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The *miR-106b-25* polycistron, activated by genomic amplification, functions as an oncogene by suppressing p21 and Bim

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Abstract

Background & Aims—Barrett’s esophagus (BE) is a highly premalignant disease that predisposes to the development of esophageal adenocarcinoma (EAC); however, the involvement of microRNAs (miRs) in BE-EAC carcinogenic progression is not known.

Methods—Esophageal cultured cells (HEEPiC, QhTERT, ChTERT, GihTERT and OE-33) and esophageal tissues (22 normal epithelia, 24 BE and 22 EAC) were studied. MiR microarrays and quantitative RT-PCR were employed to explore and verify differentially expressed miRs. Quantitative genomic PCR was performed to study genomic copy number variation at the *miR-106b-25* polycistron and *MCM7* gene locus on chromosome 7q22.1. *In vitro* cell proliferation, cell cycle, and apoptosis assays and *in vivo* tumorigenesis experiments were performed to elucidate biological effects of the *miR-106b-25* polycistron. Western blotting and luciferase assays were performed to confirm direct mRNA targeting by *miR-106b-25* polycistron.

Results—The *miR-106b-25* polycistron exerted potential proliferative, anti-apoptotic, cell cycle-promoting effects *in vitro* and tumorigenic activity *in vivo*. *MiRs -93* and *-106b* targeted and inhibited p21, while *miR-25* targeted and inhibited Bim. This polycistron was upregulated progressively at successive stages of neoplasia, in association with genomic amplification and overexpression of *MCM7*. In addition, *miRs -93* and *-106b* decreased *p21* mRNA, while *miR-25* did not alter *Bim* mRNA, suggesting the following discrete miR effector mechanisms: 1) for p21, mRNA degradation; 2) for Bim, translational inhibition.

Conclusions—The *miR-106b-25* polycistron is activated by genomic amplification and is potentially involved in esophageal neoplastic progression and proliferation via suppression of two target genes, p21 and Bim.

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Introduction

Barrett's esophagus (BE) is a highly premalignant disease that predisposes to the development of esophageal adenocarcinoma (EAC), one of the most rapidly increasing cancers in developed nations 1. The prevalence of BE in United States endoscopic series ranges from 0.9% (of 51,311 patients) 2 to 4.5% (of 833 patients) 3. A recent study from Sweden reported a prevalence of BE of 1.6% (among 21,610 patients) 4. These findings imply that more than 3 million individuals may harbor BE in the United States alone 5. Among these patients, EAC susceptibility is a life-threatening long-term sequela. The incidence of EAC in BE patients is estimated 1.8 per 1,000 person-years, 30-fold higher than in the general population 6.

The molecular genetics of BE and its evolution to EAC have been studied 7–10. Nonetheless, molecular mechanisms underlying the BE-EAC carcinogenic sequence remain unclear, and a more thorough understanding of the molecular basis of this process would yield several benefits. These benefits include: 1) clues to biological pathways underlying Barrett's-associated neoplastic transformation; 2) useful biomarkers of early cancer detection, disease progression, or ultimate prognosis; and 3) therapeutic targets for intervention in both the prevention and the treatment of this disease.

MicroRNAs (miRs) are a class of abundant, approximately 22 nucleotide non-coding RNAs that mediate post-transcriptional regulation of target mRNAs. Most known functions of miRs relate to negative gene regulation: miRs silence gene expression, usually by interfering with mRNA stability or protein translation. Cancer-specific miR fingerprints have been identified in every tumor type examined. Emerging evidence reveals that a number of miRs are involved in tumorigenesis and/or tumor progression 11, 12. MiR regulatory mechanisms involved in cancer progression may be viewed as previously missing pieces of a puzzle that is now essential to understanding the complete molecular genetic landscape of cancer progression. However, thus far, there has been only one report that describes miR expression in BE and EAC 13.

Herein, we describe novel findings showing that: (1) the *miR 25-93-106b* polycistron is activated in BE-associated neoplastic transformation; (2) DNA copy number variation is closely associated with activation of the *miR 25-93-106b* polycistron; and (3) the *miR 25-93-106b* polycistron exerts cell-proliferative and oncogenic effects via key downstream regulatory targets.

Materials and Methods

See also supplementary documents for full description.

Cell Culture

A human esophageal adenocarcinoma cell line, OE-33, three metaplastic BE-derived cell lines, QhTRT, ChTRT and GihTRT 14, human normal non-immortalized primary esophageal epithelial cells, HEEpiC, and human normal lung fibroblasts, WI-38 were used.

Tissue Specimens

All patients provided written informed consent under a protocol approved by the Institutional Review Boards at the University of Maryland and Baltimore Veterans Affairs Medical Centers. Clinicopathological information is displayed in Supplementary Table S1.

MicroRNA Microarrays

MiRNA Labeling Reagent and Hybridization Kits and Human miR Microarray Kits (Agilent) were used. Data was collected and normalized to non-functional small RNA internal controls.

Quantitative RT-PCR (qRT-PCR) for miR Expression

TaqMan MicroRNA Assays, Human (Applied Biosystems) were used. All qRT-PCRs were performed in triplicate. *RNU6B* was used as an internal control.

