Strand-Specific miR-28-5p and miR-28-3p Have Distinct Effects in Colorectal Cancer Cells

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BACKGROUND & AIMS: MicroRNAs (miRNAs) can promote or inhibit tumor growth and are therefore being developed as targets for cancer therapies. They are diverse not only in the messenger RNAs (mRNA) they target, but in their production; the same hairpin RNA structure can generate mature products from each strand, termed *5p* and *3p*, that can bind different mRNAs. We analyzed the expression, functions, and mechanisms of miR-28-5p and miR-28-3p in colorectal cancer (CRC) cells. **METHODS:** We measured levels of miR-28-5p and miR-28-3p expression in 108 CRC and 49 normal colorectal samples (47 paired) by reverse transcription, quantitative real-time polymerase chain reaction. The roles of miR-28 in CRC development were studied using cultured HCT116, RKO, and SW480 cells and tumor xenograft analyses in immunodeficient mice; their mRNA targets were also investigated. **RESULTS:** miR-28-5p and miR-28-3p were down-regulated in CRC samples compared with normal colon samples. Overexpression of miRNAs in CRC cells had different effects and the miRNAs interacted with different mRNAs: miR-28-5p altered expression of *CCND1* and *HOXB3*, whereas miR-28-3p bound *NM23-H1*. Overexpression of miR-28-5p reduced CRC cell proliferation, migration, and invasion in vitro, whereas miR-28-3p increased CRC cell migration and invasion in vitro. CRC cells overexpressing miR-28 developed tumors more slowly in mice compared with control cells, but miR-28 promoted tumor metastasis in mice. **CONCLUSION: miR-28-5p and miR-28-3p are transcribed from the same RNA hairpin and are down-regulated in CRC cells. Overexpression of each has different effects on CRC cell proliferation and migration. Such information has a direct application for the design of miRNA gene therapy trials.**

Keywords: Transcript Regulation; Gene; RNA Processing.

Colorectal (CRC) cancer is the third most commonly diagnosed cancer in men and the second in women.[1] In the United States, it is the third leading cause of death by cancer, with 51,371 estimated deaths and 142,570 estimated newly diagnosed cases in 2010.[2] Therefore, new therapeutic approaches and prognostic markers are needed. In 2002, new players in cancer biology were identified: microRNAs (miRNAs).[3] These are a large family of small noncoding RNAs with approximately 20-nt length that regulate gene expression post-transcriptionally by inhibition of translation or messenger RNA (mRNA) degradation.[4] miRNA targeting occurs by binding to 3′-untranslated regions, coding sequences, or 5′-untranslated regions of target mRNA that can be involved in diverse biological processes, such as proliferation, apoptosis, inflammation, differentiation, and metastasis.[4] miRNAs can function as either oncogenes or tumor suppressor genes, depending on the type of tumor or the cellular context.[5] In CRC, miRNAs have been involved in tumor susceptibility (as polymorphisms in miRNA-binding sites have been associated with CRC risk) and in diagnosis (as miRNAs can be detected in feces or blood and used as biomarkers).[6] In addition, miRNA expression is dysregulated in CRC, as well as in other cancer types, and miRNAs have emerged as potential new therapeutic targets.[6,7] Therefore, understanding the role of miRNAs in CRC is crucial for the development of new therapies.

In the miRNA biogenesis pathway, long primary transcripts transcribed from the genome are processed by the cellular RNase enzyme III Drosha into a structure of 60 to 110 nt called precursor miRNA (pre-miRNA), which is then exported to the cytoplasm by an Exportin 5-dependent mechanism.[4] The pre-miRNA is cleaved by the RNase III enzyme Dicer-1 producing a short, imperfect, double-stranded miRNA duplex, which is unwound by a helicase, creating a mature miRNA.[4] In some cases, 2 mature miRNAs can be excised from the same stem-loop pre-miRNA.[8] These 5p and 3p miRNAs, although generated from a single primary transcript, have different sequences and therefore tar-

Abbreviations used in this paper: CRC, colorectal cancer; miRNA, microRNA; MSS, microsatellite stable; mRNA, messenger RNA; MSI, microsatellite unstable; PCR, polymerase chain reaction; SCR, scrambled negative control; PARP1, poly(adenosine diphosphate-ribose) polymerase 1; pre-miRNA, precursor miRNA.

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get different mRNAs. In humans, 2 different mature miRNA sequences are excised from opposite arms of the stem-loop pre-miR-28 and generate 2 different miRNAs—hsa-miR-28-5p and hsa-miR-28-3p. Despite nearly a decade of studies on miRNA roles in cancer,[3] the comparative roles of strand-specific mature miRNAs that originated from the same stem-loop precursor (5p and 3p) have not yet been fully studied.

To our knowledge, the roles miR-28-5p and miR-28-3p play in CRC have never been described. Therefore, the purpose of our study was to analyze miR-28-5p and miR-28-3p expression and to use in vitro and in vivo approaches to understand, for the first time, the functions and mechanisms of these 2 miRNAs in CRC.

