223 (D) expression patterns were further analyzed in cardiac, BE-ND, HGD, and additional EAC specimens and matched NSE specimens. The highest median expression level in EAC was noted for miR-199a-5p (B), whereas miR-223 was the only miR showing stepwise up-regulation during the multistep progression towards EAC. Significantly higher miR-223 levels were detected in cardiac, BE-ND, HGD, and EAC compared to the paired NSE tissues (D). Notably, expression levels in cardiac mucosa and BE-ND showed great similarities across the four miRs. In these figures each dot represents a patient. The bar indicates the median expression level, whereas the asterisk (*) depicts that the median significantly differs ($P < 0.05$) from the median in the matched NSE tissue.

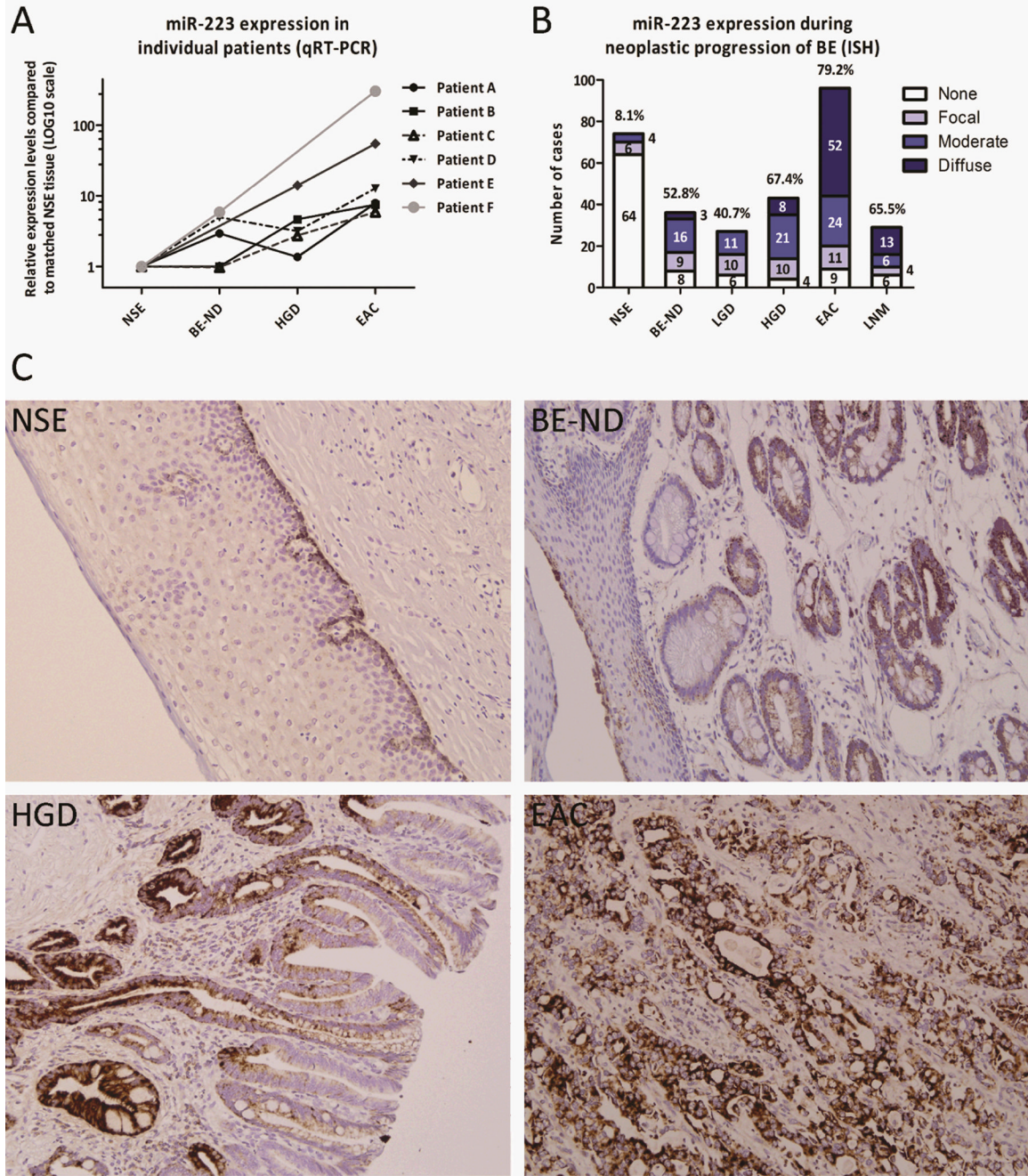


Figure 3. miR-223 in esophageal adenocarcinoma carcinogenesis. Panel (A) shows that miR-223 expression is up-regulated in a stepwise manner in six individuals (qRT-PCR). MiR-223 expression analysis in a large EAC cohort using ISH revealed miR-223 presence in 8.1%, 52.8%, 40.7%, 67.4%, 79.2%, and 65.5% of the NSE, BE-ND, LGD, HGD, EAC, and LNM tissues, respectively (B). The percentages above the columns in graph B indicate the total percentage of tissues exhibiting either moderate or diffuse miR-223 expression. Representative ISH photos in panel (C) illustrate the stepwise increased miR-223 expression from NSE, to BE-ND, to HGD, and ultimately EAC. MiR-223 expression was limited to the

(non-)dysplastic/neoplastic epithelial cells, and completely undetectable in inflammatory and stromal cells.