Quantitative RT-PCR for mRNA Expression and Quantitative Genomic DNA PCR

iQ5 Multicolor Real-Time PCR Detection System and iQ SYBR Green Supermix (BIO-RAD) were used for both quantitative RT-PCRs of mRNA expression and quantitative genomic DNA PCRs. Primer sequences are available in Supplementary Table S2. All PCRs were performed in triplicate. *Beta actin* was used to normalize mRNA expression levels. An *Alu* sequence¹⁵ was used to normalize quantitative genomic DNA PCR.

Transfection of miR Mimics and Inhibitors

Synthesized RNA duplexes of miR mimics, miR inhibitors and small interfering RNA duplex against *p21* were purchased from Dharmacon. Cells were transfected with 60nM of each miR mimic, inhibitor or siRNA.

Cell Proliferation Assay

Cells were plated onto 96-well plates at day 0. At every other day until day 5, Cell Proliferation Reagent WST-1 (Roche) was added to each well, incubated at 37°C for 1 hour and an optical density was measured.

Ki-67 staining

OE-33 cells were transfected with miR-25-INH, miR-93-INH, miR-106b-INH, or NSC-INH. 48 hours after transfection, cells were fixed in 100% acetone. Ki-67 positive cells were detected by using anti-Ki-67 antibody (Invitrogen) and an UltraVision Detection System (Thermo Scientific Inc.).

In Vivo Tumorigenesis

Suspensions of 2×10^6 OE-33 cells transfected with miR inhibitors were mixed with BD Matrigel™ Basement Membrane Matrix (BD). Cells were injected subcutaneously into both flanks of athymic nude mice (Harlan) at day 0. Tumor growth was estimated by averaging the volumes of tumors at eight sites. Tumor volume was calculated according to the following formula: $L^2/2 \times W$, where L is the length and W is the width of the tumor. All animal procedures were performed in accordance with institutional guidelines under a protocol approved by the Johns Hopkins Animal Care and Use Committee.

Western Blotting

For CDKN1A (p21^{Cip1/WAF1}), rabbit anti-p21^{Cip1/WAF1} from Invitrogen was used, while for BCL2L11 (Bim), rabbit-anti-Bim from Cell Signaling Technology was used. For an internal control, mouse anti-beta actin monoclonal antibody was used.

Luciferase Reporter Assay

Full-length *p21* 3'-untranslated region (3'-UTR) and truncated *Bim* 3'UTR, common to both *Bim EL* and *Bim L*, flanking miR responsive sites were amplified. We obtained plasmid clones pGL4.13 (Promega) containing correctly oriented inserts (pGL4.13-P21UTR and pGL4.13-BimUTR). Plasmid clones that contained reversely oriented inserts (pGL4.13-revP21UTR and pGL4.13-revBimUTR) were used as universal negative controls for the assay. We also constructed plasmids containing p21-3'UTR with mutated seed regions for the predicted miR-93/miR-106b binding sites (pGL4.13-mutP21UTR), along with plasmids containing the Bim-3'UTR with mutated seed regions for the predicted miR-25 binding sites (pGL4.13-

mutBimUTR). Primer sequences are available in Supplementary Table S2. The constructed pGL4.13 vector and an internal control pRL-CMV (Renilla luciferase) vector were co-transfected 24 h after miR mimic or inhibitor transfection. 24 hours after plasmid vector transfection, the luciferase reporter assay was performed using a Dual-Glo luciferase assay kit (Promega). The luminescence intensity of Firefly luciferase was normalized to that of Renilla luciferase.

Statistical Analysis

Results of experiments were displayed as mean $\pm$ standard deviation in the figures. To evaluate statistical significances, Student's unpaired *t* test was used, otherwise noted.

RESULTS

MiR Microarrays Define Novel EAC-specific Candidate Tumor-suppressive miRs and Oncogenic miRs

As a first approach to study the involvement of miRs in BE-associated neoplasms, we performed miR microarray investigations of non-immortalized primary normal esophageal (NE) epithelial cells (HEEpiC), BE-derived cell lines (QhTRT, ChTRT and GihTRT), and an EAC-derived cell line (OE-33). Representative results of these experiments are displayed in Table 1. Several miRs were aberrantly regulated in BE or EAC cells *vs.* NE cells. In particular, we found that *miRs* -25, -93 and -106b, all of which form a polycistron on chromosome 7q22.1 (the *miR*-106b-25 polycistron), were significantly upregulated in OE-33 cells relative to BE and HEEpiC cells, suggesting possible involvement of this polycistron as an oncogene in BE-associated neoplastic transformation.

Quantitative Reverse-Transcriptase PCR (qRT-PCR) Verifies miR Microarray Results

Next, we validated miR microarray results for 7 miRs that were differentially up- or downregulated in BE or EAC cells (*miRs* -25, -93, and -106b in Figures 1A–1C; *miRs* -19b, -100, -125b and -205 in Supplementary Figure S1 and **bold** in Table 1). Using real-time qRT-PCR, we analyzed the same EAC and BE, and NE cells that were assessed on miR microarrays. Results of these qRT-PCRs verified those obtained by miR microarray assays, as shown.

