Regulation of the Adenomatous Polyposis Coli Gene by the miR-135 Family in Colorectal Cancer

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Abstract

Inactivation of the adenomatous polyposis coli (APC) gene is a major initiating event in colorectal tumorigenesis. Most of the mutations in APC generate premature stop codons leading to truncated proteins that have lost β-catenin binding sites. APC-free β-catenin stimulates the Wnt signaling pathway, leading to active transcription of target genes. In the current study, we describe a novel mechanism for APC regulation. We show that miR-135a&b target the 3′ untranslated region of APC, suppress its expression, and induce downstream Wnt pathway activity. Interestingly, we find a considerable up-regulation of miR-135a&b in colorectal adenomas and carcinomas, which significantly correlated with low APC mRNA levels. This genetic interaction is also preserved in full-blown cancer cell lines expressing miR-135a&b, regardless of the mutational status of APC. Thus, our results uncover a miRNA-mediated mechanism for the control of APC expression and Wnt pathway activity, and suggest its contribution to colorectal cancer pathogenesis. [Cancer Res 2008;68(14):5795–802]

Introduction

Inactivating mutations in the tumor suppressor gene Adenomatous Polyposis Coli (APC), a key component of the Wnt signaling pathway, cause formation of adenomas in the large intestine, which are precursors of colorectal cancer (CRC; ref. 1). The malignant transformation process is induced due to subsequent accumulation of genetic alterations, which often include KRAS and p53 mutations, chromosomal gains (e.g., 20q, 13q, and 8q) and losses (17p and 18q), and epigenetic changes (2–4). In the intestine, the canonical Wnt pathway maintains the proliferative compartment at the crypts as well as cell fate and position of the epithelial cells to bind the TCF/LEF transcription factors, resulting in transcriptionally active transcription of target genes. In the current study, we describe a novel mechanism for APC regulation. We show that miR-135a&b target the 3′ untranslated region of APC, suppress its expression, and induce downstream Wnt pathway activity. Interestingly, we find a considerable up-regulation of miR-135a&b in colorectal adenomas and carcinomas, which significantly correlated with low APC mRNA levels. This genetic interaction is also preserved in full-blown cancer cell lines expressing miR-135a&b, regardless of the mutational status of APC. Thus, our results uncover a miRNA-mediated mechanism for the control of APC expression and Wnt pathway activity, and suggest its contribution to colorectal cancer pathogenesis. [Cancer Res 2008;68(14):5795–802]

Materials and Methods

Cell culture. HeLa, MCF-7, HEK293T, CaCo2, Colo205, DLD-1, HCT15, HT29, LS174T, SW48, SW480, WiDr, and L-cells were cultured in DMEM supplemented with 10% FCS and antibiotics. For miR-135 inhibitor treatment, cells were grown to 30% confluency, after which the tissue culture medium was replaced with medium containing either 25 μmol/L...
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(24 wells) or 75 μmol/L (6 wells) of miR-135 or control (miR-372) inhibitors. Sequences (Dharmacon) were as follows: 135, CH-UAG AAA CAC AUA GGA AUG AAA AGC CAU AGA GAA; and 372, Chl-CGG UGA CGC UAA UAU GUC GCA CUC UCU ACC.

L-cells and L-cells stably expressing Wnt3A were a kind gift from A. Berns (NKI, Amsterdam, The Netherlands). Wnt-conditioned medium was harvested as previously described (29).

For the miRNA-library screen, transfection and virus harvesting steps were carried out on a Hamilton ML as described before (27).

**Patient material.** Frozen and paraffin-embedded tissues of 43 colorectal tumors (23 adenomas and 20 carcinomas) and 18 colorectal normal tissues of archival paraffin-embedded tissue as isolated using TRIzol according to the ethical guidelines of our institution concerning informed consent about the use of patient’s material after surgical procedures. DNA from the colorectal tumors was isolated from archival paraffin-embedded tissue as previously described (3, 30). Total RNA was isolated using TRIzol (Invitrogen) according to manufacturers protocol with some modifications, described in detail on the VUMc Web site. All tumor samples were reviewed by an expert gastrointestinal pathologist (GAM) and classified according to standard criteria. In addition, tissue areas for DNA and RNA isolation were histologically verified to contain a minimum of 70% tumor.