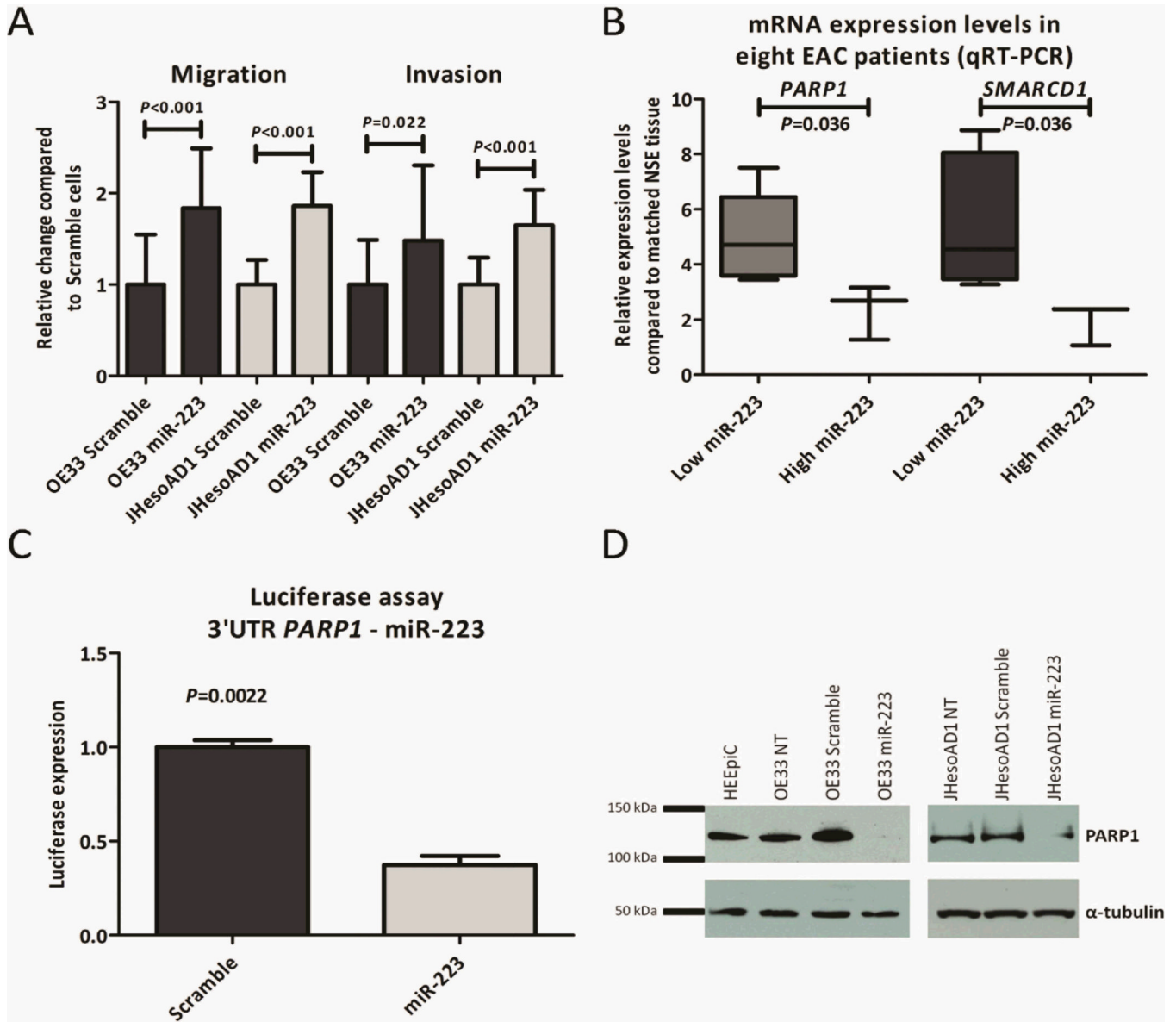


Figure 4. MiR-223 functional studies in esophageal adenocarcinoma cell lines. MiR-223 EAC cells showed significantly increased migratory and invasive potential compared to scramble EAC cells (A). Validation of the Illumina microarray results in OE33 and JHesoAD1 cells by qRT-PCR confirmed that *PARP1* and *SMARCD1* are prone to be direct target genes of miR-223 in the esophagus. Correlation of miR-223 and target gene expression in eight EAC patients 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 co-transfected 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 standard error of the mean.