Materials and Methods

Colorectal Samples

Eighty-five CRC samples and 26 normal colorectal tissue samples (of which 24 were paired) were collected between 2003 and 2008 at the University Hospital of Ferrara in Ferrara, Italy (first sample set). Forty-two tumors were classified as microsatellite stable (MSS), and 43 tumors were classified as microsatellite unstable (MSI) (Supplementary Methods). For a confirmation set of samples, we obtained 23 paired samples of tumor and adjacent colorectal tissue that were collected between 2002 and 2005 at the Istituto per lo Studio e la Cura dei Tumori della Romagna in Meldola, Italy (second sample set). Tumors were classified according to the World Health Organization pathologic classification system. All patients provided informed consent, and collection of the samples was approved by the institutional review board at each institution. Patients did not receive any therapy before surgery. Upon resection, fresh surgical specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C. Total RNA from tissue samples was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instructions (Supplementary Methods).

Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction

RNA purity was assessed by measuring absorbance at 260, 280, and 230 nm. Mean 260/280 ratio was 1.97 ± 0.05, with a range between 1.86 and 2.05, and mean 260/230 ratio was 2.17 ± 0.11, with a range between 2.00 and 2.31. In addition, as recommended by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines,[9,10] we analyzed RNA integrity by gel electrophoresis and clearly defined 28S and 18S ribosomal RNA bands were visualized. Samples with low quality that did not meet these criteria were excluded. We quantified miR-28-5p and miR-28-3p expression with real-time quantitative polymerase chain reaction TaqMan miRNA assays (Applied Biosystems, Foster City, CA), namely assay 000411 for miR-28-5p, assay 002446 for miR-28-3p, and assay 001973 for U6 snRNA (Supplementary Methods). The efficiency of the Taqman assays used in this study was determined (Supplementary Figure 1 and Supplementary Table 1). Relative expression levels were calculated using the $\Delta\Delta C_t$[11] and the Pfaffl method.[12]

In Vitro Cell Proliferation Assays

HCT116 and RKO cells transfected with scrambled negative control (SCR), miR-28-5p, or miR-28-3p were seeded onto a 12-well plate at 1×10^5 cells/well in triplicate. Cells were harvested and counted at 0, 24, 48, 72, and 96 hours after transfection using the Vi-CELL cell viability analyzer (Beckman Coulter, Brea, CA). In order to further confirm our results, a 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed (Supplementary Methods). The experiment was repeated twice independently.

In Vitro Cell Migration and Invasion Assays

To determine the effect of miR-28-5p and miR-28-3p on cell migration, we used 6.5-mm diameter Transwell chambers with 8-μm pore size polycarbonate membranes (Corning Incorporated, Lowell, MA). To determine the effect of these miRNAs on cell invasion, we used BioCoat growth-factor reduced Matrigel invasion chambers (BD Biosciences, Bedford, MA). Cells transfected with SCR, miR-28-5p, or miR-28-3p were resuspended in serum-free medium and plated on the top of the Transwell chambers. Fetal bovine serum was used as a chemoattractant on both assays. Each assay was performed in triplicate and in 2 independent experiments. Additional details are described in Supplementary Methods.

In Vivo Studies of Tumorigenesis and Metastatic Potential

For the in vivo tumorigenesis assay, 1.5×10^6 HCT116-pBABE-miR-28 or HCT116-pBABE-empty cells were subcutaneously injected into the flanks of NOD-SCID-IL2R−deficient mice (n = 9; stock #005557; The Jackson Laboratory, Bar Harbor, ME). Tumor size was measured every 2 days. Animals were sacrificed 21 days after injection, and final tumor volume was determined. Tumor size was determined by digital caliper measurements (length and width in mm), and tumor volume (mm^3) was estimated using the following formula: tumor volume = ½ (length × $width^2$).

For the in vivo tumor-metastasis assay, 4×10^6 HCT116-pBABE-miR-28 and HCT116-pBABE-empty cells were injected into the tail vein of NOD-SCID-IL2R–deficient mice (n = 11/group). Thirty-five days after injection the mice were sacrificed. All of the organs were examined at necropsy. Tumors were sectioned, stained with H&E and anti−green fluorescent protein antibody (Ab13970; Abcam, Cambridge, MA), and examined histologically.

All animal care and handling was approved by The University of Texas MD Anderson Institutional Animal Care and Use Committee.

Statistical Analysis

Shapiro–Wilk test was used to verify the clinical samples' distribution. Differences were analyzed using the nonparametric test Mann–Whitney–Wilcoxon. To compare the paired groups, paired *t* test was used. For in vitro and in vivo studies, the differences between groups were analyzed using Student *t* test (2-tailed), assuming unequal variance. Discrete variables were compared with the Fisher exact test. Graphics represent the mean ± standard deviation, unless otherwise stated. Statistical analysis was performed in R (version 2.11.0). Statistical significance was considered if $P < .05$.

Additional methods, including cell culture, STR DNA fingerprinting, and miRNA mimics transfection, apoptosis quantification, caspase activity, cell cycle analysis by flow cytometry, establishment of miR-28–expressing cell line, miRNA target prediction, Western blot, and luciferase reporter assays, are available in Supplementary Methods.