** Constructs.** GFP-APC-3’ UTR was constructed by cloning the 3’UTR of APC between EcoRI and BamHI restriction sites of the green fluorescent protein (GFP) sensor vector as described in (27). The 3’UTR of APC was amplified from genomic DNA using forward and reverse primers. Primers used for this PCR were GCG ACG TCT TAA AAG AGA GGA AGA ATG AAA CTA AG and CAC CGG TGC ATG TAT CTC CAT TGT TTA TGG as forward and reverse primers, respectively. Luc-APC-3’UTR DM was created using GGA TAT CAC AGA GAT CCA TGA CCT CCA GCA AAA CCC and GAC ACA TAC TGG ACT CAT CTA TTA TTT TTT TCC TAA CCA TC as primers. Italicized is the miR-135 antisense sequence converted to BamHI.

The APC3’was designed to target the sequence AAAT CCT CCG TGC TTA TGG and was constructed as previously described (31).

**TOPFLASH and FOPFLASH constructs.** A kind gift of A. Berns. GFP-APC-3’UTR was produced by subcloning the 3’UTR of APC into the plg3 vector (Promega) downstream of the luciferase gene as means of PCR. Primers used for this PCR were GCC ACG GAC TTA AAG AGA GGA AGA ATG AAA CTA AG and CAC CGG TGC ATG TAT CTC CAT TGT TTA TGG as forward and reverse primers, respectively. Luc-APC-3’UTR DM was created using GGA TAT CAC AGA GAT CCA TGA CCT CCA GCA AAA CCC and GAC ACA TAC TGG ACT CAT CTA TTA TTT TTT TCC TAA CCA TC as primers. Italicized is the miR-135 antisense sequence converted to BamHI.

** Western blot analysis and antibodies.** Proteins were analyzed by SDS-PAGE and Western blotting using Immobilon-P transfer membrane (Millipore). For loading control, an antibody against tubulin was used (YLI/2; European Collection of Animal Cell Cultures). Antibodies against β-catenin, active β-catenin (dephosphorylated), and APC were a kind gift of M. Fornerod (NKI, Amsterdam, The Netherlands).

**Luciferase assay.** Luciferase assays were performed as described before (34). In short, MCF-7 cells were transfected using PEI (Polysciences, Inc.). For Luc-APC-3’UTR reporter assays, cells were cultured in 24-well plates and transfected with 5 ng Luc-APC-3’UTR (or DM), 5 ng Renilla, and 0.5 μg of miR-135a, miR-135b or APC3’UTR. For TOP/FOP assays, HEK293T cells were cultured in 24-well plates and transfected either with 100 ng TOP- or FOPFLASH, 5 ng Renilla, and 0.5 μg of miR-135a, miR-135b, or APC3’UTR.

**APC mutation analysis.** APC mutation status was analyzed by sequencing the mutation cluster region (MCR) between codons 1286 and 1513 in all colorectal tumor and control samples. To cover the MCR region, four primers sets (set 1: Fwd GAG GAT AAT AGA GGA AA, Rev GCC TCC TGA AA AAT TCC AAC; set 2: Fwd GAG GCT GCC AGC AGA ACT, Rev GAA GGT CCA GCA GTG CAC C; set 3, Fwd GCT GAG ACA AAA AGT CT, Rev ATT TTT AGG TAC TTC TCG CTT G and set 4, Fwd AA ACA CCT CCA CCA CTC C, Rev C ATT CCC ATT GTC ATT TCT C) were used for PCR reactions as previously described (2). The PCR products were inspected by running on a 2% agarose gel, after which the products were purified using SAP and EXO enzymes. Subsequently, samples were prepared for sequencing using the ABI PRISM BigDye terminator cycle sequencing ready kit (Applied Biosystems) according to manufacturers protocol. Sequencing was done on a 3130 DNA sequencer (Applied Biosystems) and analyses were carried out with the sequencer navigator and the Vector NTI (Invitrogen).

**APC promoter methylation analysis.** Promoter methylation of the APC gene was established by methylation-specific PCR (MSP). Before MSP, samples were subjected to sodium bisulfite treatment according to the EZ DNA Methylation kit (Zymo Research Corporation) using 500 ng of DNA as starting material. Primer sequences to amplify the methylated alleles DNAMethylation kit (Zymo Research Corporation) using 500 ng of DNA as starting material. Primer sequences to amplify the methylated alleles were designed to amplify a 500 bp fragment of the APC promoter region as described before (2). The MSP products were inspected by running on a 2% agarose gel containing ethidium bromide.
Results

APC expression is suppressed by miR-135a and miR-135b.