Clinical Significance of Aberrant miR Expression

We also performed qRT-PCRs of these differentially expressed miRs (except *miR*-19b, Figures 1D–1F and Supplementary Figure S2) in primary human esophageal tissue specimens to address the clinical significance of their aberrant expression. In all cases examined, tissue miR expression results correlated closely with results obtained in cell lines. In particular, all three members of the *miR*-106b-25 polycistron were not significantly upregulated in BE relative to NE tissues (*p* = 0.14, 0.26 and 0.35 respectively), however, notably, these miRs were markedly upregulated in EAC relative to NE and BE. These results implied progressive involvement of the *miR*-106b-25 polycistron in BE-EAC neoplastic evolution.

Regulatory Mechanisms Underlying miR-25, -93 and -106b Activation

Since the *miR*-106b-25 polycistron is located within intron 13 of the minichromosome maintenance protein 7 (*MCM7*) gene on chromosome 7q22.1, we hypothesized that activation of these miRs should correlate with *MCM7* “mother gene” activation. To investigate this hypothesis, we first examined *MCM7* mRNA levels in the same cell lines using expression microarrays and qRT-PCR (Figure 2A). Indeed, we found that *MCM7* mRNA expression was upregulated in BE-derived *vs.* HEEpiC NE cells; and notably, *MCM7* was more markedly upregulated in OE-33 EAC cells than in BE-derived cells, reflecting the same stage-related activation pattern seen with the *miR*-106b-25 polycistron. Analogous *MCM7* mRNA

expression results were obtained in tissue specimens (Figure 2B). Secondly, we performed quantitative genomic DNA PCR of the *MCM7* mother gene to evaluate whether DNA copy number variation (CNV) constituted a mechanism underlying its activation. Interestingly, this experiment revealed a progressive increase in DNA copy number at the *MCM7* locus during neoplastic evolution, proceeding from NE-derived, to BE-derived, and finally to EAC-derived cells (Figure 2C). Similarly, we observed significant DNA copy number increases at the *MCM7* locus in primary esophageal tissue specimens (NE vs. BE: $p=0.0022$; NE vs. EAC: $p=0.00013$, Figure 2D). We also discovered significant *de novo* CNV of the *MCM7* locus in paired NE and EAC tissue specimens from 11 patients ($p=0.0037$ by paired *t* test in Figure 2E). Figure 3 showed a close relationship between miRs -25, -93 and -106b expressions and CNV at the *MCM7* locus in tissue specimens (Pearson's correlation coefficient test; $p=0.0014$, 0.011 and 0.019 respectively).

Biological Impact of miR-25, -93 and -106b

In Vitro Effects—To elucidate cancer-relevant biological effects of *miRs* -25, -93 and -106b, we first performed cell number assays using WST-1 reagent. Inhibition of *miRs* -25, -93 and -106b induced significantly decreased numbers of OE-33 EAC cells (Figure 4A). We also performed *miR*-25-, -93- and -106b-mimic transfection of a BE-derived cell line (ChTRT), as well as of WI-38 normal lung fibroblasts. WI-38 cells were used as representative non-neoplastic cells because HEEpiC cells were not adequate for these experiments, due to their resistance to nucleic acid transfection and slow growth (data not shown). We also chose WI-38 cells because their expression levels of *miRs* -25, -93, and -106b were as low as those in HEEpiC cells (data not shown). Interestingly, miR mimic transfection induced significantly increased numbers of both BE and WI-38 cells, although the three miR mimics exerted this effect to differing degrees (Figures 4B and 3C). Ki-67 staining showed that the number of Ki-67 positive cells was diminished in OE-33 cells transfected with miR-25-INH, miR-93-INH or miR-106b-INH relative to the number in cells transfected with NSC-INH (Supplementary Figure S7). Next, we performed experiments to assess the effects of these miRs on cell cycle progression. Inhibitors (INHs) of *miRs* -93 and -106b increased slightly but significantly the proportion of cells in G1 phase (nonspecific control inhibitor, NSC-INH: 51.46%, miR-93-INH: 53.38%, $p=0.0028$, and miR-106b-INH: 55.14%, $p=0.032$) in OE-33 cells. Similar effects of *miRs* -93 and -106b were observed with *miR*-93-MM and *miR*-106b-MM in ChTRT, GihTRT and QhTRT cells (data not shown). Next, to gauge the effects of these miRs on programmed cell death, we performed apoptosis assays using miR inhibitors. In these assays, *miR*-25-INH slightly but significantly augmented the proportion of cells in early apoptotic phase (NSC-INH: 5.00%; *miR*-25-INH: 5.76%, $p=0.011$).

In Vivo Tumorigenesis—Our discovery that the *miR*-106b-25 polycistron induced increased cell numbers *in vitro* prompted us to evaluate its effects *in vivo*. As shown in Figure 4D, inhibitors of *miRs* -25, -93 and -106b reduced *in vivo* tumorigenesis in nude mice. For all three miR inhibitors, the most marked inhibitory effects were observed at 5 days post-transfection (Day 4 in Figure 4D).