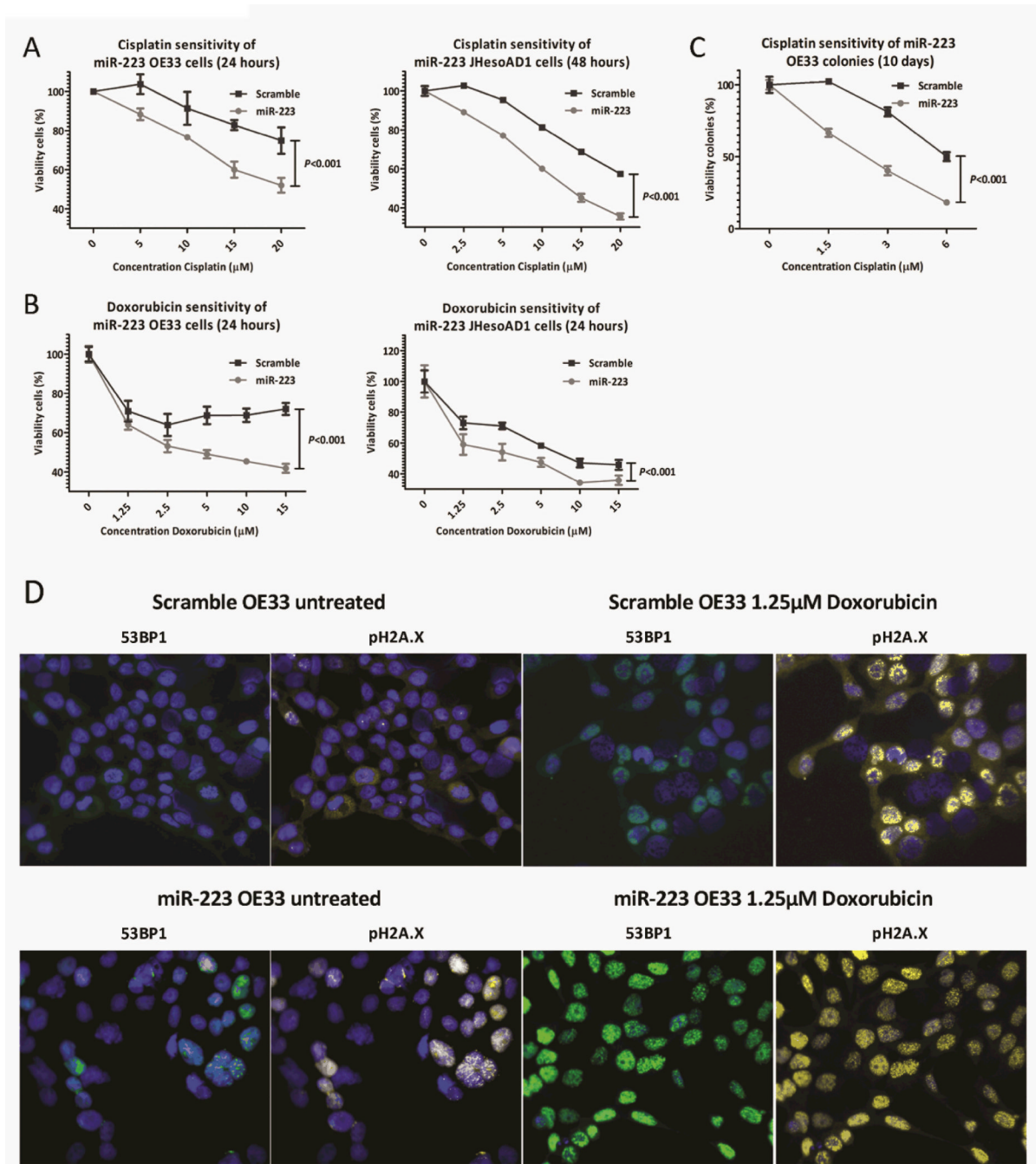


Figure 5. Since *PARP1* inhibition has been previously linked to increased chemotherapy sensitivity, we tested Cisplatin (A) and Doxorubicin(B) sensitivity of miR-223 OE33 and JHesoAD1 cells. MiR-223 OE33 and JHesoAD1 cells were significantly more sensitive to both chemotherapeutics compared to scramble cells in short-term MTS assays (B, 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 de 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). Panel (D)

shows example photomicrographs of the immunofluorescence experiment. Co-localization 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 to scramble transfected OE33 cells ($P < 0.05$).

Putative target genes of miR-223. 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 down-regulated in OE33 cells upon miR-223 transfection. Expression of *PARP1*, *CYB5A*, and *SMARCD1* was significantly inhibited in miR-223 compared to scramble JHesoAD1 cells.

Table 1

Gene	Full name	Average fold change miR223 vs Mock OE33 cells	Binding of miR-223 to 3'UTR gene predicted by:			Fold change qRT-PCR validation	