To identify miRNAs that directly regulate the expression of APC, we made use of a previously developed sensor vector (27) and cloned the 3'UTR of APC downstream of the GFP gene. HeLa cells were transduced with this vector, and a single clone stably expressing GFP-APC-3'UTR was isolated and expanded. Subsequently, we transduced the cell line with the miRNA vectors (miR-Vec) from our miR-Lib, drug selected, and pooled all stable clones. We assumed that miRNAs that regulate APC expression through its 3'UTR will reduce the fluorescent signal, enabling us to distinguish between APC-targeting and nontargeting miRNAs (Fig. 1A). After 2 weeks in culture, the low-GFP-expressing cells were fluorescence-activated cell sorting (FACS)-sorted from the total GFP-expressing cell population, and genomic DNA was extracted from both populations. By comparing the relative abundance of miR-Vec inserts between low and total GFP-expressing populations using our miR-Arrays, we were able to identify 5 miR-Vecs that were reproducibly enriched in the low-GFP-expressing population (Fig. 1B).

We next tested the effect of each of these miRNAs by comparing their regulatory capacity on either GFP-APC-3'UTR or a GFP-control-3'UTR. Whereas miR-135a&b inhibited GFP-APC-3'UTR expression by ~35%, no effect was observed toward a control GFP-3'UTR construct (Fig. 1C). For the other obtained hits (miR-19b, 199a, and 371), no significant down-regulation of the GFP-APC reporter could be detected (Supplementary Fig. S1). Subsequently, we subcloned the 3'UTR of APC downstream of a luciferase gene, creating luc-APC-3'UTR. Transient expression of this vector revealed that miR-135a&b reduce luciferase expression levels by ~30% to 40% when compared with control miR-Vec (Fig. 1D).

To determine whether miR-135a&b affect endogenous APC expression, we transduced HEK293T cells, which possess a wild-type Wnt signaling pathway, with the miR-Vec constructs. The expression of miR-135a and miR-135b was verified using quantitative reverse transcription-PCR (qRT-PCR; Supplementary Fig. S2). Examination of endogenous APC protein levels from HEK293T cells stably expressing miR-135b revealed a clear down-regulation of APC protein compared with cells expressing a control construct (Fig. 2A). A corresponding reduction in APC mRNA levels was detected by qRT-PCR, suggesting mRNA decay as a mechanism involved in miR-135a&b–mediated APC regulation (Fig. 2B).

Examination of the APC 3'UTR for miR-135a&b binding sites revealed two potential target sequences. TargetScan 4.0 predicted the first site, whereas the second site was obtained using RNA22 software (Fig. 2C). To show specificity of miR-135 function, we mutated both sites, generating a double mutant (DM) of the luciferase reporter (Fig. 2D). Cotransfection experiments revealed that the DM is completely refractory to miR-135a&b–mediated
repression, indicating the direct suppression of APC by miR-135a&b (Fig. 2D). Altogether, we have identified miR-135a&b as potent regulators of APC expression.

**miR-135a and miR-135b induce β-catenin signaling.** Next, we investigated the functional relevance of the interaction between miR-135a&b and APC by determining the effects of their expression on the activity of the Wnt pathway. First, we examined effects of miR-135 on TCF transcriptional activity. Therefore, we transfected HEK293T cells with luciferase reporter constructs harboring either three optimal TCF binding sites (TOPFLASH) or three mutated TCF binding sites (FOPFLASH). Addition of Wnt ligand to cells containing TOPFLASH, but not FOPFLASH, resulted in a 2-fold increase in luciferase counts, demonstrating the activity and specificity of these reporter constructs (Fig. 3A). Interestingly, transfection of either miR-135a or miR-135b significantly increased TOPFLASH reporter activity by 1.5-fold, in the absence of Wnt ligand. In comparison, transfection of a functional shRNA construct targeting APC (APCkd) similarly increased TOPFLASH reporter activity by 1.5-fold (Fig. 3A). Thus, the expression of miR-135a&b activates the Wnt pathway in a manner similar to APC loss and comparable with addition of Wnt ligand.

**miR-135a&b suppress APC in CRC cell lines.** In all the experiments performed thus far, miR-135a&b were exogenously introduced. To examine whether endogenous miR-135a&b regulate APC in a full-blown cancer environment, we first determined the expression level of miR-135a&b in a set of nine CRC cell lines, as APC is frequently deregulated in CRC. These cell lines harbor different types of APC mutations (listed in Supplementary Table S1). Figure 4A shows that all of the CRC cell lines we examined express high levels of miR-135a and miR-135b relative to the control cell lines HeLa and HEK293T.