The miR-106b-25 Polycistron Directly Regulates p21 and Bim

We next focused our attention on mRNA targets of *miRs* -25, -93 and -106b. To identify these targets, we employed two principal strategies: 1) focusing candidate mRNA searches on results of our phenotypic experiments, *e.g.*, cell cycle- and apoptosis-related genes; 2) performing database searches in miR target prediction engines, such as miRBase (<http://microrna.sanger.ac.uk/cgi-bin/targets/v4/search.pl>), TargetScan (http://www.targetscan.org/vert_40/) and PicTar (<http://pictar.bio.nyu.edu/>). In our cell cycle experiments, *miRs* -93 and -106b exerted internally consistent effects on the G1/S checkpoint: Inhibitors of *miRs* -93 and -106b both increased the proportion of cells in G1 phase (*i.e.*, induced

G1 arrest) in OE-33 EAC cells. This finding prompted us to search for G1/S-regulatory proteins, and in combination with 3'UTR homology searches, we ultimately identified a CDK inhibitor and known tumor suppressor gene, CDKN1A (p21^{Cip1/WAF1})¹⁶. Western blotting revealed that p21 protein expression was sharply reduced by *miR-93* and *miR-106b* mimics, and correspondingly, that p21 protein expression was sharply increased by *miR-93* and *miR-106b* inhibitors (Figures 5A and 5B). To evaluate direct binding to and silencing of p21 by *miRs* -25, -93, and -106b, we then performed luciferase assays of the *p21* 3'UTR. Luciferase reporter activity was significantly reduced by *miRs* -93 and -106b ($p=0.0015$ and 0.0049 for *miR-93* and $p=0.00078$ and 0.00087 for *miR-106b* in Figures 5C and 5D, respectively) but not by *miR-25* (Supplementary Figures S6A and S6B), suggesting that *miRs* -93 and -106b bind directly to the *p21* 3'UTR and inhibit p21 protein expression.

Similarly, in our apoptosis experiments, miR inhibitors increased the population of apoptotic cells, thus, in combination with 3'UTR homology searches, we identified the pro-apoptotic tumor suppressor gene, BCL2L11 (BCL2-like 11 or Bim)¹⁷. Bim protein exists in two major isoforms: Bim EL and Bim L, and both of them have the same 3'UTR sequence. Western blotting revealed that the expression of both Bim isoforms was reduced by a *miR-25* mimic and increased by a *miR-25* inhibitor (Figures 6A and 6B). Luciferase reporter activity was significantly reduced by *miR-25* ($p=0.0012$ and 0.0096 in Figures 6C and 6D, respectively) but not by *miRs* -93 and -106b (Supplementary Figures S6C and S6D).

Regulatory Effects of the miR-106b-25 Polycistron on p21 and Bim

Next, to investigate whether suppression of p21 and Bim protein expression was due to mRNA degradation, we performed qRT-PCRs of *p21* and *Bim* mRNA after transfecting mimics of *miRs* -25, -93, and -106b. An siRNA directed against *p21* served as a positive control. qRT-PCRs showed that *p21* mRNA expression was diminished approximately 30% by the *miR-93* mimic and 40% by the *miR-106b* mimic, vs. 94% by the control *p21*-siRNA (Figure 7A). *Bim* mRNA expression did not change following transfection of a *miR-25* mimic (Figure 7B).

Discussion

Although the first microRNA, *lin-4*, was discovered in 1993¹⁸ and the second miR, *let-7*, in 2000¹⁹ in *Caenorhabditis elegans*, only recently has the study of this class of small regulatory RNAs in humans become more widespread²⁰. Experimental evidence that has accumulated in recent years has led oncologists to speculate that unrevealed molecular actors, particularly non-coding RNAs previously been classified as "junk," play important roles in carcinogenesis. In fact, approximately 70% of the genome has been found to be transcribed in mammals²¹. Thus, it is a natural extension of this logic to explore novel noncoding RNA-based mechanisms underlying cancer-related gene dysregulation, even mechanisms unanticipated prior to the discovery of *lin-4* and *let-7*. MicroRNAs (miRs) are now intensively studied in cancer research, and these molecules are predicted to control as much as 30% of all gene expression²².

To our knowledge, the current findings constitute the first report documenting (1) activation of the *miR-106b-25* polycistron by DNA copy number variation (CNV); (2) miR biologic effects in esophageal cells; and (3) *in vivo* tumorigenic properties of the *miR-106b-25* polycistron.

Several of the miRs listed in Table 1 have been previously implicated in carcinogenesis. Among miRs upregulated in the current study, *miR-200a* in ovarian cancer²³ and *miR-15b* in gastric cancer²⁴ have been reported as potential oncomiRs. Among miRs that were downregulated in the current study, members of the *let-7* family have been implicated in lung cancer²⁵, as have members of the *miR-34* family in colorectal cancer²⁶ and of the *miR-125* family in prostate

cancer²⁷. The *miR-17-5p-92* and *106a-92* polycistrons have also been documented to function as oncomiRs and are highly homologous to each other, as well as to the *miR-106b-25* polycistron¹². Surprisingly, members of the *miR-17-5p-92* and *106a-92* polycistrons (*viz.*, *miRs-17-5p*, *-19b*, *-20a*, *-20b*, *-92* and *-106a* in Table 1) were *downregulated* in EACs in the current study, suggesting the possibility that the *miR-106b-25* polycistron, with its overexpression and high homology to these other polycistrons, may actually *compensate* for a “perceived” deficit in oncogenic activity of the (downregulated) *miR-17-5p-92* and *106a-92* polycistrons.