			Micro Cosm	Target Scan	Match miR-223 and 3'UTR gene	OE33*	JHesoAD1*
PARP1	Poly (ADP-ribose) polymerase 1	-2.42	Yes	Yes	8mer	-2.71**	-1.42**
MTIE	Metallothionein IE	-2.27	Yes	Yes	7mer-1A	-1.90**	-1.09
CYB5A	Cytochrome b5 type A (microsomal)	-1.91	Yes	Yes	8mer	-2.43**	-1.55**
SLC11A2	Solute carrier family 11, member 2	-1.84	Yes	Yes	8mer	-1.82**	-1.17
SLC39A1	solute carrier family 39, member 1	-1.84	Yes	Yes	7mer-m8	-1.79**	-1.21
SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1	-1.79	No	Yes	8mer	-1.98**	-1.52**
STMN1	Stathmin 1	-1.75	Yes	Yes	7mer-m8	-1.75**	1.15
GFPT1	Glutamine-fructose-6-phosphate transaminase 1	-1.65	Yes	Yes	8mer	-1.73**	-1.13
LMO2	LIM domain only 2 (rhombotin-like 1)	-1.60	No	Yes	8mer	-1.36	1.38**
KIAA1279	KIAA1279	-1.56	Yes	Yes	7mer-m8	-1.77**	-1.22

Key notes and abbreviations:

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';

* average of two biological replicated experiments;

** indicates $P < 0.05$;

3'UTR: 3' untranslated region.