Next, to inactivate miR-135a&b, we designed one antagoniR that is able to target both miR-135 family members. We tested the effectiveness and specificity of antagoniR-135 on the down-regulation of luciferase-APC-3’UTR by miR-135a&b. Supplementary Fig. S3 shows that treatment of antagoniR-135, but not control antagoniR-372, inhibited both miR-135a and miR-135b function, indicating that antagoniR-135 is effective and specific.
Having validated antagoniR-135 as a potent and specific inhibitor of both miR-135a&b, we applied it to the CRC cell lines for 72 hours. Figure 4B shows that in all cases, APC mRNA levels were increased 1.5- to 2.5-fold after treatment with antagoniR-135. The observed effect is specific, as APC mRNA levels were not altered after antagoniR-135 treatment in cells that do not express miR-135a&b (HeLa and HEK293T). This shows that APC is still under control of the miR-135 family members in colorectal cell lines expressing miR-135a&b, regardless of the mutational status of the gene.

miR-135 family expression is increased during CRC progression. To further investigate the role of miR-135a and miR-135b in CRC tumorigenesis, the expression of miR-135a and miR-135b was determined in 43 colorectal tumors (20 adenocarcinomas and 23 adenomas) and 18 controls (normal colon epithelium). The tumors showed an increased expression of both miR-135a and miR-135b compared with normal colon epithelium. We observed a 4.8-fold higher expression of miR-135a (P < 0.0001; Fig. 5A) and 10-fold higher expression of miR-135b (P < 0.0001; Fig. 5B), in accordance with the previously detected high levels of miR-135a&b expression in the cell lines. Furthermore, we detected a 3.2-fold increase in miR-135a expression levels in colorectal adenomas compared with the controls (P < 0.0001), whereas adenocarcinomas showed 6.7 times increased expression (P < 0.0001) compared with the control group. Moreover, we found a 2.1 times higher expression of miR-135a in colorectal carcinomas compared with adenomas (P = 0.007; Fig. 5C). For miR-135b, we observed a 6.6-fold higher expression in adenomas (P < 0.0001) and a 14 times increased expression in the carcinomas (P < 0.0001) compared with the control group. The expression of miR-135b was 2.1-fold higher in carcinomas than in adenomas (P = 0.02; Fig. 5D). These results imply a role of both miR-135a and miR-135b in the pathogenesis of CRC.

To confirm the connection of the miR-135 family with APC gene expression that was observed in the in vitro experiments, we measured APC mRNA expression levels in this same panel of colorectal samples. As expected, APC expression levels were 20 times higher in the normal controls than in the adenomas (P < 0.0008) and in carcinomas (P = 0.01), which is inverse to the pattern that we observed for miR-135a and miR-135b (Fig. 5C and D).

To explore a possible association between the presence of mutations and/or hypermethylation of the APC gene and the levels of the miRNAs, we compared the expression levels of both miRNAs in tumors without any aberrations in the APC gene to tumors showing mutations and/or hypermethylation events in the APC gene. No significant associations between the presence of APC mutations and/or promoter hypermethylation, and the expression levels of miR-135a&b in the tumor samples were found (data not shown).

In conclusion, increasing expression of miR-135a and miR-135b from normal colorectal epithelium, through adenomas to adenocarcinomas, is correlated with decreasing expression of APC gene during colorectal tumorigenesis. This association is independent of the status of APC mutation or promoter hypermethylation in the tumors.

Discussion

Using a functional genetic screening approach, we have uncovered a novel mechanism for the control of Wnt pathway activity. We identified the miR-135 gene family as a regulator of APC expression and showed its potential to activate the Wnt pathway in the absence of Wnt ligand. Using CRC cell lines, we showed direct and causal suppression of endogenous APC by miR-135a&b. Furthermore, in a significant number of colon tumors, we observed high levels of miR-135a&b that negatively correlated with APC expression.

miRNAs in control of Wnt signaling. Our study reveals the effect of miR-135 on Wnt signaling in human cells. Recently, Silver and colleagues (36) also identified a miRNA capable of regulating the Wnt pathway in Drosophila. Using an unbiased approach to identify miRNAs involved in the Wingless-Wnt pathway, miR-315 was shown to regulate both Axin and Notum, thereby activating the signaling cascade. However, the regulation of the Drosophila APC by miR-135a&b was not identified as, these miRNAs are not conserved. Additionally, the regulation of APC by miR-135a&b seems a regulatory mechanism specific to humans, as the target