Among BE tissue specimens in the current study, there were 12 BE metaplasias, 4 low-grade dysplasias (LGDs), and 8 high-grade dysplasias (HGDs; Supplementary Table S1). Expression levels of *miRs -25*, *-93* and *-106b* did not differ significantly between NE and any histologic grade of BE. Moreover, all clinical characteristics, including prognosis (not shown), did not correlate with expression levels of *miRs -25*, *-93*, or *-106b*.

We found that expression of *miRs -25*, *-93*, *-106b*, and *MCM7* mRNA in NE, BE and EAC correlated closely in both cell lines and tissue specimens (Figures 1, 2A and 2B); however, relative levels of these three miRs differed from those of *MCM7* mRNA, particularly in EAC cells and tissues. Specifically, in EAC, the fold-change of *MCM7* mRNA expression was higher than that of the *miR-106b-25* polycistron (Figures 1A–1C *vs.* 2A, and Figures 1D–1F *vs.* 2B). This contrasting result suggests that unique regulatory mechanisms may also underlie *miR-106b-25* polycistron *vs.* *MCM7* mRNA expression in EAC.

One novel finding was that CNV of *MCM7* locus correlated closely with *miR-106b-25* polycistron expression, not only in NE, BE and EAC cell lines (Figures 1A–1C *vs.* 2C), but also in tissues (Figures 1D–1F *vs.* 2D and 3). CNV of *MCM7* in the three metaplastic BE-derived cell lines (QhTERT, ChTERT and GihTERT) averaged 1.4-fold higher than in HEEpiC NE cells, while CNV of *MCM7* in OE-33 EAC-derived cells was much higher (5.8-fold; Figure 2D). These findings suggest that cumulative CNV occurs during BE neoplastic transformation, possibly at the transition between HGD and EAC. We also observed a close correlation between *miR-106b-25* polycistron expression and CNV in matched RNA-DNA pairs from the same individuals ($p=0.0014$, 0.011 and 0.019 , respectively; Figure 3). These findings also support the conclusion that the *miR-106b-25* polycistron is activated by genomic copy number variation at least in a proportion of cases. To our knowledge, this is the first direct evidence of a relationship between *miR-106b-25* polycistron activation and CNV during neoplastic evolution of any human cancer. There have been several articles describing concomitant *de novo* genomic amplification, a type of CNV, and miR overexpression^{28, 29}. Recent reports have noted that although most CNV is inherited as a polymorphic trait³⁰, *de novo* CNV can also occur³¹. Figure 2E shows that among our paired normal-tumor DNA samples, most manifested *de novo* CNV, *i.e.*, somatic DNA amplification, at the *MCM7* locus. Although there were only two paired BE-EAC samples in the current study, we observed the same trend in *de novo* CNV for *MCM7* in these two paired BEs and EACs (Relative amplification: 1.293 to 1.504 and 1.159 to 1.177). Further studies are needed to ascertain whether inherited polymorphic CNV occurs in normal subjects, since only normal tissues from EAC patients were included in the current study. Taken together, these results strongly imply that genomic DNA copy number variation at the *MCM7* locus was at least partially responsible for coordinate activation of both the *miR-106b-25* polycistron and of the *MCM7* gene, and that this CNV increased progressively during BE-associated neoplastic evolution.

We also studied cancer-related biologic effects of the *miR-106b-25* polycistron. All three members of this polycistron induced increased cell numbers in normal esophageal, BE-derived and EAC-derived cell lines. These three miRs exhibited unique degrees of cell type-specific targeting in their cell number-increasing effects (Figure 4): *i.e.*, *miRs-93* and *-106b* induced

greater increases in cell numbers in normal fibroblasts than did *miR-25*, while *miR-25* induced greater increases in cell numbers in BE-derived cells, and *miR-93* was the most but *miR-25* the least influential in EAC-derived cells. Thus, which member of the *miR-106b-25* polycistron most markedly affected cell numbers depended on cell type. The *miR-25* mimic effect rapidly decreased from days 3 to 5 only in WI-38, but not in ChTRT, QhTRT and GihTRT cells (QhTRT and GihTRT not shown in Figure 4). In contrast, the effects of *miR-93* and *-106b* persisted at least until day 5 in all cell lines examined. We were unable to determine exactly what caused this unique difference in effects; however, we speculate that WI-38 may have degraded or inactivated *miR-25* more rapidly than did the other cell lines we examined. One notable feature of oncomiR polycistrons, including *miR-106b-25*, is that coordinated activation involving the entire polycistron may enhance its overall oncogenic effect. Moreover, *MCM7* itself is oncogenic in prostate cancer³². Thus, DNA copy number variation occurring at the *MCM7* locus may enhance *miR*-derived oncogenesis by activating *MCM7* simultaneously with the *miR-106b-25* polycistron.

OE-33 EAC cells grow slowly in athymic nude mice and are mildly rejected during the course of several weeks (Supplementary Figure S3) with peak tumor size usually occurring within 7 days and maximal tumor volume reaching only 40 mm³ on average, a size at which experimental effects are difficult to discern. We overcame this difficulty by introducing Matrigel reagent, which accelerated cell growth and appeared to protect these cells from their potentially hostile *in vivo* environment. According to qRT-PCR measurement of *miR-25*, *-93* and *-106b* following *miR* mimic transfections, levels of these *miR*s remained quite high (24.0-, 23.6-, and 40.0-fold change, respectively) at 6 days post-transfection (Supplementary Figure S4), indicating that these transfected small RNAs continued to be biologically effective for at least 6 days post-transfection. As shown in Figure 4D, antagonists of *miR-25*, *-93* and *-106b* exerted inhibitory effects on *in vivo* tumorigenesis in nude mice, further supporting the *in vitro* cell number-increasing effects of these *miR*s.

Negative regulation of *miR* target mRNAs may be due to either protein translation inhibition or to mRNA degradation. Figures 7A show that *miR-93* and *-106b* caused at least some degree of *p21* mRNA instability (though not as much as that caused by *p21* siRNA), while *miR-25* did not affect *Bim* mRNA levels (Figure 7B). These findings were further corroborated by Ivanovska *et al.*³³, who found that *miR-106b* reduced *p21* mRNA by approximately 40% in human mammary epithelial cells. However, our data only partially agrees with a recent study of gastric cancers, which showed no apparent effects of *miR-25*, *-93* and *-106b* on RNA stability³⁴. Taken together, our findings suggest that these two mRNA targets are regulated by discrete *miR* effector mechanisms: 1) for *p21*, mRNA degradation; 2) for *Bim*, translational inhibition.

Identifying *miR* target mRNAs poses a challenge to investigators. Target prediction engines often yield hundreds of potential candidate mRNAs, compelling investigators to struggle with the choice of which targets to focus on. One promising strategy to overcome this challenge is to compare global *miR* and mRNA expression profiles in the same starting materials³⁵. Judging from our own results (Figures 7A and 7B), *p21* would have been detected by such a comparison, while *Bim* would not. Such global comparisons are useful only when *miR*s effect a decrease in their target mRNA expression levels; moreover, this strategy may also unearth mRNAs whose stability or expression are not directly targeted by particular *miR*s. Thus, more efficient and innovative approaches to identify mRNA targets of *miR*s are still needed.

In conclusion, the *miR-106b-25* polycistron exerted potential proliferative, anti-apoptotic, cell cycle-promoting effects *in vitro*, tumorigenic properties *in vivo* and was progressively activated progressively at successive stages of neoplasia from NE to BE and finally to EAC, potentially due to *de novo* genomic DNA copy number variation of the *MCM7* locus at chromosome

7q22.1. The *miR-106b-25* polycistron thus represents a potential oncogene in BE-EAC carcinogenic evolution, as well as in other human cancers, and may act via regulation of two specific target genes, p21 and Bim.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BE	Barrett's esophagus
CNV	copy number variation
EAC	esophageal adenocarcinoma
INH	inhibitor
MM	mimic
NE	normal epithelium

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Figure 1. Quantitative RT-PCR of *miR-106b-25* polycistron in esophageal cultured cells and tissues (A–C) Microarray and qRT-PCR results for *miRs* -25, -93 and -106b are shown in esophageal cells. The values of HEEpiC are set at 1 for both experiments. As shown, results of qRT-PCR correlated closely with those of microarrays. Both methods revealed that *miR-106b-25* polycistron expression was gradually upregulated progressively at successive stages of neoplasia from NE to BE to EAC cells. (D–F) 22 NE (normal esophageal epithelial tissue), 24 BE, and 22 EAC tissues were studied. The average value of NE is set at 1. Horizontal bars indicate average levels. *MiRs* -25, -93 and -106b were significantly upregulated in EAC relative to NE and BE.

Figure 2. *MCM7* mRNA expression and CNV in esophageal cultured cells and tissues
(A) qRT-PCR and gene expression microarray results of *MCM7* are shown in cultured esophageal cells. The value of HEEpiC is set at 1. Both methods revealed that *MCM7* expression was gradually upregulated during neoplastic progression from NE to BE to EAC cells. *MCM7* exhibited an expression pattern similar to that of the *miR-106b-25* polycistron (Figures 1A–1C). **(B)** 22 NE, 24 BE, and 22 EAC tissues were studied. The average value of NE is set at 1. Horizontal bars indicate average levels. *MCM7* was significantly upregulated in EAC relative to NE and BE. **(C)** qPCR of *MCM7* locus is shown in cultured esophageal cells. The value of HEEpiC is set at 1. The CNV pattern of the *MCM7* locus in the esophageal cultured cells correlated with those of both *miR-106b-25* polycistron expression (Figures 1A–1C) and *MCM7* mRNA expression (Figure 2A). **(D)** 24 NE, 22 BE, and 24 EAC tissues were enrolled. The average value of NE is set at 1. Horizontal bars indicate average levels. CNV of the *MCM7* locus in tissues showed a gradual and significant upregulation during neoplastic

progression from NE to BE to EAC. CNV ratio in EAC averaged 1.37-fold across all specimens, but 29.2% (7/24) of EACs exhibited significant genomic copy number increase (≥ 1.5 -fold *vs.* NE). (E) CNV of the MCM7 locus in 11 paired tissue specimens. The MCM7 locus was statistically significantly amplified in EAC tissues relative to paired NE tissues. Paired average fold change (EAC/NE) equalled 1.67 ($p = 0.0037$, paired t test).