Figure 3. Effects of miR-135a&b expression on Wnt pathway activity. A, dual luciferase experiment demonstrating the effect on TOPFLASH/FOPFLASH reporter activity in HEK293T cells. Values were normalized to a Renilla transfection control and standardized to the control vector. The figure shown is a representative of at least three independent experiments. Columns, mean of a triplicate transfection; bars, SE. B, Western blot showing staining of ABC in HEK293T cells stably expressing the indicated constructs. ABC levels were normalized to a tubulin loading control using Tina 2.0 software, and standardized to the control. C, dual luciferase assay showing the effect of miR-135a&b expression on a c-Myc reporter construct in HEK293T cells. A representative figure is shown of at least three independent experiments; columns, mean of a triplicate transfection; bars, SE.
sites for miR135a&b are not conserved in other species (data not shown). Altogether, the observations of Wnt pathway regulation by miR-315, as well as miR-135a&b family, add additional layers to the control of Wnt pathway activity.

Deregulated expression of miR-135 gene family in CRC. To fully understand the role of miR-135a&b in colorectal pathogenesis, it will be necessary to identify the mechanisms regulating expression of these miRNAs. Interestingly, miR-135b is located in the first intron of the LEM domain containing 1 (LEMD1) gene. Although the function of this gene is not known, it has been reported to be highly expressed in CRC compared with control tissue (37). It seems likely that miR-135b will follow the same expression pattern as its host gene and is elevated by the same mechanism. As miR-135b is located on 1q32.1, which frequently shows DNA copy number gain in CRC progression (38, 39), a gene dosage effect could explain the increase in its expression. miR-135a, on the other hand, is encoded by two copies in the human genome, which are located in the first intron of the stabilin 1 (STAB1) gene on 3p21 and in intron 5 of the rhabdomyosarcoma 2-associated transcript (RMST) gene on 12q23, respectively. To our knowledge, these genes have not been reported to be differentially expressed in CRC or associated with DNA copy number changes during CRC progression. Therefore, the mechanism by which the miR-135 family is up-regulated in CRC pathogenesis remains to be explored. Nevertheless, our findings suggest that elevation of miR-135 levels is a commonly occurring event in CRC pathogenesis.

Pathway addiction and the miR-135 family. Our results indicate that even in fully developed cancer cells, the presence of miR-135a&b suppresses APC expression even when APC mutations are present. According to the Knudson hypothesis and the caretaker/gatekeeper model, two hits would suffice to knock down APC function and up-regulate Wnt signaling as a first step toward CRC. The present data, however, suggest that miR-135a&b up-regulation modifies Wnt signaling independent of accumulation of APC mutation or promoter methylation status. The observation that high miRNA-135 expression is also found in samples bearing biallelic APC mutations might suggest that other targets of this miRNA could also be important for CRC pathogenesis. Additionally, miR-135 expression might work in synergy with APC mutations, to inhibit APC function to a greater extent. Similar observations supporting this hypothesis have been made for other regulators of Wnt signaling, such as SFRP1. SFRP1 is frequently inactivated, which is also independent of APC mutation or promoter hypermethylation status and results in increased Wnt signaling (40). The term pathway addiction has been suggested for this phenomenon.

Figure 4. The effect of inhibition of the miR-135 family on APC mRNA levels and cellular growth. A, relative expression of miR-135a&b in the indicated cell lines as determined by qPCR. Levels were obtained from triplicate PCRs and normalized to the expression levels observed in HeLa cells. Expression of miR-135a and miR-135b is indicated in different colors. mRNA expression is depicted on a logarithmic scale. B, relative APC mRNA expression 72 h after addition of control or miR-135 antagomiRs as determined by qPCR. Figure is a representative of three independent qPCRs. Columns, mean of a triplicate PCR, standardized to the APC levels of control antagomiR-treated cells; bars, SE.
where multiple (epi)genetic alterations cooperate to shift signaling pathways toward a pro-oncogenic direction (41, 42). Our data might imply that elevated miR-135a and/or miR-135b expression is another player in the pathway addiction mechanism, and possibly cooperates with the currently known mechanisms for APC deregulation, or is a prerequisite for their efficient occurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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