Figure 3. Expression levels of miRs -25, -93 and -106b correlated significantly with CNV levels in primary esophageal tissues (Pearson's correlation coefficient test, $p = 0.0014, 0.011$ and 0.019 , respectively). The average value of NE was set at 1 for both miR expression and CNV determinations. 11 NE, 5 BE, and 9 EAC specimens were analyzed.

Figure 4. Cell Proliferation Assessed by WST-1 and *in vivo* Tumorigenesis Assays

OE-33 cells were transfected with either a nonspecific control inhibitor (NSC-INH), *miR-25*-INH, *miR-93*-INH or *miR-106b*-INH. WI-38 and ChTRT cells were transfected with either NSC mimic (NSC-MM), *miR-25*-MM, *miR-93*-MM or *miR-106b*-MM. WI-38 cells were also transfected with a miR cocktail (20nM each of *miRs* -25, -93 and -106b). Each experiment was performed in 6 replicates. (A) *Mir-25*, -93 and -106b inhibitors significantly inhibited proliferation in OE-33 cells (* $p = 1.3 \times 10^{-5}$, ** $p = 6.6 \times 10^{-9}$, *** $p = 3.6 \times 10^{-7}$, at Day 5, respectively). (B) *Mir-25*, -93 and -106b mimics significantly increased the number of ChTRT cells ($^{\dagger}p = 2.3 \times 10^{-9}$, $^{\dagger\dagger}p = 7.1 \times 10^{-6}$, $^{\dagger\dagger\dagger}p = 1.6 \times 10^{-7}$, at Day 5, respectively). (C) *Mir-93*, -106b, and miR cocktail mimics significantly increased the number of WI-38 cells ($^{\$}p = 8.2 \times 10^{-7}$, $^{\$ \$}p = 7.5 \times 10^{-7}$, $^{\$ \$ \$}p = 3.3 \times 10^{-7}$, at Day 5, respectively), while *miR-25* significantly increased cell numbers at Day 3 ($^{\$ \$ \$ \$}p = 1.0 \times 10^{-6}$), but not at Day 5. (D) 2×10^6 OE-33 cells transfected (Day -1) with either *miR-25*, -93, -106b or NSC-INH were implanted (Day 0) subcutaneously into the flanks of nude mice (N=8 each). *Mir-25*, -93 and -106b inhibitors significantly inhibited tumorigenesis of OE-33 cells relative to NSC-INH in nude mice.

Figure 5. p21 Western Blotting and Luciferase Assays

(**A** and **B**) Western blotting for p21. WI-38 cells were transfected with *miR-93*, *-106b* or NSC-MM, while OE-33 cells were transfected with *miR-93*, *-106b* or NSC inhibitors-INH. (**C** and **D**) Luciferase assays for *p21* 3'UTR. ChTERT and GihTERT cells were performed. Each procedure was performed in triplicate. The average values of NSC-MM were set at 1. *p21* protein expression was reduced by *miR-93* and *-106b*-MM (**A**), and increased by *miR-93* and *-106b*-INH (**B**). *Mir-93*-MM and *miR-106b*-MM significantly decreased luciferase activity in both ChTERT (**C**) and GihTERT (**D**) cells.

Figure 6. Bim Western Blotting and Luciferase Assays

(**A** and **B**) Western blotting for Bim. WI-38 cells were transfected with *miR-25* or NSC-MM, while OE-33 cells were transfected with *miR-25* or NSC-INH. (**C** and **D**) Luciferase assay for *Bim* 3'UTR. GihTRT cells and OE-33 cells were used. Procedure was the same as Figure 5. Bim protein expression was reduced by *miR-25*-MM (**A**) and increased by *miR-25*-INH (**B**). (**C**) *MiR-25*-MM significantly decreased luciferase activity in GihTRT. (**D**) *MiR-25*-INH significantly increased luciferase activity in OE-33 cells.

Figure 7. p21 and Bim mRNA Expression Following miR Mimic Transfection

WI-38 cells were transfected with NSC, *miR-25*, *-93* or *-106b*-MM as well as an siRNA against *p21* mRNA (*p21*-siRNA) for comparison. *p21* and *Bim* mRNA expression levels were assayed by qRT-PCR. Data was normalized to *beta-actin*. The value of NSC-MM was set at 1. (A) *p21* mRNA was significantly (approximately 40%) decreased by *miR-106b*-MM, while *p21*-siRNA decreased *p21* mRNA by approximately 94%. (B) *Bim* mRNA was not significantly changed by *miR-25*-MM.

MiRs Up- or Downregulated in EAC Cells

MiRs that were at least two-fold upregulated or downregulated in the EAC cell line OE-33 relative to normal esophageal epithelial cells (NE, HEEpiC) are shown. **Bold** lettering indicates miRs and fold changes that were individually verified by qRT-PCR (Figures 1A–C and Supplementary Figure 1). Intensities in BE represent averages of three BE cell lines.

Table 1

Upregulated miRNAs in EAC						
Gene ID	Signal Intensity			Fold changes		
	NE	BE	EAC	BE/NE	EAC/NE	EAC/BE
hsa-miR-625	2.427	15.472	60.471	6.375	24.917	3.908
hsa-miR-25	19.320	43.905	176.964	2.273	9.160	4.031
hsa-miR-93	123.097	166.889	999.810	1.356	8.122	5.991
hsa-miR-106b	222.536	328.722	1643.634	1.477	7.386	5.000
hsa-miR-155	11.069	37.825	49.596	3.417	4.481	1.311
hsa-miR-345	7.941	20.186	32.616	2.542	4.107	1.616
hsa-miR-130b	70.430	101.451	235.714	1.440	3.347	2.323
hsa-miR-429	29.220	0.133	97.754	0.005	3.346	735.873
hsa-miR-186	32.248	41.120	107.330	1.275	3.328	2.610
hsa-miR-590	19.182	22.582	63.096	1.177	3.289	2.794
hsa-miR-339	62.318	137.244	190.642	2.202	3.059	1.389
hsa-miR-151	16.512	24.195	42.669	1.465	2.584	1.764
hsa-miR-28	26.015	26.736	67.193	1.028	2.583	2.513
hsa-miR-30a-5p	96.976	544.846	245.660	5.618	2.533	0.451
hsa-miR-200a	126.864	0.613	280.950	0.005	2.215	458.480
hsa-miR-301	53.618	18.079	117.880	0.337	2.199	6.520
hsa-miR-15b	58.416	125.848	122.633	2.154	2.099	0.974
hsa-miR-765	46.595	86.992	95.532	1.867	2.050	1.098

Downregulated miRNAs in EAC						
Gene ID	Signal Intensity			Fold changes		
	NE	BE	EAC	BE/NE	EAC/NE	EAC/BE
hsa-miR-138	78.652	18.056	0.130	0.230	0.002	0.007
hsa-miR-130a	591.094	334.128	1.258	0.565	0.002	0.004

Gene ID	Upregulated miRNAs in EAC					Fold changes		
	Signal Intensity							
	NE	BE	EAC	BE/NE	EAC/NE	EAC/BE		
hsa-miR-377	57.836	0.133	0.130	0.002	0.002	0.002	0.977	
hsa-miR-34c	57.986	3.918	0.231	0.068	0.004	0.004	0.059	
hsa-miR-100	46.127	51.461	0.429	1.116	0.009	0.009	0.008	
hsa-miR-125b	147.561	124.285	1.751	0.842	0.012	0.012	0.014	
hsa-miR-136	60.717	0.337	0.794	0.006	0.013	0.013	2.354	
hsa-miR-125a	101.390	90.420	2.026	0.892	0.020	0.020	0.022	
hsa-miR-34b	121.161	7.363	4.758	0.061	0.039	0.039	0.646	
hsa-miR-205	4425.770	0.133	190.119	<0.001	0.043	0.043	1431.173	
hsa-miR-503	47.259	2.633	2.591	0.056	0.055	0.055	0.984	
hsa-miR-203	61.533	0.174	3.490	0.003	0.057	0.057	20.078	
hsa-miR-424	219.416	36.410	17.791	0.166	0.081	0.081	0.489	
hsa-let-7e	298.152	300.862	38.384	1.009	0.129	0.129	0.128	
hsa-miR-34a	468.722	31.102	76.107	0.066	0.162	0.162	2.447	
hsa-miR-193a	29.086	132.574	6.795	4.558	0.234	0.234	0.051	
hsa-miR-92	527.562	768.209	127.465	1.456	0.242	0.242	0.166	
hsa-miR-146a	37.491	39.839	10.162	1.063	0.271	0.271	0.255	
hsa-miR-630	35.513	89.212	10.726	2.512	0.302	0.302	0.120	
hsa-miR-22	2747.800	955.157	873.014	0.348	0.318	0.318	0.914	
hsa-miR-574	49.703	47.840	16.318	0.963	0.328	0.328	0.341	
hsa-miR-27a	2979.200	1549.889	1020.512	0.520	0.343	0.343	0.658	
hsa-miR-19b	846.009	1243.164	306.367	1.469	0.362	0.362	0.246	
hsa-miR-20b	135.788	180.632	50.567	1.330	0.372	0.372	0.280	
hsa-miR-23a	788.543	486.468	295.407	0.617	0.375	0.375	0.607	
hsa-miR-17-3p	98.531	108.599	37.361	1.102	0.379	0.379	0.344	
hsa-miR-20a	466.738	596.429	177.354	1.278	0.380	0.380	0.297	
hsa-let-7c	485.420	646.144	192.216	1.331	0.396	0.396	0.297	
hsa-let-7a	1127.190	1306.387	460.268	1.159	0.408	0.408	0.352	
hsa-miR-31	2639.880	268.650	1121.611	0.102	0.425	0.425	4.175	
hsa-miR-106a	414.097	431.847	182.995	1.043	0.442	0.442	0.424	
hsa-miR-17-5p	328.521	366.471	147.929	1.116	0.450	0.450	0.404	

Upregulated miRNAs in EAC							
Gene ID	Signal Intensity			Fold changes			
	NE	BE	EAC	BE/NE	EAC/NE	EAC/BE	
hsa-let-7f	588.235	728.934	278.346	1.239	0.473	0.382	
hsa-let-7b	1303.260	1788.424	634.358	1.372	0.487	0.355	
hsa-miR-26a	67.126	89.710	33.217	1.336	0.495	0.370	